Production and evaluation of pseudotyped viruses for Hepacivirus and Flavivirus studies

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Kamilla Toon

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Flaviviridae is a diverse family of RNA viruses that includes many important human pathogens such as dengue virus, West Nile virus, Japanese encephalitis virus (JEV), and hepatitis C virus (HCV). Handling many of these pathogens requires high containment level biosafety laboratories and some cannot replicate in cell culture. The use of replication deficient pseudotyped viruses (PV) circumvents these issues. This thesis shows the development of two *Flaviviridae* PVs, GB Virus B (GBV-B) and JEV, for serological assay development and virus-host interaction studies.

HCV is a major global concern with over 70 million people chronically infected. Until the recent discovery of related animal hepaciviruses, GBV-B was the only known homolog of HCV and is used in new world monkeys as an animal model. However, there is still little known about the viral-host interactions. This thesis first describes the production and optimization of GBV-B PV to develop a neutralization assay for screening sera from experimentally infected tamarins.

Viral entry mechanisms were investigated using the GBV-B PV. Cells without Claudin 1 (CLDN1), a known HCV receptor, had no detectible GBV-B infection. CLDN1 was subsequently confirmed to be an entry factor when restoration of expression conferred susceptibility to GBV-B PV. Chimeric claudin proteins consisting of the permissive CLDN1 and non-permissive CLDN9 were created to investigate the region of importance for GBV-B and CLDN1 interaction. The important region was identified as extracellular loop 2, or downstream, which is different from the dependence of CLDN1-restricted HCV strains on regions in extracellular loop 1.

Research with JEV requires high containment level biosafety labs which are often unavailable in resource limited areas. Described here is the production of JEV PV using two alternative pseudotyping methods, *in vitro* assembly of subviral particles with envelope-less lentiviral particles and dengue virus core, after failed attempts at using traditional systems.

Impact Statement

The research presented in this thesis shows the development and optimization of pseudotyped viruses harbouring the outer proteins of two viruses within *Flaviviridae*: GB virus B (GBV-B) and Japanese encephalitis virus (JEV). The development of these tools has many potential impacts on public health.

Despite the recent success of directly acting antivirals that have success rates of over 90%, HCV is still a major global concern. The key to eradicating HCV is likely the development of a vaccine. The GBV-B PV can be used to, for the first time, screen the sera from experimentally-infected animals. This assay will be able to decipher the role that neutralization antibodies play in the clearance of GBV-B, which is generally cleared in the acute phase. Characterizing the immune response that results in the clearance of a hepacivirus could provide valuable insight into potential HCV vaccine design. The development of an HCV vaccine has the potential to save hundreds of thousands of lives per year.

The expected publication of the research regarding the discovery of an entry factor for GBV-B should inspire peers to study entry factors of other hepaciviruses. Characterization of more hepaciviruses will help to determine how these viruses move into new hosts, including humans. This information is valuable for preventing future zoonotic infections. Further to that, animal models are needed for preclinical vaccine evaluation, and full characterization of the candidate models will aid in determining the appropriate models to use. This is particularly important as the numerous recently discovered animal hepaciviruses mean that there are more potential models than ever.

Development/optimization of JEV PV will allow the National Institute of Biological Standards and Control (my funder) to screen candidate sera for neutralizing

antibodies in a high-throughput and low containment environment to more quickly develop reference materials for the WHO. These materials allow for reduction of variability and harmonization of assays performed from different labs. Furthermore, as the precise mechanisms of cell entry for JEV are currently unknown, this provides a safe tool for researchers to determine essential entry factors. Manufacturers can then create therapeutics that target these interactions, which are needed as there are currently no licenced treatments for JEV. Even without knowing the mechanisms of entry the JEV PV platform allows screening of already used therapeutic compounds for cell entry inhibition. Moreover, there are public health concerns regarding the geographical spread of JEV and the PV allows for serosurveillance in a low containment setting to help predict and mitigate outbreaks in naïve populations.

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Table of contents

Contents

List of Figures

List of Tables

Abbreviations

ΔENV$_{(DENV)}$- dengue virus core with no glycoproteins

ΔENV$_{(HIV)}$- HIV core virus with no glycoproteins

ΔENV$_{(MLV)}$- MLV core virus with no glycoproteins

ΔENV$_{(VSV)}$- VSV core virus with no glycoproteins

aa- amino acid

Ab- antibody

ADE- antibody dependent enhancement

ALT- alanine aminotransferase

apoE- apolipoprotein E

BHK- Baby hamster kidney

BSL- biosafety level

C- core protein

CD14- cluster of differentiation 14

CD81- cluster of differentiation 81

CD4+ - cluster of differentiation 4 positive

CD8+ - cluster of differentiation 8 positive

CLDN1- claudin 1

CLDN1/9- claudin 1 and 9 chimera

CLDN6- claudin 6

CLDN9- claudin 9

cDNA- Complementary DNA

cLDs- cytosolic lipid droplets

CLEC5A- C-type lectin domain family 5, member A

CRFK- Crandell-Rees Feline Kidney Cell

CSF- cerebrospinal fluid

DAA- directly acting antivirals

DAPI- 4′,6-diamidino-2-phenylindole

DC- dendritic cells

DC-SIGN- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DENV- dengue virus

DMEM- Dulbecco’s Modified Essential Medium

DNA- Deoxyribonucleic acid

E- envelope protein (JEV)

E1- envelope protein 1 (HCV)

E2- envelope protein 2 (HCV)

EDTA- Ethylenediaminetetraacetic acid

EGFR- epidermal growth factor receptor

EL- extracellular loop

ELISA- Enzyme linked immunosorbent assay

EphA2- Ephrin receptor A2

ER- endoplasmic reticulum

FACS- fluorescence-activated cell sorting

FBS- fetal bovine serum

GBV-A- GB virus A

GBV-B- GB virus B

$GBVB_{(DENV)}$- dengue core pseudotyped with GB virus B envelope proteins

$GBVB_{(HIV)}$- HIV core pseudotyped with GB virus B envelope proteins

$GBVB_{(MLV)}$- MLV core pseudotyped with GB virus B envelope proteins

$GBVB_{(VSV)}$- VSV core pseudotyped with GB virus B envelope proteins

GFP- green fluorescent protein

GLDH- glutamate dehydrogenase

GRP78- 78-kDa glucose-regulated protein

HCV- hepatitis C virus

$HCV_{(HIV)}$- HIV core pseudotyped with HCV envelope proteins

$HCV_{(MLV)}$- MLV core pseudotyped with HCV envelope proteins

$HCV_{(VSV)}$- VSV core pseudotyped with HCV envelope proteins

HEK- human embryonic kidney cells

HEPES- N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

HI- hemagglutination inhibition

HIV- human immunodeficiency virus

HLA- human leukocyte antigen

HSC70- heat shock cognate protein 70

HSP70- 70 kilodalton heat shock protein

HSP90- Heat shock protein 90

HVR1- hypervariable region 1

Huh7/7.5- human hepatoma-derived cells 7/7.5

HVR1- hypervariable region 1

ICTV- International Committee on Taxonomy of Viruses

IFI16- Interferon-gamma induced protein 16

IFN- interferon

Ig- immunoglobulin

IL- interleukin

IRES- internal ribosome entry site

JAK/STAT- Janus kinase/signal transducer and activator of transcription protein

JEV- Japanese encephalitis virus

$JEV_{(DENV)}$- dengue core pseudotyped with JEV envelope proteins

$JEV_{(HIV)}$- HIV core pseudotyped with JEV envelope proteins

JEV_{NAK}- Nakayama JEV strain

$JEV_{(VSV)}$- VSV core pseudotyped with JEV envelope proteins

JFH1- Japanese Fulminant Hepatitis HCV genotype 2a strain

Kb- kilobases

kDa- kilodaltons

KO- knock out

LB- Luria-Bertani broth

LDLR- Low-density lipoprotein receptor

LR- laminin receptor

LSECtin- liver and lymph node sinusoidal endothelial cell C-type lectin

Luc- luciferase

LVP- lipoviral particle

M- membrane protein (JEV)

MAVs- Mitochondrial antiviral-signaling protein

MHC- major histocompatibility complex

miRNA- microRNA

miR-122- microRNA-122

MLV- murine leukemia virus

MOI- multiplicity of infection

mosGCTL- mosquito galactose-specific C-type lectin

MR- mannose receptor

mRNA- messenger

MW- membranous webs

NAb- neutralizing antibody

NanoLuc- NanoLuc luciferase

NIBSC- National Institute for Biological Standards and Control

NIID- National Institute of Infectious Diseases

NK- natural killer cells

NPC1L1- Niemann-Pick C1-Like 1

NPHV- Non-primate hepacivirus

NrHV- Norway rat hepacivirus

NS- non-structural

NTR- nontranslated region

OCLN- occludin

PAMPs- pathogen-associated molecular patterns

PBS- phosphate-buffered saline

PCR- Polymerase Chain Reaction

PEG-IFN- pegylated interferon

PEI- polyethylenimine

prM- pre-membrane protein (JEV)

PRNT- plaque reduction neutralization assay

PV- pseudotyped virus

PVNA- pseudotyped virus neutralization assay

RAV- Rous associated virus

RCF- Relative centrifugal field

RdRp- RNA-dependent RNA polymerase

RIG-I- retinoic acid-inducible gene-I

RLR- retinoic acid-inducible gene-I-like receptor

RLU- relative light units

RNA- Ribonucleic acid

RSV- Rous sarcoma virus

rVSV- recombinant Vesicular stomatitis virus

SOC- Super Optimal broth with Catabolite repression

SR-B1- Scavenger Receptor Class B type 1

TAE- Tris-acetate-EDTA

TBK1- TANK-binding kinase 1

TE- Tris-EDTA

Th1- Type 1 T helper

TLR3- Toll like receptor 3

TNF- tumor necrosis factor

TRIF- Toll/IL-1R domain-containing adaptor-inducing IFN-β

TRIS- trisaminomethane

VEP- viral envelope protein

VSV- vesicular stomatitis virus

$VSV_{(HIV)}$- HIV core pseudotyped with VSV envelope proteins

$VSV_{(MLV)}$- MLV core pseudotyped with VSV envelope proteins

VSV-ΔG- vesicular stomatitis virus without the gene for the glycoprotein-G

WNV- West Nile virus

YFV- yellow fever virus

ZIKV- zika virus

Chapter 1:
INTRODUCTION

1. Introduction

This thesis focuses on pseudotyping two viruses in the *Flaviviridae* family, GB Virus B (GBV-B) and Japanese encephalitis virus (JEV) and some of the downstream uses of the GBV-B pseudotype. GBV-B is generally studied as a model virus for hepatitis C virus (HCV), a human pathogen. Therefore, this introduction covers Flaviviridae and its members HCV, GBV-B and JEV.

1.1 Flaviviridae

Flaviviridae is a diverse viral family of important animal and human pathogens. Viruses in *Flaviviridae* are globally distributed and with significant morbidity and mortality. Prominent members of the family such as yellow fever virus (YFV), West Nile virus (WNV), dengue virus (DENV), Japanese Encephalitis virus (JEV), Zika virus (ZIKV), and hepatitis C virus (HCV) are major public health concerns, with epidemic potential, and/or associated with significant socioeconomic burden. Animal viruses such as classical swine fever virus and bovine viral diarrhoea virus cause large economical losses and are a significant concern for the livestock industry. Flaviviridae contains 4 genera: *Hepacivirus, Flavivirus, Pestivirus and Pegivirus* (Figure 1.1.) [1,2]

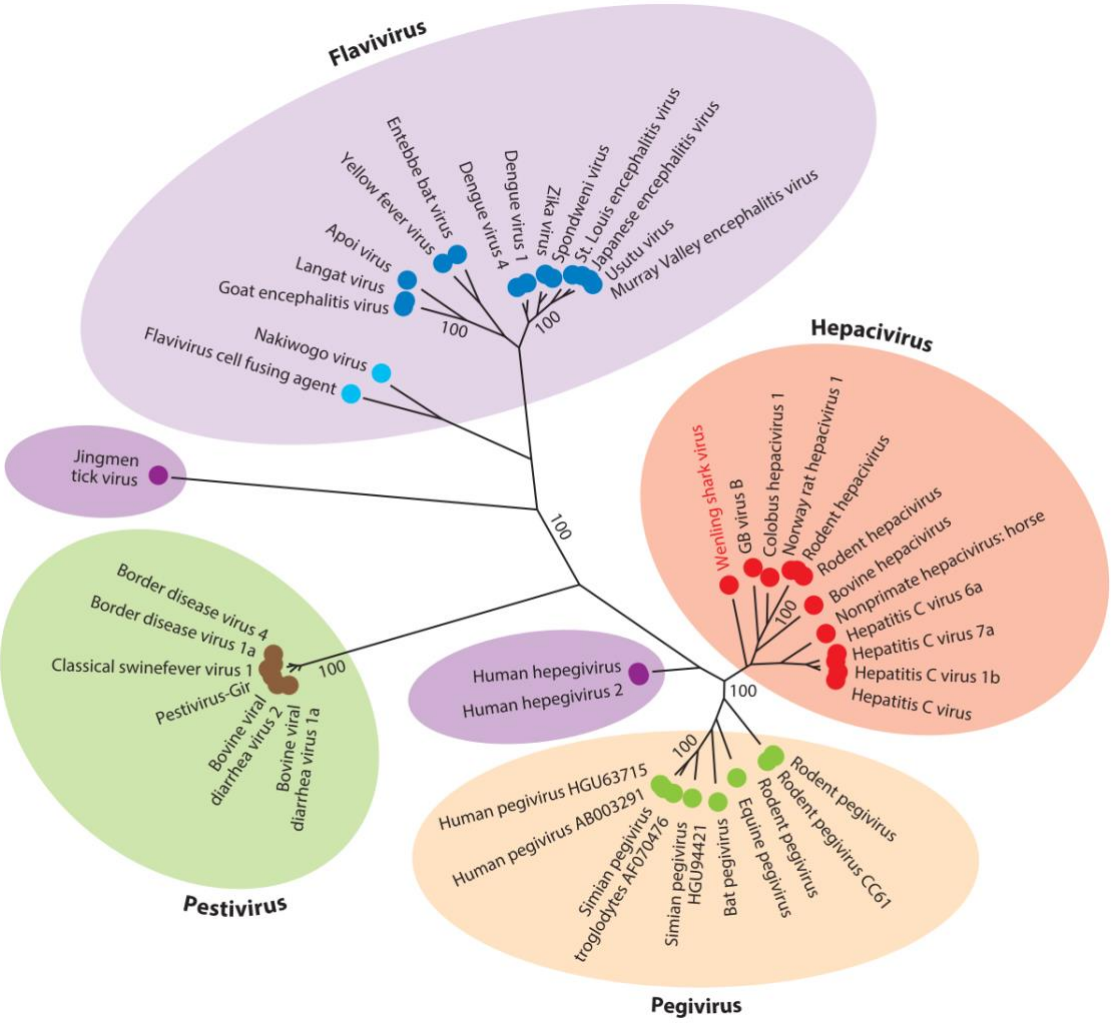
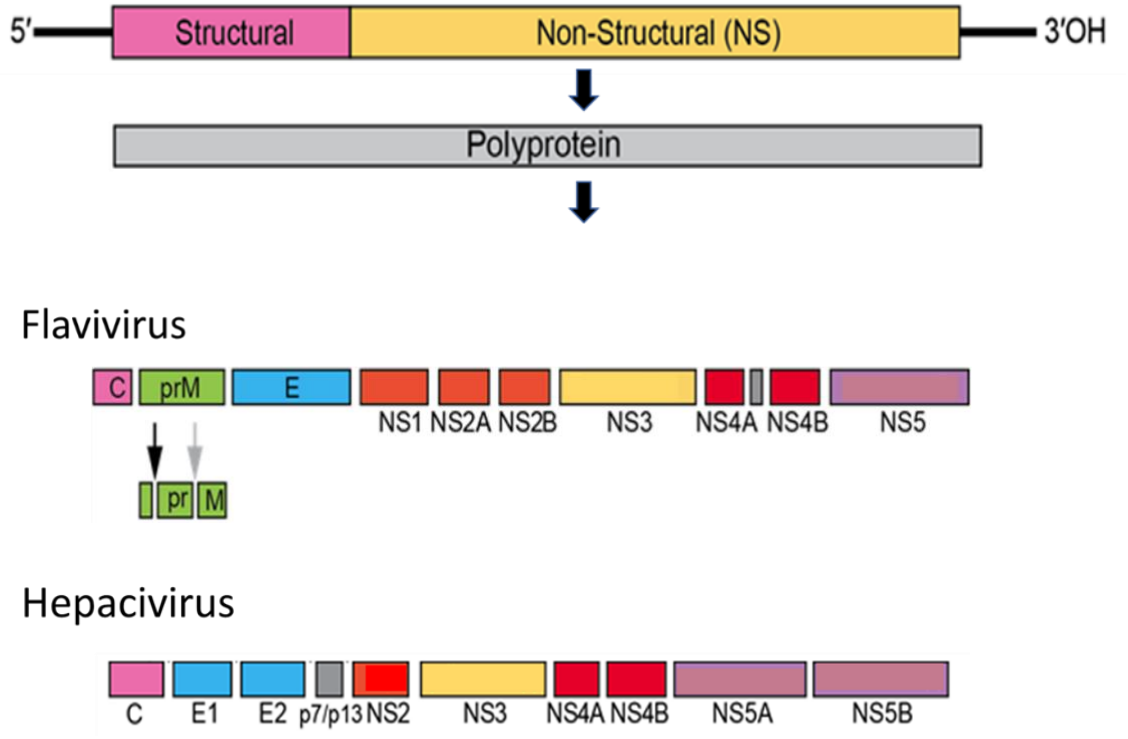


Figure 1.1. Flaviviridae is classified into 4 genera

Phylogenetic tree based on the analysis of the helicase gene of representative members of the 4 Flaviviridae genera: Flavivirus, Hepacivirus, Pestivirus, Pegivirus. The evolutionary history was inferred using the maximum likelihood method based on the Le and Gascuel 2008 model. The percentage of trees in which the associated taxa clustered together is shown next to each branch. The tree is drawn to scale with branch lengths measured in number of substitutions per site. Figure adapted from Hartlage et al [3].

1.1.1 Structure

Members of *Flaviviridae* are RNA viruses with genomes of 9,000 to 13,000 bases. A single copy of the genome is surrounded by core/capsid protein, which is enveloped by lipid bilayer a with two (*Hepacivirus, Flavivirus, and Pegivirus*) or three (*Pestivirus*) envelope proteins. The genome is a single stranded positive sense RNA with a single open reading frame flanked by 5′ and 3′ non-translated regions. The open reading frame encodes one polyprotein, with the structural proteins at the N-terminus and the non-structural proteins at the C-terminus, with the exception being the *Pestiviruses* which have one non-structural protein preceding the structural proteins (Figure 1.2) [4]. Figure 1.2 shows how the polyproteins are processed. None of the non-structural proteins are packed into the virions but as described in the next sections they have integral roles in replication, assembly, and immune evasion.



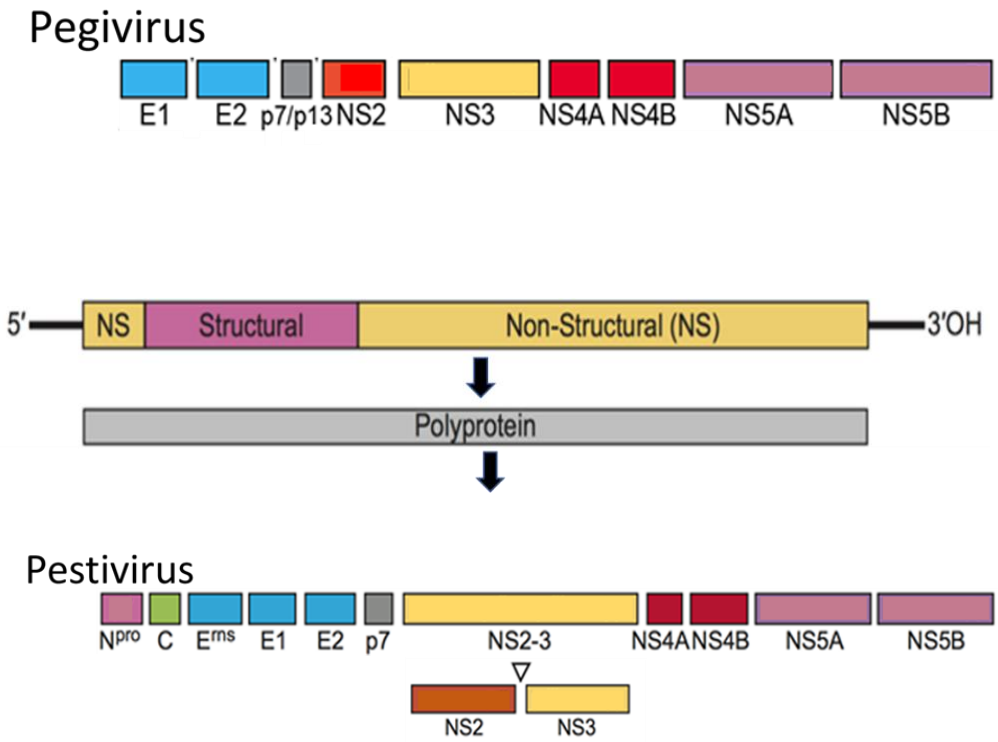


Figure 1.2. Flaviviridae genome structure and processing of the polyprotein

Schematic of Flaviviridae genome organization in reference to structural and non-structural proteins and processing of the polyprotein into the proteins of their respective genus. Figures adapted from ICTV website [5–8].

1.1.2 Lifecycle

Flaviviridae members share a similar replication scheme. They enter the cell via receptor mediated endocytosis. Low pH of the endosome triggers fusion of the viral envelope and endosomal membrane, releasing viral RNA into the cytosol where it serves as the messenger RNA (mRNA) for translation. Translation of the genome polyprotein occurs via cap dependent (*Flavivirus*) or internal ribosome entry site (IRES)-mediated translation [9–11]. Cellular and viral proteases process the polyprotein both co- and post-translationally into 9–12 mature proteins [12]. *Flavivirus* and *hepacivirus* non-structural proteins remodel the endoplasmic reticulum (ER) to create double membrane vesicles that house replication

departments where RNA amplification occurs. The newly synthesized RNA leaves the replication departments and associates with capsid protein for encapsidation [13]. Envelope proteins accumulate in the ER membrane were the nucleocapsids bud through to acquire its lipid envelope and glycoproteins. Assembled virions in the ER use cellular machinery to move along the secretory pathway to exit the cell through exocytosis [14–16].

1.2 Hepatitis C Virus

Hepatitis C virus (HCV) is a hepatotropic virus that infects humans and is a major cause of liver cancer worldwide. Prevalence varies across the globe with some regions, such as Egypt, having more than 5% of the adult population seropositive; global prevalence is estimated at 1.0% [17]. HCV is a blood borne virus that is mainly transmitted through contact with an infected person's blood or bodily fluids, such as use of intravenous drug use equipment, reuse of medical equipment, and blood transfusions. Less commonly, HCV can also be transmitted sexually and from mother to baby [18]. Approximately 80% of new HCV infections are asymptomatic. Those that do exhibit acute symptoms may have fever, fatigue, abdominal pain, dark urine, and jaundice. In the acute stage of infection, approximately 30% of patients are able to clear the virus without treatment in under 6 months. In the remaining 70% of infections, the body is unable to clear the virus and chronic HCV infection develops. Chronic infection can lead to persistent hepatitis, which in turn can lead to cirrhosis and hepatocellular cancer. HCV infections are found globally in all regions with 58 million people chronically infected and 299,000 deaths per year resulting from HCV related liver damage [19]. To combat viral hepatitis, the World Health Organization has set goals to

reduce new viral hepatitis infections by 90%, including HCV, and reduce deaths due to viral hepatitis by 65% by 2030 [20].

1.2.1 Discovery

In 1975, Feinstone and colleagues [21], discovered that following blood transfusions, 22 patients developed hepatitis but neither hepatitis A nor hepatitis B virus was the causative agent- thus terming the illness non-A, non-B hepatitis. This non-A, non-B hepatitis exhibited different clinical characteristics than hepatitis A or B. Hepatitis A is rarely transmitted by blood transfusion. The acute stage of infection generally induced a milder disease than hepatitis B did, but often had similar clinical chronic infections as hepatitis B. However, chronic disease was seen more frequently in non-A, non-B hepatitis than with hepatitis B [22]. It was then shown that the sera from patients with non-A, non-B hepatitis contained an infectious agent and could be used to infect chimpanzees and induce chronic hepatitis [23]. A small positive sense RNA virus isolated from the serum of an experimentally infected chimpanzee was determined to be the causative agent of non-A, non-B hepatitis which was then termed hepatitis C virus (HCV) [24]. The subsequent development of an assay to screen for HCV antibodies showed that HCV was the major causative agent of both post transfusion and community acquired non-A, non-B hepatitis around the world [25].

1.2.2 Structure

The viral RNA genome, which is 9.6 kb, encodes 3 structural proteins at the N-terminus and 7 non-structural proteins at the C-terminus as seen in Figure 1.2. A single copy of the genome is surrounded by core protein, which is enveloped by lipid bilayer in which the viral envelope proteins, E1 and E2, are displayed [26]. HCV has 8 genotypes, in which genome sequence diversity between any 2

genotypes is more than 30%. Most genotypes are further divided into subtypes that differ from each other by up to 25% [27]. HCV particles range in size from 40 to 100 nm and are structurally irregular [28]. They have characteristically low buoyant density due to their interaction with lipids and lipoproteins (Figure 1.3) to form lipoviral particles (LVP). It has not yet been elucidated whether the structure of the LVPs is comprised of virions transiently associating with lipoproteins or create a hybrid wherein the virions are contained within the lipoproteins and share a membrane [29].

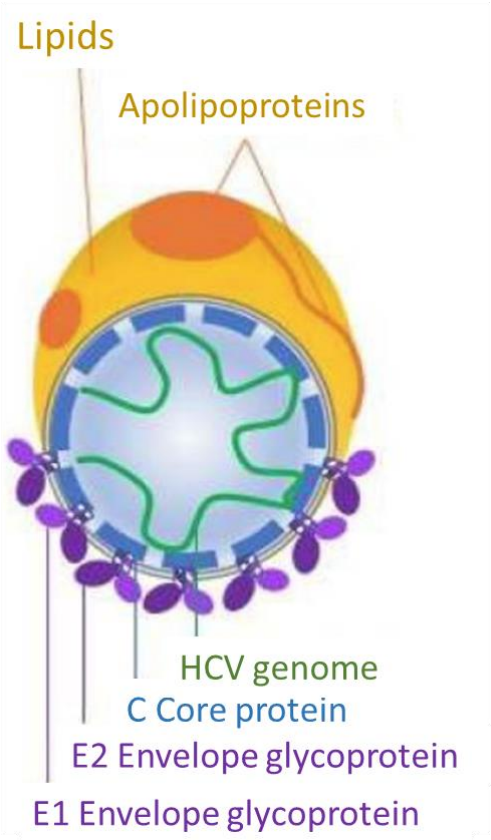


Figure 1.3. Model of HCV lipoviral particle

HCV core protein encapsidates and associates with the positive strand RNA genome. The core is enveloped by a membrane derived from the ER in which the E1 and E2 glycoproteins are embedded. The viral particles are tightly associated with lipids and apolipoproteins, which accounts for its characteristically low density. Figure adapted from Alazard-Dany et al [26].

1.2.3 Cell Entry

HCV has evolved a complex mechanism for cell entry, involving at least four host factors, and it is still not fully understood. The virion initially attaches to hepatocytes at the basolateral surface via heparan sulfate proteoglycans. E1E2 glycoproteins then engage SR-B1 and CD81 (Figure 1.4) [31,32]. CD81 engagement is thought to initiate a signalling cascade that leads to intracellular actin remodelling, driving the translocation of the CD81-tethered virus toward the tight junction. HCV then interacts with claudin-1 (CLDN1) during transit to or at the tight junction where the receptor complex also binds occludin (OCLN) [33,34]. Many strains of HCV are able to utilize CLDN6 and CLDN9 in addition to CLDN1 which may have evolved as an escape of CLDN1 therapeutic strategies, while others such as J6 have a narrow tropism, only using CLDN1 [35,36]. Finally, the virion is internalized via clathrin-mediated endocytosis and the low pH environment of the early endosome promotes E1E2-catalysed fusion between the viral and endosomal lipid bilayers [37]. Other entry factors such as the low-density lipoprotein receptor (LDLR), epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and Niemann-Pick C1-like 1 (NPC1L1) have been identified as co-factors for entry [38–41]. However, the exact mechanisms by which these factors contribute to HCV entry are yet to be elucidated.

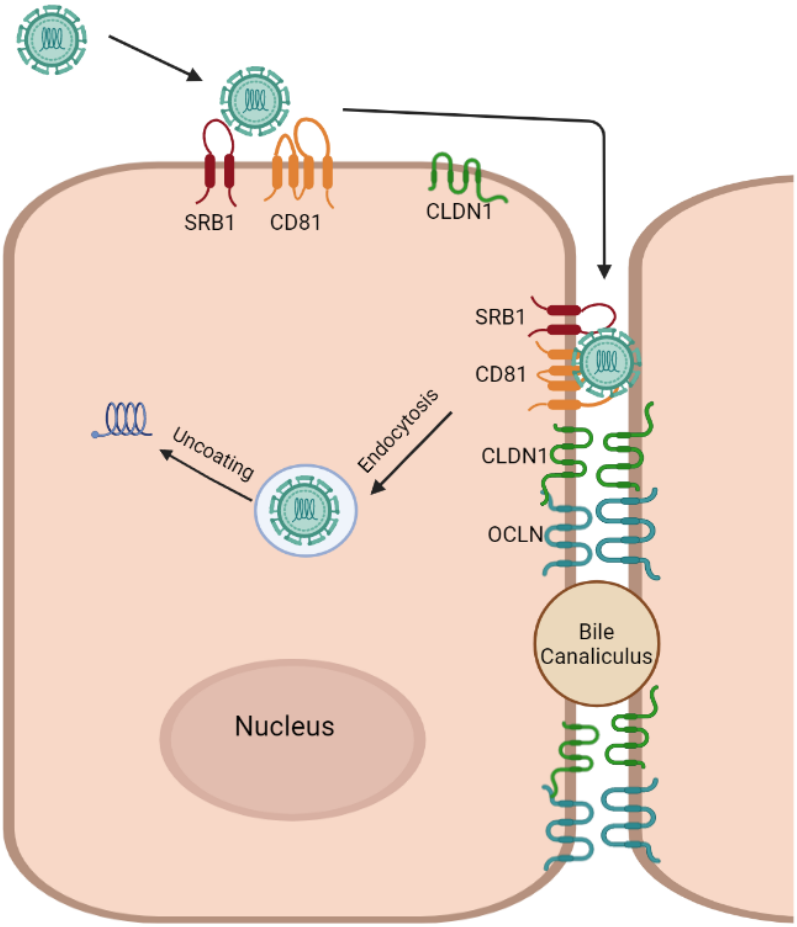


Figure 1.4. HCV cell entry

The virus engages SRB1 and CD81 at the basolateral surface of the cell. CD81 engagement drives translocation of the CD81-tethered virus toward the tight junction. HCV then acquires CLDN1 during transit to or at the tight junction where it acquires OCLN, ultimately culminating in internalization via clathrin-dependent endocytosis and release of the genome into the cytosol. Image produced in Biorender.

1.2.4 Replication, Assembly, and Egress

The 5' NTR has an IRES for direct binding of host ribosomes for translation of the viral genomic RNA by a cap independent mechanism [26]. The 5′ NTR also harbours two binding sites for microRNA-122 (miR-122), a liver specific microRNA. Unlike most miRNA that repress gene expression, miR-122 is a positive regulator that enhances replication (Figure 1.5) and protects viral RNA from degradation [42]. The polyprotein is translated at the endoplasmic reticulum (ER) and processed by cellular and viral proteases to produce 10 viral proteins: 3 structural proteins (core, E1, E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A NS4B, NS5A, NS5B). NS2 and NS3 are both proteases involved in processing the viral polyprotein. NS4A is a protease co-factor that anchors with NS3 to the ER membrane. After cleavage to form the mature proteins, a replicase complex is formed with NS3, 4A, 4B, 5A, and 5B. NS4B and NS5A have major roles in biogenesis of 'membranous webs' derived from ER membrane that serve as the location for replication complex, as seen in Figure 1.5 [43,44]. NS3 has a helicase domain important in RNA replication and NS5B is the RNA dependent RNA polymerase (RdRp) responsible for RNA replication. After translation, the core proteins are trafficked to cytosolic lipid droplets (cLDs) [45] and E1E2 are anchored in the ER as heterodimers by a retention signal in their transmembrane domains [46,47]. NS5A is involved in delivery of newly synthesised RNA genomes to the core proteins on cLDs [44,48]. NS2 and p7 have a role in recruiting E1E2 heterodimers, and NS3/4A complexes to the sites of assembly. P7/NS2 interacts with NS3/4 to recruit core protein to virus assembly [49]. Once all viral factors needed for assembly are located at the assembly site, the particles acquire their envelope and glycoproteins by budding from the ER. Newly formed particles traffic through the secretory pathway, and in the Golgi apparatus post

translational glycosylation of the glycoproteins take place [50]. In addition, during this transit the particles interact with lipoproteins, such as apoE, to obtain their characteristic low buoyant density [29]. Non-structural protein p7 acts as a viroporin and protects virions as it moves through the pathway by equilibrating pH gradients [51].

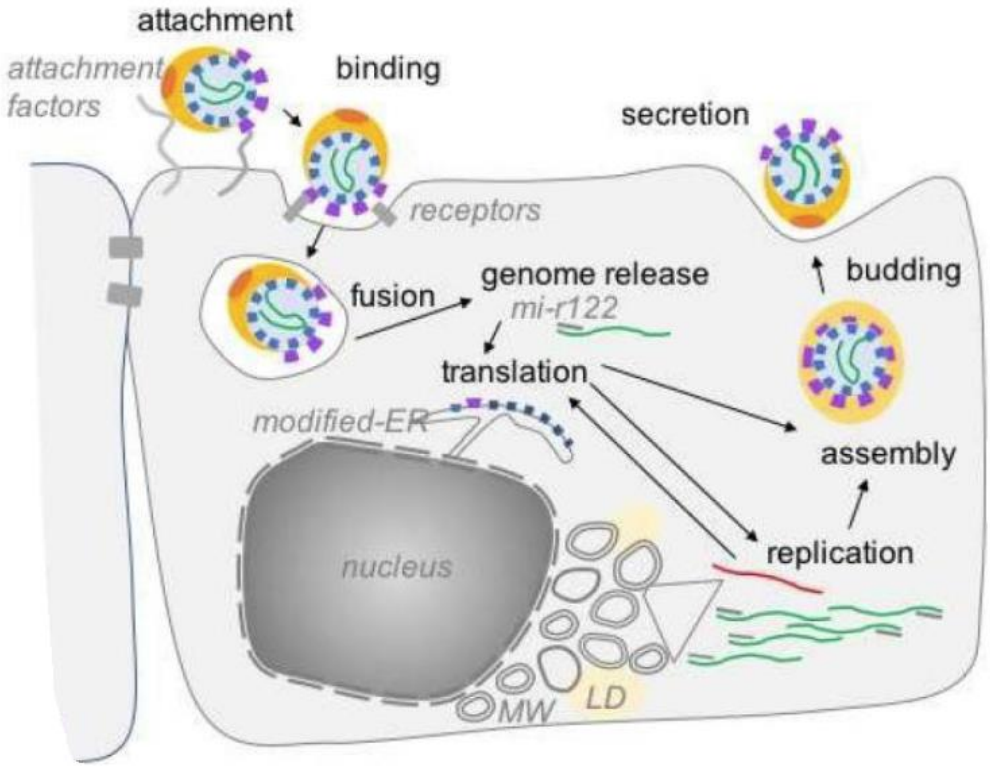


Figure 1.5. HCV life cycle

After attachment, binding, and fusion, the viral RNA is released and binds to miR-122, then the genome (green) is translated at the ER. NS proteins form the membranous webs (MW), where genome replication takes place (intermediate negative RNA strand in red) and viral components interact with lipid droplets (LD). After assembly the particles move through the secretory pathway where they interact with lipoproteins before being released by exocytosis. Figure adapted from Alazard-Dany et al [30].

1.2.5 Immune Responses

HCV infection triggers both the innate and adaptive immune response. Understanding the responses that are successful in clearing the virus and the mechanisms by which viral components hamper that immune response are key to elucidating the mechanisms of persistence and developing an effective vaccine. Below is a brief overview of the immune responses elicited by HCV infection.

1.2.5.1 Innate

One to two weeks after infection with HCV, viral RNA can be detected in the blood and an immune response is triggered [52]. The innate immune response, otherwise, known as the non-specific immune response, is the first line of defence after infection. In an infected cell, Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) sense the invading virus and induce a type I and III interferon (IFN) response to inhibit viral replication and activate natural killer (NK) cells [53,54]. HCV RNA also activates NK cells through type I IFN produced by plasmacytoid dendritic cells (DC). NK cells produce IFN-γ and tumor necrosis factor (TNF)-α, which inhibit viral replication as well as induce DC maturation. Mature DC cells secrete interleukin (IL)-12 and IL-15 that further activate NK cells. NK cells destroy HCV infected hepatocytes by releasing cytolytic enzymes perforin/granzymes, which can cause damage to host tissues, initiating hepatitis [55,56].

1.2.5.2 Adaptive

1.2.5.2.1 Cellular

Activated DC cells initiate the adaptive immune response by stimulating the differentiation of CD4+ and CD8+ T cells into Th1 and cytotoxic T cells, respectively [54]. CD4+ T cells inhibit HCV replication and further activate DCs by secreting IFN-γ and TNF-α. Additionally, IFN-γ induces differentiation of B cells to produce antibodies [56]. Th1 cells prime CD8+ T cells through IL-2 secretion. CD8+ T cells recognize infected hepatocytes by antigens presented on human leukocyte antigen (HLA) class 1 and respond with both cytolytic and noncytolytic functions [55]. Secretion of IFN-γ and TNF-α inhibits viral replication without killing infected cells [57]. Cytotoxic T cells secrete perforin/granzymes to kill infected cells [58,59]. Viral clearance in the acute phase is associated with a strong CD4+ and CD8+ T cell response. CD8+ T cells without a strong CD4+ response leads to T cell exhaustion in the presence of high viral loads; thus, both are required for the effective clearance [52,60–63].

1.2.5.2.2 Humoral

Antibodies appear approximately 6–12 weeks after infection [56,62,64]. Antibodies that bind the virus and prevent cell entry, known as neutralizing antibodies (NAb), are directed at HCV E1 and E2 envelope proteins. Appearance of NAb can coincide with seroconversion or be delayed by many months [64,65]. Early appearance of NAbs in acute infections is associated with clearance of the virus, and delayed appearance associated with progression to chronic infection [66–69]. Pestka et al. [68] studied a cohort of patients from a single source HCV outbreak and demonstrated that patients that cleared the virus in the acute phase were more likely to have rapidly developed neutralizing antibodies. Patients who

progressed to chronic infection were associated with low or absent NAb titres in the acute phase despite subsequent onset of NAb in the chronic phase of infection. There is also some evidence that treatment with NAbs can protect from infection in animal models [70–72]. However, the presence of NAbs are not always required for clearance of the virus [52,67,68,73]. As particularly demonstrated by spontaneous clearance in agammaglobulinemic patients [74]. Therefore, it is likely that clearance of the virus in the acute phase is mediated by both humoral and cellular immune responses.

1.2.6 Establishment of chronic infection

Approximately 70% of HCV infections cannot be cleared by patient immune defences and progress to the chronic form of disease. This suggests that the virus can efficiently evade or suppress the host immune defences. HCV achieves this through a number of strategies including suppression of immune responses through viral protein interactions with immune factors, induction of immunosuppressive factors, high genetic variability, epitope shielding, and cell to cell transmission.

Structural and non-structural proteins interfere with host immune responses during both the innate and adaptive responses. The NS3/4A protease impairs IFN production by cleaving mitochondrial antiviral-signaling protein (MAVS) and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF), which are vital for TLR3 and RLR pathways [75,76]. NS4B also inhibits the TLR3 pathway by degradation of TRIF [77]. NS3/4A also impairs TLR3 pathway by binding to TANK-binding kinase 1 (TBK1), inhibiting a transcription promoter for INF production. Additionally, NS3/4A impairs the E3 ligase Riplet, which is required for the activation of the RIG-I pathway [78]. Furthermore, NS3/4A can inhibit HLA

class I trafficking to the cell surface in infected cells, resulting in reduced recognition by HCV specific CD8+ T cells [79]. NS5A inhibits INF production by impairing the TRL3 and RLR pathways by inhibiting gene transcription and binding to viral RNA to shield it from recognition [80,81]. Core protein modulates the Janus kinase/signal transducer and activator of transcription protein (JAK/STAT) pathway which impairs the expression of interferon stimulated genes [82]. HCV core proteins also interact with DC cells to supress production of IL-2 and IL-12, which in turn supresses CD8+ T cell proliferation and impairs differentiation of CD4+ T cells to Th1 cells, respectively [83]. Core protein also down regulates major histocompatibility complex (MHC) on DC cells inhibiting their ability to prime HCV specific CD4+ and CD8+ T cells resulting in the increased production of IL-10, an immunosuppressive cytokine [84]. Moreover, core protein can block NK cell mediated cytolysis by upregulation of MHC class I in infected cells [85,86]. P7 is also indicated in having a role in immune evasion by inhibiting IFI16-16, an interferon stimulated gene [87]. Due to the many strategies HCV employs to evade first the innate and then the adaptive immune response, the majority of infections reach the chronic phase. The consistently high viral load in chronic infections leads to T cell exhaustion; therefore, resulting in reduced cytotoxicity, impaired proliferation, reduced INF production, and apoptosis [56].

Chronic HCV infections are also associated with an increase in Treg cells [88,89]. These cells as well as immunosuppressive cytokines such as IL-10 suppress HCV specific T cell response, hampering cytotoxic ability. However, this may also play a role in attenuating tissue damage in the liver resulting from prolonged immune responses [56,62].

Lack of proofreading by the RNA dependant RNA polymerase (RdRp) leads to high genetic variation and emergence of quasispecies in infected individuals. This continual evolution of the virus is effective in escaping the adaptive immune response. Mutations in regions targeted by HCV CD4+ and CD8+ specific T cells leads to escape mutants by reduced T cell receptor recognition, which in return further contributes to T cell failure [90,91]. The hypervariable region (HVR1) on E2, a highly immunogenic site, is a flexible region under little functional constraint and can tolerate many mutations without affecting functionality thus able to escape NAb [92]. Pressure from the immune responses is the driving force behind the quickly mutating envelope proteins, resulting in chronic infections where the immune response is lagging behind the quickly changing virus [93,94]. Studies have shown that NAbs fail to neutralize the current dominant circulating virus in patients but are capable of neutralizing virus from earlier time points [95].

HCV also escapes NAbs by a number of additional strategies. Another function of the HVR1 is to act as an immunogenic decoy and shield more conserved regions of E2 responsible for CD81 interaction, which are key epitopes for neutralization [96,97]. HCV E1E2 are also highly glycosylated proteins; these glycans limit the availability of the proteins to NAbs by masking epitopes [98]. Similarly, epitopes can be masked by the lipoproteins that HCV is closely associated with in the LVP [99]. Furthermore, it has been suggested that HCV may evade neutralization by induction of non-neutralizing Ab that bind E2 and interfere with Ab that bind to neutralizing epitopes [100,101]. However, other studies have not observed this interference [102]. Lastly, HCV is capable of cell to cell transmission, thus avoiding exposure to the extracellular humoral responses [103,104].

For clearance in the acute phase of infection a strong, rapid, multi-faceted immune response is needed. However, as described above, HCV has many strategies to suppress and evade both the innate and adaptive immune responses allowing the virus to persist into chronic infection. Approximately 1 in 3 people who sustain a chronic infection are likely to develop liver cirrhosis, which in turn increases risk of hepatocellular carcinoma [105].

1.2.7 Treatments

Before the recently developed directly acting antivirals (DAA), treatment for HCV infections was limited to pegylated interferon (PEG-IFN), which are interferon molecules conjugated with a polyethyleneglycol molecule that increases half-life, and ribavirin, a synthetic guanosine analogue which is incorporated into the viral genome thus inhibiting replication [106]. These treatments were only effective in less than half of patients. Moreover, these treatments were associated with significant side effects, such as, flu-like symptoms, hematologic abnormalities, and depression [107], which often led to discontinuation of treatment. Consequently, more effective and tolerable antivirals were developed. Discovery of essential steps in the HCV life cycle elucidated antiviral targets for drug development. DAA, as the name implies, target specific viral proteins vital for replication. They are classified based on the protein they target, which includes: NS3/4A, NS5B, and NS5A inhibitors [108]. DAAs have been shown to be safer and more effective than previous treatments for HCV. This advancement in antivirals has been a great leap forward in the treatment of HCV, with cure rates above 90% [108]. However, they are not without their limitations. There has been an emergence of DAA resistant HCV variants, which has implications for reducing transmission and efficacy of the treatment [109]. Moreover, the high cost of these

antivirals makes them unaffordable in resource-limited areas where HCV prevalence is high. Furthermore, clearance of HCV with these therapies also does not provide protection against reinfection [110,111]. As such, development of an effective vaccine that provides protection through sterilizing immunity will be key to eradication or significant reduction of infections.

1.2.8 Vaccines

Currently there are no approved HCV vaccines, prophylactic or therapeutic. However, research is still ongoing in this area, with a number of candidates having progressed to clinical trials. However, none of the candidates have progressed to phase III [112,113]. Many of the candidates have encountered issues such as eliciting weak or narrow immunological responses [114]. Approximately 80% of people who spontaneously clear HCV in the acute phase, attain viral clearance again upon reinfection; this is significantly higher than the 30% clearance rate in primary infections [115]. The subsequent infections have shorter infection periods and lower RNA titres [116], suggesting that the adaptive immune response is responsible for clearance. This makes a strong case that exposure to viral antigens through vaccination can lead to immunological memory that can protect from chronic infection.

The exact mechanisms by which the protection is afforded have not been elucidated. More research into this area will be vital to determine the precise immune response that correlates with protection and what viral antigens elicit these responses. As seen above, both cellular and humoral responses are likely to be key players in clearance of the virus in the acute phase. Therefore, a vaccine that can elicit strong humoral and cellular reposes will be key. However, one of the main challenges in developing a vaccine for HCV include extremely

high genetic diversity, due in part to the error prone RdRp [117]. Consequently, humoral and cellular responses are often genotype or even strain specific and do not confer cross reactive immune reposes to heterologous isolates [118]. Thus, creating a vaccine that confers broad protection to multiple if not all genotypes is a main challenge. Moreover, as detailed above, HCV employs many strategies to evade the immune response. Further emphasizing the need to fully understand the mechanisms of successful viral clearance to rationally design a vaccine that elicit a similar response.

1.2.9 Tools to study HCV

There are a number of tools that have been employed to characterize HCV infection and the resulting immune response to elucidate mechanisms of persistence and inform vaccine design. Below is an overview of tools available to study HCV.

1.2.9.1 In vitro

Initially, sub genomic HCV replicons were used to study the replication cycle of the virus *in vitro,* as HCV could not be propagated in cell culture [119]. Viral-like particles carrying the core, E1, and E2 proteins were also developed to study viral entry and these could induce a humoral response to HCV [120]. Neither of these systems produced infectious particles. Generation of HCV pseudotyped retroviral particles, bearing the E1 and E2 glycoproteins of HCV, marked a significant leap forward in viral entry investigation. HCV pseudotypes enabled the identification of receptors, host range, and screening of entry inhibitors [121–124]. As pseudotypes only contain the glycoproteins of the virus, they cannot be used to investigate any steps downstream of entry in the viral life cycle. HCV isolate JFH1 was discovered to be able to replicate *in vitro* and produce infectious particles

after transfection of the full-length genome [125,126]; this isolate has been invaluable to the study of the whole HCV life cycle and identification of antiviral targets.

1.2.9.2 In vivo

While *in vitro* systems are useful to study many aspects of HCV life cycle, they are artificial conditions and do not necessarily mimic what happens *in vivo*. Animal models are needed to more closely recapitulate the complex host responses to infection and preclinical assessments of vaccine candidates [127]. HCV has a narrow host range, with only humans, chimpanzees, and tree shrews known to be susceptible to infection. Chimpanzees have been an important model that has led not only to the discovery and characterization of HCV, but to the assessment of many antiviral therapies [128,129]. While chimpanzees are considered the best model for HCV research, ethical concerns have led to the ban of chimpanzees for research in most countries; the European Union has banned their use since 2010 [130,131]. Tree shrews have been shown to be susceptible to HCV with some studies showing more than 80% of the test animals becoming infected with 20% leading to chronic infections [132]. This is a promising model as there are fewer ethical concerns with tree shews than with chimpanzees; but they are not widely available, they are genetically heterogenous and difficult to genetically manipulate, which can be challenging for HCV pathogenesis analysis [127,133].

In addition to chimpanzees and tree shrews, zebra fish and rodents have been explored as *in vivo* models. The zebra fish has recently been developed as a model for HCV genomic replication. The HCV replicon, delivered via microinjection into larvae, proliferates in the liver and a similar pathology to

human HCV infection is observed. This is a small and easy to use model that is promising for HCV replication studies and potentially in identifying new antiviral targets [134]. Rodents have been widely used as *in vivo* models for HCV. Transgenic mice expressing viral proteins have been used to study viral protein-host cell interaction [135,136]. Immunocompromised mice that have been transplanted with human hepatocytes to create chimeric livers have been utilized to study HCV related pathogenesis in liver cells as well as evaluate potential drugs [137–150]. The necessity of immunosuppressing the mice to prevent rejection of the human cells has the drawback of not being able to determine the immune response to infection, therefore, they are not usable for vaccine studies [133]. To overcome this, a tolerized rat model was developed by injecting Huh7 cells *in utero* into fetal rats so that they become tolerant of these human cells. This negated the need for immunosuppression [151]. The drawbacks of this model are low HCV viremia even though replication and hepatitis are present, and a mismatch of human MHC antigens on the infected liver cells and the rat immune system, so there will be no recognition of antigens by the rat immune cells [133,151,152]. To that end, mouse models that have been transplanted with human hepatocytes and human hematopoietic stem cells to induce a human immune system are promising as they support HCV infection, have a human immune response, and recapitulate clinical signs such as liver fibrosis and tumorigenesis [153–155]. Drawbacks of these include low human chimerism rates and the suboptimal human immune system that is achieved in transplanted mice. While they are useful in studying aspects of HCV immunopathogenesis, they are unlikely to be used for vaccine development as they lack of functional B-lymphocytes antibody production [127,133]. Adaptation of an HCV virus to use mouse CD81 as a receptor has been achieved but uptake of the virus is low and

persistent infection was not observed [133,156]. Finally, immunocompetent mice have been humanized to express human HCV receptors. Chen et al., produced a transgenic mouse model expressing human CD81 and OCLN that is permissive to HCV infection, sustains infection for more than 12 months, and results in the development of liver disease [157]. This is a promising model for studying persistent HCV infections and possible vaccine candidates.

Another approach to *in vivo* models for HCV is the use of HCV homologs in susceptible animals; this allows the study of pathogenesis and immune responses in animals with an immunocompetent system. HCV homologs used for animal models include non-primate hepacivirus (NPHV), Norway rat hepacivirus (NrHV) and GB virus B (GBV-B). NPHV was first discovered in canines, but horses were later shown to be the natural host [158,159]. NPHV in horses is a valuable immunocompetent model for HCV as it demonstrates persistent infection in the liver, albeit at a lower rate of less than 40%, and elicits a similar immune response to that in humans [160]. However, due to the size of these animals, the costs associated with care and housing are a major drawback [133]. NrHV, which was discovered during a metagenomic analysis of feral Norway rats (*Rattus norvegicus*) in New York city, was shown to be a hepatotropic virus [161]. Mouse models with this rodent hepacivirus have been developed, but rats have been shown to be the best model [162,163]. In the Holtzman rat, many characteristics of hepatic inflammation seen in human HCV infection are exhibited, and as this is an immunocompetent model it is suitable for immunity studies in addition to pathogenesis [163]. GBV-B, the most extensively characterized model virus, is reviewed in the next section.

1.3 GB Virus B

1.3.1 Discovery

In 1966, GBV-B, as well as GBV-A, were isolated from tamarin monkeys with acute hepatitis after being experimentally inoculated with the serum of a surgeon with the initials G.B. who was experiencing acute hepatitis [164]. Initially referred to as GB agent, Simons and colleagues [165] discovered the tamarin serum contained two flavivirus-like genomes: GBV-A and GBV-B, with GBV-B being the causative agent of hepatitis in the tamarins. However, GBV-B cannot infect chimpanzees [166,167] and it has never been isolated from humans, indicating that the virus did not come from the surgeon. It has yet to be isolated from any non-experimentally infected monkeys; its natural host remains an enigma [168]. GBV-A has since been determined to be a tamarin virus and classified as a *Pegivirus* [1,169]. Before the recent discovery of many hepaciviruses [159,170,171], GBV-B was the only known homolog and closely related virus to HCV. Therefore, GBV-B has been used in small new world primates, such as tamarins and marmosets, as a surrogate HCV model [167,172].

In 2011, a novel hepacivirus was found in the nasal swab from a dog with a respiratory illness [159]. Since then many more hepaciviruses have been discovered in a diverse range of species including dogs, horses, bats, monkeys, rodents, cows, ticks and sharks [160,171,173–177]. Since the discovery of these hepaciviruses, we now know that HCV is more closely related to other genus members such as the equine and rodent hepaciviruses than to GBV-B (Figure 1.6). Nevertheless, GBV-B is the most well characterized of the animal hepaciviruses. Studying these related viruses has increased understanding of host range and have given some clues into the origins of HCV [3]. More

characterization of these viruses as well as identification of more novel hepaciviruses will be the key to understanding how they cross species barriers, including into humans. Additionally, further characterization is needed to assess their utility as models for HCV infection, as animal models are needed to closely recapitulate the complex host responses to infection and preclinical assessments of HCV vaccine candidates [127].

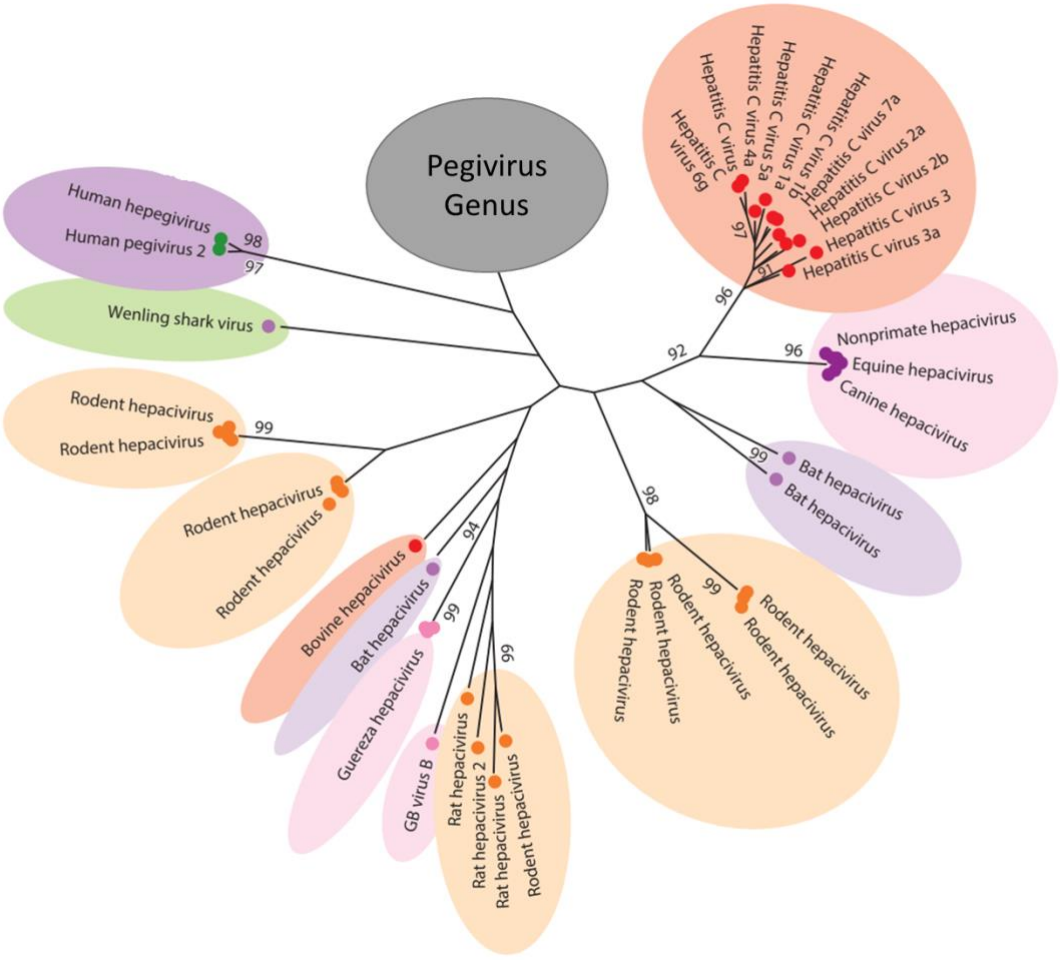


Figure 1.6. Phylogenetic Tree of Hepaciviruses

Phylogenetic tree based on the analysis of the helicase gene of Hepacivirus and Pegivirus. The evolutionary history was inferred using the maximum likelihood method based on the Le and Gascuel 2008 model. The percentage of trees in which the associated taxa clustered together is shown next to each branch. The tree is drawn to scale. Figure adapted from Hartlage et al [3].

1.3.2 Structure and lifecycle

GBV-B and HCV share a similar genome organization as seen in Figure 1.2. They contain a single stranded positive sense RNA genome with a single open reading frame encoding one polyprotein, with the structural proteins (Core, E1, and E2) at the N terminus and the non-structural proteins (p13, NS2, NS3, NS4A, NS4B, NS5A, NS5B) at the C terminus [1]. However, instead of the HCV p7, GBV-B encodes a larger p13 that is also indispensable for replication [178]. The overall homology between HCV and GBV-B at the amino acid level is 25–30% [4]. However, NS2, NS3, and NS5A, and NS5B are shown to have homologous functional roles [179]. The NS3 protease of GBV-B can correctly cleave HCV polyproteins [180]. They both have a type 3 IRES in the 5′ UTR for translation of the viral genomic RNA [1]. Although both utilize an error prone RdRp for replication, it was initially thought that GBV-B developed far fewer mutations limiting variation seen in infected animals [181]. Furthermore, no hypervariable regions have been observed in E2 of GBV-B [181]. However more recent studies of GBV-B into the chronic phase show greater genetic variability than initially thought. Mutations accumulate over the course of infection, with rates of mutation similar to those observed for HCV, suggesting viral evolution is linked to persistence [182,183]. There is a lack of research into GBV-B lifecycle, in large part due to the inability of the virus to be propagated in immortalized cells [184]. However, like HCV, GBV-B core proteins have been shown to be directed to lipid droplets [185], this, coupled with similar genome structure and functional homology between proteins, suggests that GBV-B has a similar life cycle to HCV.

1.3.3 Infection and Pathology

GBV-B, like HCV, is associated with hepatitis and is primarily found in the liver of the infected host [167,186]. GBV-B can infect new world monkeys including multiple tamarin *(Saguinus)* species, common marmoset (*Callithrix jaccus*), and owl monkeys (*Aotus trivirgatus*) [167,187]. Unlike HCV, GBV-B generally only causes an acute infection that is cleared in under 6 months [167,168,188,189]. Although, there have been reported cases of tamarins and marmosets developing persistent viremia, for over 4 years, and liver pathologies like those associated with HCV infection after being inoculated intra-hepatically with GBV-B RNA [182,190–192]. Host factors are likely the be the main determinant of persistent infection, as virus isolated from chronically infected monkeys has not been shown to cause a chronic infection in experimentally infected animals [172,179]. Infection is generally characterized by a rapid rise in viremia within 3 weeks then a plateau before clearance. Serum enzymes alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH), which are biomarkers that indirectly measure liver damage, increase during acute infection and peak immediately before viral clearance [94,186,187,193]. Pathology associated with GBV-B hepatitis include dilated portal tracks due to lymphocyte filtration with spill over into the parenchyma and hepatocyte necrosis [172,190,194]. Tamarins and marmosets have both been shown to develop fibrosis following prolonged hepatitis that mimics HCV disease progression [182,192,193].

1.3.4 Immune responses

Similar to HCV, cellular immunity plays an important role in GBV-B clearance. Appearance of GBV-B specific T cells have been detected in the blood of infected

marmosets at time points that coincide with clearance of viremia in the acute phase. Additionally, detection of INF-γ coincided with 1000-fold reduction in viremia [195]. Furthermore, specific T cells have been observed in the liver of marmosets during infection and post clearance of GBV-B [172,195]. Similarly, an increase in T cells in the liver and peripherally in the blood has been observed in tamarins infected with GBV-B [194,196]. Following rechallenge with GBV-B, both marmosets and tamarins rapidly clear the virus [195–197]. Protection is associated with a sharp increase in T cells, indicating mobilization of memory T cell responses [195].

Humoral responses to GBV-B infections have not been well characterized, primarily due to lack of *in vitro* serological assays. There are many reports of antibodies targeting structural (core) and non-structural (NS3 and NS5) proteins during GBV-B infection. Although not extensively studied, there is limited evidence showing an association between delayed antibody response and persistent infection [191,198]. Antibodies to both structural and non-structural targets peak just prior to viral clearance, indicating a possible role in the clearance [182,186,188,199,200]. There are, however, conflicting reports on how long antibodies are maintained after clearance of the virus and their role in protection upon rechallenge [186,197,199]. These discrepancies could be due to variable sensitivities of the assays detecting anti-GBVB antibody. Thus far, anti GBV-B antibodies have been characterized by ELISA or western blot. These methods cannot distinguish non neutralizing from NAbs. All the antibodies identified thus far to GBV-B are not targeting the envelope proteins, and therefore it is unlikely that they are neutralizing. Indeed, there is very little research into the role of NAbs in GBV-B infection. Bukh et al. demonstrated that convalescent sera from a tamarin mixed with virus before inoculation did not protect against infection [197].

However, without the development of a robust *in vitro* assay the presence or role of NAbs cannot be determined.

1.3.5 Utility as a model virus

The GBV-B infection model in new world primates does generally diverge from HCV infection persistence and there are costs and ethical concerns with using primates for biomedical research; nevertheless, it has been a useful research tool. It has helped discover the functional importance of HCV genome features such as the microRNA-122 binding site [201]. GBV-B has also been a helpful tool in screening HCV antivirals such as ribavirin and NS3 protease inhibitors [187,202]. Although GBV-B and HCV have a very similar genome structure they have limited shared amino acid identity. These differences pose challenges for drug and vaccine assessments as therapeutics are often highly specific to the target virus [127]. To improve this model, HCV/GBV-B chimeras have been developed to address these issues. Chimeras containing all of the structural HCV genes or just the glycoproteins are able to infect marmosets and induce persistent infections as well as liver pathology seen with HCV infections [203]. Therefore, marmoset disease progression could be monitored to assess candidate vaccine efficacy.

1.4 Japanese encephalitis virus

JEV is the most common cause of encephalitis in Asia. In endemic areas, it is primarily of disease of childhood, although infections in adults can happen [204]. There are an estimated 68,000 clinical cases of JEV infections per year [205]. The majority of infections are mild with fever and headache or asymptomatic. However, about 1 in 250 cases result in severe illness. After an incubation period of 4–15 days patients develop rapid onset of symptoms including fever,

headache, and vomiting. Mental and neurological deficits including movement disorders and seizures occur in the few days following. Of the cases that result in severe illness, there is a mortality rate of up to 30%. Further to that, up to 50% of those that survive encephalitis result in permanent neurologic or psychiatric damage that can manifest as recurrent seizures, paralysis, mutism, and more [206].

1.4.1 History

The first clinical JEV infection was described in Japan in 1871, followed by notable outbreaks in 1924, 1927 and 1935 [207]. In 1934, the virus was first isolated from a fatal case of encephalitis in Japan and characterized as the prototype strain (Nakayama) [208]. Observations from previous outbreaks and the seasonal occurrence of the disease pointed to a mosquito vector, which was confirmed with the isolation of the virus from *Culex tritaeniorhynchus* mosquitoes in 1938 [209]. JEV has spread to affect most countries in south Asia, southeast Asia, and the Asian pacific rim [210].

1.4.2 Transmission

Humans are a dead-end host for JEV; viremia is generally not high enough for onward transmission [211]. JEV can infect numerous vertebrates but wading/water birds and pigs are important amplifying reservoirs for JEV transmission into humans [212]. Pigs and birds are generally asymptomatic carriers, with the exception of new-born birds, and JEV infection in pregnant pigs often results in stillborn piglets [213,214]. Humans are infected through bites from infected mosquitoes. *Culex tritaeniorhynchus* is the primary mosquito vector, although, JEV has been isolated from many other *Culex* species [215]. Not all mosquito species are competent for transmission, but other mosquito species

such as *Aedes* and *Anopheles* can be experimentally infected with JEV raising questions about their potential as vectors in certain environmental conditions [216–218].

1.4.3 Structure

JEV virions contain a single stranded 10.9 kb RNA genome. Virions are spherical and approximately 50 nm in diameter. Similar to other members of the genus *Flavivirus*, the JEV genome encodes 7 non-structural proteins and 3 structural proteins; the envelope proteins are called Envelope (E) and precursor Membrane (prM), which is cleaved into the mature, infectious M protein [2]. During egress from cells, the prM covers the E protein, preventing premature fusion in the low pH environment of the secretory pathway [219,220]. Cleavage of the prM protein induces a structural rearrangement of the envelope proteins into in its fusogenic state. In the mature form, 30 ‘rafts’, each made up of 3 sets of E dimers, are organized into a herringbone pattern that lie on the viral membrane with the M proteins buried underneath (Figure 1.7) [221].

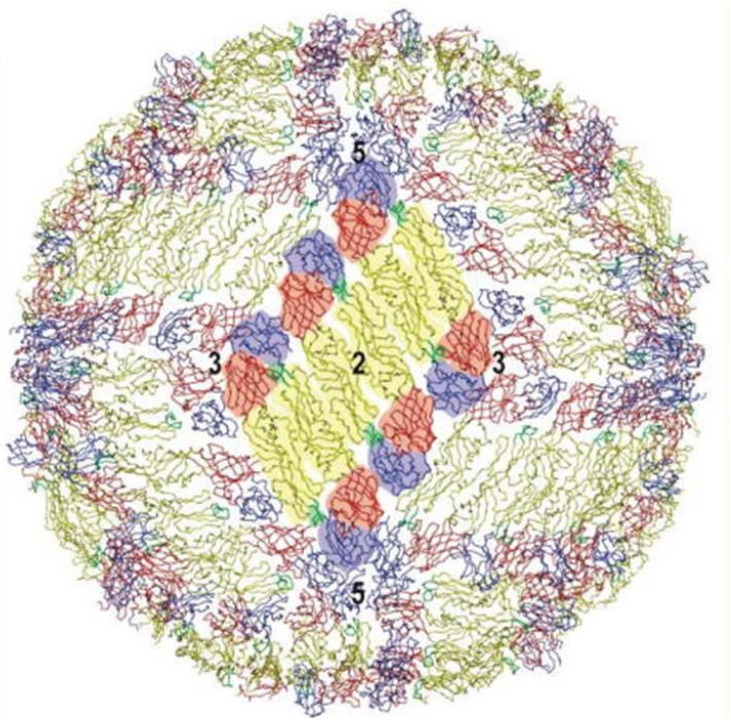


Figure 1.7. Flavivirus virion structure

Mature flavivirus virion showing the herringbone pattern of the E proteins. Symmetry axes are labelled. Figure adapted from Zhang et al [220]

1.4.4 Cell entry

JEV can infect numerous vertebrates and mosquitoes, and in *vitro* can infect and replicate in wide range of vertebrate and invertebrate cell types, suggesting a well conserved receptor or that there may be multiple host factors involved in viral entry [222]. Indeed, there are many cellular factors that have been indicated in JEV attachment or entry. Glycosaminoglycan has been shown to serve as an initial attachment factor to concentrate viral particles at the cell surface by binding to the E protein [223–227]. C-type lectins, which are glycan binding proteins, have also been indicated to be involved in the early steps of infection. DC-SIGN has been identified as important for viral binding in dendritic cells. C-type lectin domain family 5, member A (CLEC5A) and the liver and lymph node sinusoidal

endothelial cell C-type lectin (LSECtin) have been shown to be involved in JEV entry of myeloid cells. Additionally, mosquito galactose-specific C-type lectins, have been shown to mediate infection in mosquitoes [228]. There is also evidence that integrins play a role in JEV cell entry into Vero and hamster kidney cells, as pre-treatment with anti-integrin $\alpha_v\beta_3$ antibodies inhibits entry [229,230]. Several other host factors such as heat shock proteins, CD4, CD14, Vimentin, LDLR, and others have been indicated in roles for cell entry [222]. Table 1.1 summarizes the host factors that have been associated with JEV cell entry. As highlighted by the number of cellular factors identified for JEV entry, there has not been a lack of research into the area, however a clear mechanism of interactions with cellular receptors that leads to internalization has not been elucidated yet. Many of the host factors mentioned appear to serve in a cell type dependent way indicating that there may be several different paths and cellular factors depending on the host cell. Moreover, flaviviruses have been observed to fuse synthetic membranes without cellular protein [231,232]; therefore, it is conceivable that a highly conserved, ubiquitous non-protein receptor may be involved/effective in cell entry.

Table 1.1. Cellular factors indicated in JEV cell entry

Cellular component	Host Cell	Reference
Heparan sulfate	Mammal	[233,234]
DC-SIGN	Mammal	[235,236]
MR	Mammal	[237]
CLEC5A	Mammal	[238,239]
LSECtin	Mammal	[236]
mosGCTL-7, 1, and 3	Mosquito	[228]
αvβ3	Mammal	[229,230]
HSP70/HSC70/GRP78	Mammal, Mosquito	[240–242]
HSP90	Mammal	[243]
37/67-kDa LR	Mammal	[244]
CD4	Mammal	[244]

CD14	Mammal	[244]
Vimentin	Mammal	[245,246]
LDLR	Mammal	[247]

1.4.5 Replication, Assembly, and Egress

Like other members of the Flaviviridae family, JEV undergoes the general process described in section 1.1.2 for fusion, replication, assembly, and egress. One of the key differences in the lifecycle between hepaciviruses and flaviviruses like JEV is that flaviviruses leave the ER in an immature state with the E protein and complete prM protein. In the trans Golgi network, furin or furin-like proteases cleave the prM precursor. This allows the rearrangement of the E proteins to form the icosahedral pattern observed in flaviviruses [248,249].

JEV as well as other flaviviruses produce subviral particles as part of the natural lifecycle [2,250,251]. Subviral particles are mature E and M proteins embedded in a lipid membrane but are devoid of core protein or genetic material, making them non-infectious. They have the same post-translational modifications and follow the same path through the secretory pathway for exocytosis as infectious virions. Recombinant subviral particles can be produced in mammalian cells by only expressing the E and prM proteins. As they have the same outer proteins and fusion activities of infectious particles they can be used as immunogens to elicit as protective immunity [248,252].

1.4.6 Immune responses

JEV infection activates both the humoral and cellular branches of the immune response. This response generally results in long-lasting or even lifetime immunity [253]. Below is a brief overview of the immune response elicited.

1.4.6.1 Humoral

Humoral responses are well documented for JEV infection. IgM antibodies appear within 5–7 days in the blood/serum and cerebrospinal fluid (CSF) of most symptomatic patients [254]. In symptomatic patients, early elevated levels of serum anti-JEV IgM are associated with mild disease outcomes and those who fully recover. Conversely, low IgM in the sera is associated with death or severe disease progression resulting in neurological sequelae [255]. A robust humoral response is thought to prevent viral replication before the virus can cross the blood brain barrier. Indeed, virus can only be isolated from patients that fail to produce an effective IgM response [256]. For surviving patients, class switching to IgG occurs within 30 days [254]. Those that have had previous exposure to flavivirus pathogens exhibit an anamnestic response to common antigens and generally have a more favourable outcome [254,255,257]. NAbs generally target the E protein, blocking cell entry, and making the E protein a promising target for therapeutics (e.g. monoclonal antibody treatment [258]) and vaccine development [259]. Immune responses to the E protein are sufficient to confer protection from infection [258,259].

1.4.6.2 Cellular

The cellular immune response is less well characterized than the humoral response. However, both CD4+ and CD8+ T cells have been shown to proliferate in response to JEV infection [260,261]. Additionally, spider monkeys that normally present with subclinical JEV infection become symptomatic when T cell function is suppressed [262]. Studies with animal models have shown conflicting outcomes with some studies showing a subsidiary role of CD8+ T cells that are dispensable for JEV recovery [263] and with others showing that adoptive transfer of primed T

cells can protect against lethal challenge [264], even without JEV-specific antibodies [265]. A study by Turtle et al. concluded that asymptomatic infections had a predominately CD8+ response to NS3, NS4 and NS5, and that patients who recovered from disease mounted mostly a CD4+ response that targeted structural proteins and NS1 [266]. Moreover, the nature of the CD4+ response is the strongest indicator of disease outcome with a robust polyfunctional CD4+ response associated with recovery and poor outcome associated with a significant TNF-α+-only CD4+ T cell population. Multiple studies have indicated a mechanism where T cell responses are responsible for the damaging inflammation observed with JEV infection [263,267].

1.4.7 Prevention/Treatment

Japanese encephalitis is a vaccine preventable disease for which a number of effective vaccines have been developed [268]. The first JEV vaccine available was a formalin-inactivated virus derived from mouse brains. This vaccine had a protective efficacy of up to 97.5% [269]. Side effects, animal ethics concerns, complex manufacturing and the need for multiple doses led to the discontinuation of its use. Other inactivated vaccines have been grown in Vero or BHK cells [270,271]. A live attenuated vaccine has also been developed by passaging the virulent SA14 strain through primary hamster kidney cells, mouse brain, and hamsters [272]. The cell culture system for creating this live attenuated vaccine did not meet the standards of many country's regulatory agency prompting the creation of the Chimerivax-JE vaccine. This is a live attenuated chimeric vaccine with the E and prM of JEV inserted into the yellow fever 17D vaccine strain [273]. This vaccine has gone through rigorous testing *in vitro* and in animal models and

clinical trials and its safety has been confirmed. In general, all JEV vaccines are safe and provide impressive protection [268].

Through vaccination programs and other preventative measures such as pesticides in rice paddies, JEV has been drastically curbed in some East Asian countries such as South Korea and Japan. Nevertheless, in recent decades there has been an increase in incidence in some regions such as India and Pakistan and geographic spread of the virus including to Australia [274,275]. Furthermore, JEV RNA has been isolated from birds and mosquitos in Italy, further raising concerns for global public health [276,277].

Currently there are no licensed treatments specific to JEV. The treatment consists of management of symptoms. Clinical trials for candidate therapeutics have not shown significant improvement in patient outcome [278]; however, all these studies are small and underpowered. There are a number of existing compounds, some of which are regularly used in humans, that have been shown to have anti-JEV activity, through either *in vitro* or in animal models. For example, pentoxifylline can inhibit JEV replication *in vitro* and can also protect mice against challenge [279]. More of these compounds are reviewed in [278]. While there are some promising preliminary results regarding treatment for JEV, they will all need go through the extensive clinical trials before use in humans.

1.5 Pseudotyped Viruses

1.5.1 Definition

A pseudotyped virus (PV) is a virus with enzymatic proteins and structural core of one virus and at least one envelope protein not encoded by its genome, usually

from a heterologous virus. Pseudotypes were discovered in the 1960's to occur naturally when chicken cells infected with both Rous sarcoma virus (RSV) and Rous associated virus (RAV) produced virions with the genome of RSV but the 'outer coat' of RAV [280]. Nowadays, PVs are generally engineered to undergo only one round of replication, as the genome of PVs does not encode envelope proteins so upon entry into target no further infectious particles can be produced [281]. This renders them a safe tool that can be handled in BSL1 or BSL2 laboratory. PVs are particularly useful for hazard group 3 and 4 pathogens requiring high containment level - which hampers accessibility to research especially in resource limited areas where these facilities are rarely available. There are, for example, only around than 50 BSL4 laboratories in operation worldwide [282]. Additionally, PVs play an important role in serological research for viruses that cannot be propagated in cell culture, such as most strains of HCV or emerging viruses that do not have cell culture systems established yet [283,284]. For this purpose, they contain a reporter gene that is expressed after cell entry. Reporter genes have included firefly luciferase, fluorescent proteins, β-galactosidase and others [285,286]. Expression of these genes can be quantified by various methods with reporter gene expression often being directly proportional to the number of cells infected.

Several viruses have been engineered for pseudotyping. Mammalian retroviruses murine leukemia virus (MLV) and human immunodeficiency virus (HIV) were found to incorporate heterologous glycoproteins [287–290]; and since have been utilized extensively as the backbone for creating pseudotypes. The most prevalent viral backbone systems are based on viruses with a genome that is easily manipulated, are able to incorporate heterologous glycoprotein on their surface, and that do not require their own envelope for viral assembly and

maturation. Among several candidates, retroviruses and vesicular stomatitis virus (VSV) represent the most used platforms. VSV, a platform, that does not involve genome integration, has become increasingly utilized in recent years. More details on the development of these PV platforms are described below.

1.5.2 Retrovirus Backbone

Retroviridae are a family of single stranded RNA viruses that include the genera *alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, lentivirus* and *spumaretrovirus.* All retroviruses have the enzymes reverse transcriptase that converts viral RNA into DNA, and integrase that integrates the viral DNA into the host genome, which leads to host transcriptional machinery expressing viral genes [291,292]. This process has been manipulated to use retroviruses as vectors for stable delivery of many non-viral genes to target cells, perhaps most notably, the delivery of therapeutic genes [293–296]. Therefore, the current systems used to produce retroviral pseudotypes have close ties to the development of viral vectors for gene therapy. In addition to gene therapy, vectors can be used to deliver genes for experimental biology, as well as a number of reporter genes that simply indicate whether the cells have been infected [283,285,297]. HIV and MLV retroviruses are the most widely used platform for creating pseudotypes.

1.5.2.1 Lentivirus

HIV-1 is the most well studied lentivirus and as such the vast majority of lentiviral pseudotypes are derived from human immunodeficiency virus (HIV). The HIV genome encodes 9 viral proteins. The *gag* gene encodes the structural proteins that make up the viral core. The *pol* gene encodes the enzymatic proteins for replication. *Env* encodes the envelope protein gp160, which is then cleaved into

two subunits: gp120 and gp41. The genome also encodes accessory proteins Vif, Vpr, Vpu and Nef and regulatory proteins Rev and Tat.

The proof of the concept that HIV genome can be modified to serve as a vector for the delivery of transgenes to a host cell was done with replication competent virus with the *nef* gene replaced with a reporter gene (chloramphenicol acetyltransferase) [298]. As HIV is classified as a hazard group 3 pathogen, modifications to the system have been made to improve the safety of these vectors and reduce the containment level to handle them. The genome was split between 2 DNA plasmids, one containing proviral DNA with a deletion of the *env* gene, and a second plasmid that provides the *env* gene, or the gene of a heterologous virus envelope protein [299,300]. Transfection of these plasmids into cells resulted in single round replication lentiviral vectors that cannot create infectious particles (without recombination events) after initial infection as there is no envelope protein produced. To further increase safety and reduce the chances of recombination events that can lead to a replication competent virus, the genes for vector production were split into 3 plasmids. The *env* gene is still contained in one plasmid with the rest of the genome being split into 2 plasmids: one containing the *gag, pol,* accessory, and regulatory genes and the third being the transfer plasmid containing the gene of interest to be packaged in the viral particles and integrated in the target cell. In addition to the gene of interest, the transfer plasmid contains all the *cis*-acting elements for genome packaging, reverse transcription, and integration, such as packaging signal (Ψ), 5' and 3' long terminal repeats (LTR), rev response element (RRE). Both the envelope and packaging plasmid do not contain Ψ, so no genes encoding viral structural proteins are packaged into the viral particle. This was further improved upon by removing accessory genes from the packaging plasmid which are dispensable

for infection *in vitro*: *vif, vpr, vpu* and *nef* [301]. The 3 plasmid system is the most commonly used for PV production (Figure 1.8). The need for *tat* was removed by introducing a heterologous viral promoter to the transfer plasmid. Lastly, a 4-plasmid system was developed by introducing *rev* in a fourth plasmid [302]. Many other modifications have taken place to improve both efficiency of the gene transfer and safety of LV vector system which are reviewed in [303]. Other lentiviruses such as feline immunodeficiency virus , and simian immunodeficiency virus have been utilized to create pseudotypes [285].

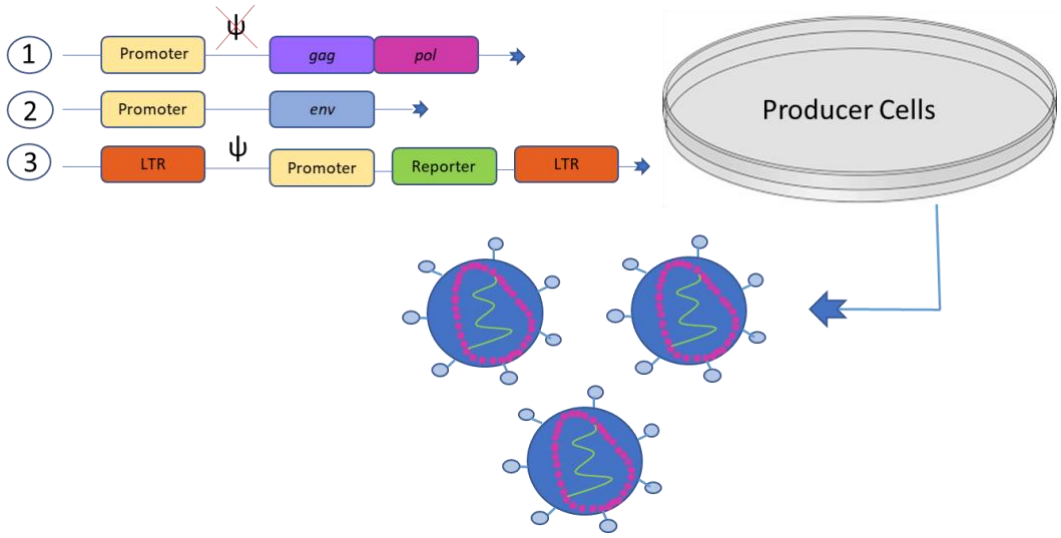


Figure 1.8. Production of retroviral PVs

Production of retroviral PV with 3 plasmids involves transfection of producer cells (generally HEK293T) with 3 plasmids containing: 1. The gag, pol, and regulatory genes. 2. The envelope gene of interest. 3. The transgene, shown here is reporter gene, and the cis-acting elements- long terminal repeats (LTR) and packaging signal (Ψ)- which are needed for genome packaging, reverse transcription, and integration. Supernatant harvested from the producer cells contain the PV.

1.5.2.2 Gammaretroviral

The packaging signal (Ψ) was first discovered in MLV to be necessary for the viral genome incorporation in the viral particles when deletion of that region resulted in viruses defective in packaging their genome [304]. It was later discovered that this signal was sufficient to package heterologous transcripts [305]. Thus, MLV vectors were also developed for gene transfer. Initially MLV vector systems were commonly produced through the use of a stable packaging cell line that continuously produces the structural and enzymatic components of the virus [306]. Similar to HIV, MLV vector systems that are comprised of 3 plasmids have been developed: (1) gag/pol (2) env (3) transfer plasmid with gene of interest [307]. Gammaretroviruses have a simpler genome than HIV; it encodes the *gag, pol,* and *env* genes but does not contain any of the accessory or regulatory protein genes [308]. Lentiviral vectors have become the preferred vector over MLV for gene therapy as they cause less insertional mutagenesis [309], and MLV cannot transduce non-dividing cells [303]. However, for many pseudotype applications (see below), integrating into non-dividing cells is not a requirement. Furthermore, in some instances, MLV backbones have been shown to produce higher titre pseudotyped viruses than HIV depending on the envelope protein used [284,310].

1.5.2.3 Envelope Incorporation

Viral envelope proteins (VEP) can be assembled on retroviral PVs in either a passive or active manner. While retroviral cores have been shown to bud in the absence of their respective Env, studies have shown that Env incorporation is an active process through the cytoplasmic tail of Env. Gag protein in the absence of Env will bud from any side of a polarized cell but in the presence of Env protein,

Gag protein is relocated to the Env containing basolateral side [311]. This type of active interaction has also been seen with core and heterologous retroviruses [312]. Passive incorporation of envelope proteins takes place through assembly by simple colocation of Env and Gag in the producer cell. This has been shown to take place in two locations, at the plasma membrane or in late endosomal vesicles [313]. There is ample research to support assembly and budding of retroviruses from the plasma membrane, particularly for HIV in lymphocytes [314–316]. While, the plasma membrane is generally accepted as the primary location of core assembly, studies have demonstrated that Gag can assemble intracellularly in a variety of cell types [313,317,318]. The lipid composition of these respective locations within different cell types may play a role in targeting Gag [316,319]

Furthermore, it has been indicated that intrinsic location of the heterologous envelope proteins in producer cells dictates the location of core and envelope protein association leading to passive incorporation of VEPs [313,320,321]. Sandrin and colleagues demonstrated that while a small proportion of HCV envelope protein was located at the cell surface of producer cells, the level of glycosylation on these proteins was different than those found on the infectious lentiviral particles, indicating another location of assembly [313]. They went on to show that HCV envelope proteins did not colocalize with Gag at the plasma membrane but only in a small proportion of late endosomes. In contrast, VSV-G was shown to be heavily colocalized with Gag at the plasma membrane [313]. For envelope proteins that do not readily or efficiently pseudotype on to retroviral vectors, modifications to the retention signals or cytoplasmic tail have been made to induce active incorporation of VEP or relocate the proteins to a more favourable location for passive incorporation [322–324]. Caution must be taken

when altering VEP as this may lead to misfolding of the proteins resulting in non-functional or antigenically altered phenotypes. Lastly, the amount of VEP in producer cells can play a role in efficient incorporation and codon optimization of envelope protein genes is often utilized to increase protein production [285,325,326]. Although more efficient PV production has been reported with reduction in envelope protein levels in some cases [323].

1.5.3 VSV Backbone

VSV is a rhabdovirus that infects livestock such as cattle and horses causing vesicular lesions [327]. It was discovered to incorporate the envelope proteins of heterologous viruses present in the same cell, to make phenotypically mixed virus [328–330]. Using reverse genetics, it was then shown that VSV particles engineered to have the glycoprotein (G) gene deleted are able to produce bald particles, indicating that the assembly and budding of the virion is independent of the G protein [331]. Replication defective VSV-ΔG has been further engineered to express reporter genes in place of the G protein [332]. This pseudotyping platform has become increasingly popular and has been used to pseudotype many envelope proteins [285,333]. A benefit of this platform over retroviral platforms is that as the VSV genome, and therefore reporter, is transcribed in the cytoplasm [334], it reduces assay times as reporter expression can be detected 24 hours after transduction. Retroviral PV reporter expression is generally detected 48–72 hours after transduction.

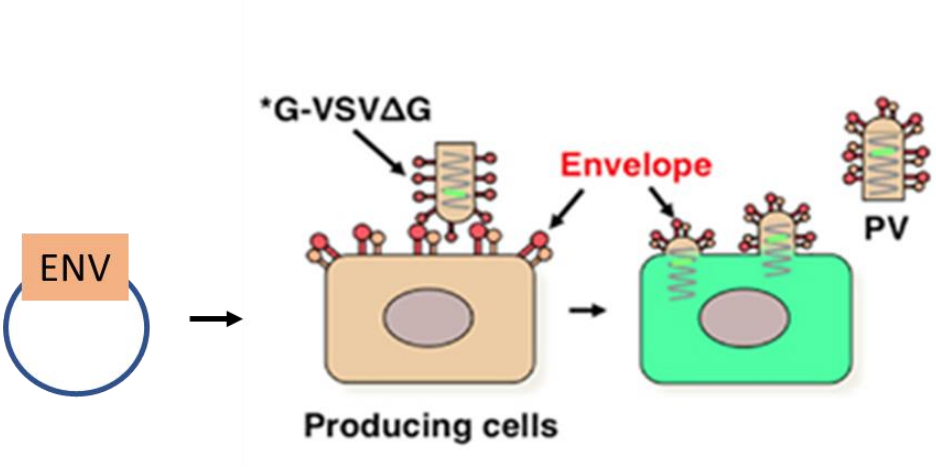


Figure 1.9. Production of VSV core PVs

*PVs with a VSV core are produced by transfection of producer cells with a plasmid containing the envelope gene/genes of interest. Then 24 hours later the producer cells are infected with a *G-VSVΔG (VSV with the gene for its G protein replaced with a reporter gene and pseudotyped with either VSV-G or a heterologous viral envelope protein). PVs are harvested from the supernatants of the producer cells. Figure adapted from Tani et al* [335].

1.5.4 Alternative Methods

Some viral envelope proteins have been difficult or impossible to pseudotype into the above-mentioned platforms, for example many members of the *Flavivirus* genus such as West Nile virus and Japanese encephalitis virus [336]. They are internally budding viruses that acquire their membrane from the ER and the main site of budding for retroviruses and VSV is the plasma membrane. As such, the core is not generally in the correct location to acquire the VEPs in their membrane. Therefore, research into other platforms has been undertaken to establish alternative backbones for internally budding viruses. Pierson et al., produced a sub-genomic replicon with the non-structural proteins of WNV and

GFP or luciferase reporter. When complemented *in trans* with expression plasmids with WNV structural proteins, reporter virus particles (RVP) are produced that are only capable of a single round of replication. These RVP were able to incorporate multiple strains of WNV [337]. A similar approach was used to make RVPs with a dengue replicon and dengue structural proteins [338]. Matsuda et al. also established single round infectious particles based on dengue/luciferase replicon, in which they also produced particles with multiple heterologous virus envelope proteins: ZIKV, JEV, WNV, YFV, tick-borne encephalitis (TBEV), and Usutu virus [339].

1.5.5 Applications

1.5.5.1 Gene therapy

Retroviral vectors are often the platform of choice for delivering therapeutic genes for many reasons. They have been engineered to be safe tools, they can integrate irreversibly into the host genome, and they can package and deliver relatively large payloads. Importantly the ability to be pseudotyped with heterologous VEPs enables altering of cellular tropism [340]. HIV vectors, for example, that harbour wildtype VEPs would be restricted to cells expressing CD4 on the surface, which would be mainly CD4+ T cells and monocytes [341]. Widening the pool of host cells susceptible to transduction by vectors was achieved by pseudotyping vectors with VSV-G. VSV has an extremely wide tropism and is able to infect many cell types such as stem cells and neuronal cells [342]. PVs with VSV-G are also stable and are able to withstand various concentration methods to increase the titre [343]. As such, VSV-G is the most ubiquitously used VEP for retroviral vectors. In addition to widening tropism heterologous, VEPs can be used to

narrow tropism to a specific cell type for transduction. For example, influenza VEPs can be used for targeted transduction of airway epithelial cells [344].

1.5.5.2 Receptor studies

Cell entry by enveloped viruses is generally mediated by interactions between the VEPs and their cognate receptors on the cell surface. Use of receptors with expression restricted to certain cell types is often responsible for the cellular tropism of viruses [345]. As PVs only harbour the VEPs of the virus of interest they are a valuable tool to study binding and entry by uncoupling them from the rest of life cycle. This is particularly valuable for viruses that cannot be propagate in cell culture, such as HCV before the discovery of the JFH1 strain. HCV pseudotypes were used to show that both CD81 and CLDN1 were essential factors for HCV cell entry [34,346]. They are also useful for high containment level viruses such as arenaviruses that cause haemorrhagic fever in humans and require a BSL4 laboratory. Arenavirus PVs of Machupo, Guanarito and Junin virus were used to determine that transferrin receptor 1 is a cellular receptor for these New World arenaviruses but not for Old World arenaviruses such as Lassa virus [347]. More recently, three weeks after SARS-CoV-2 was isolated (and available only in China), PVs were used to confirm ACE2 as a receptor [348].

1.5.5.3 Drug Screening

As stated above, VEPs are involved in binding and cell entry, and as such PVs mimic cell entry of the virus the VEPs are derived from. Therefore, PVs are used as a high throughput method to screen drugs that target these processes of the virus of interest. Influenza PVs have been used to screen a library of ~106,000 small molecules for cell entry inhibitors, identifying 2 novel inhibitors [349].

Similarly, 1280 FDA approved drugs were screened for antiviral effects against Ebola PV, identifying teicoplanin as a potential treatment [350]. Additionally, a screen of 5000 drugs using PVs identified a broad-spectrum antiviral that could inhibit entry for SARS-CoV, Ebola, Hendra, and Nipah virus PVs [351]. The molecule identified is able to inhibit entry by inhibiting cathepsin L cleavage on the glycoproteins, which is crucial for maturation into a fusion competent form. In addition to small molecules, PVs have also been used to screen herbal treatments for antiviral effects [352]. The flexibly of VEP incorporation on to PVs combined with their use in low containment level laboratories as well as easily quantifiable reporters make them a popular method for antiviral screening.

1.5.5.4 Vaccination

PVs have also been used as the platform to deliver immunogens for vaccination. Surface proteins from two subtypes of influenza on retroviral cores were used to induce a strong humoral response to mice [353]. Additionally for influenza, a PV with an influenza core and an inactivated HA which is replication defective, was pseudotyped with both homologous and heterologous HA proteins to create single round replication PV has been to vaccinate mice which resulted in homotypic and heterotypic protection against influenza A [354].

PV have also been used for serological studies. The section below elaborates on serology and the use of PVs in the field.

1.6 Serology

Serology is the study of the proteins found in blood and other secretions. In practice serology is predominantly focused on antibodies [355]. Antibodies are the key component of humoral immunity. The antibody repertoire can provide

valuable information into adaptive immunity to pathogens, as well as, past and present exposure to pathogens. This section briefly covers how antibodies come about, the role they play in infection, and serological assays to detect antibodies.

1.6.1 Production of antibodies

The body recognizes pathogenic invaders such as viruses, bacteria and parasites and mounts an immune response to clear them. The first line of defence is the innate immune response. The innate response is a nonspecific response that is triggered by detection of molecular components that are conserved features of pathogens (pathogen-associated molecular patterns (PAMPs)), such as double stranded RNA which is conserved among many RNA viruses. The innate immune system then acts to destroy the pathogens, either directly, such as ingestion by macrophages, or indirectly, such as activation of target cells by cytokines [356]. When the innate immune system fails to clear a pathogen the adaptive, or specific immune response occurs. It may take hours or days for this response to occur. The adaptive immune response is primarily made up of two cell types: T lymphocytes (T cells) and B lymphocytes (B cells). Both cell types have antigen receptors on their surface to detect antigens. After antigen detection, T cells proliferate and differentiate into cytotoxic T cells, helper T cells, or regulatory T cells to combat infection. When receptors on B cells recognize antigens, they differentiate into plasma cells that secrete antibodies that target the same antigen recognized by the B-cell receptor [357].

Every B-cell clone has antibodies imbedded in their membrane that serve as receptors that have unique antigen binding sites. There is an extensive diversity in the antibody repertoire that allows binding to vast array of antigens. When they bind to an antigen the cells are activated (with signals from helper T cells) to

proliferate into effector cells that secrete antibodies with the same antigen binding site as that of the B-cell receptor, or a subset proliferate into memory cells. The basic structure of an antibody as seen in Figure 1.10 consists of two light chain and two heavy chains and have 2 antigen binding sites. There 5 classes of human antibody: IgA, IgD, IgE, IgG, and IgM that differ by the by their heavy chain, which determine the characteristic properties of each class [358]. Antibodies go through affinity maturation wherein they acquire improved antigen binding throughout the immune response. B-cells with better affinity to the antigens are preferentially expanded, and they undergo somatic hypermutation, in which point mutations, insertions and deletions are introduced to the binding site creating more antibody sequence diversity, resulting in a subset which will be better binders to be preferentially expanded [359]. During maturation antibodies also go through class switching: DNA recombination where the genes for the heavy chain's constant regions are replaced with those of another class resulting in a different class of antibody with the same antigen binding sites. The antibodies that are elicited during an immune response are dependent on the nature of the antigen [360].

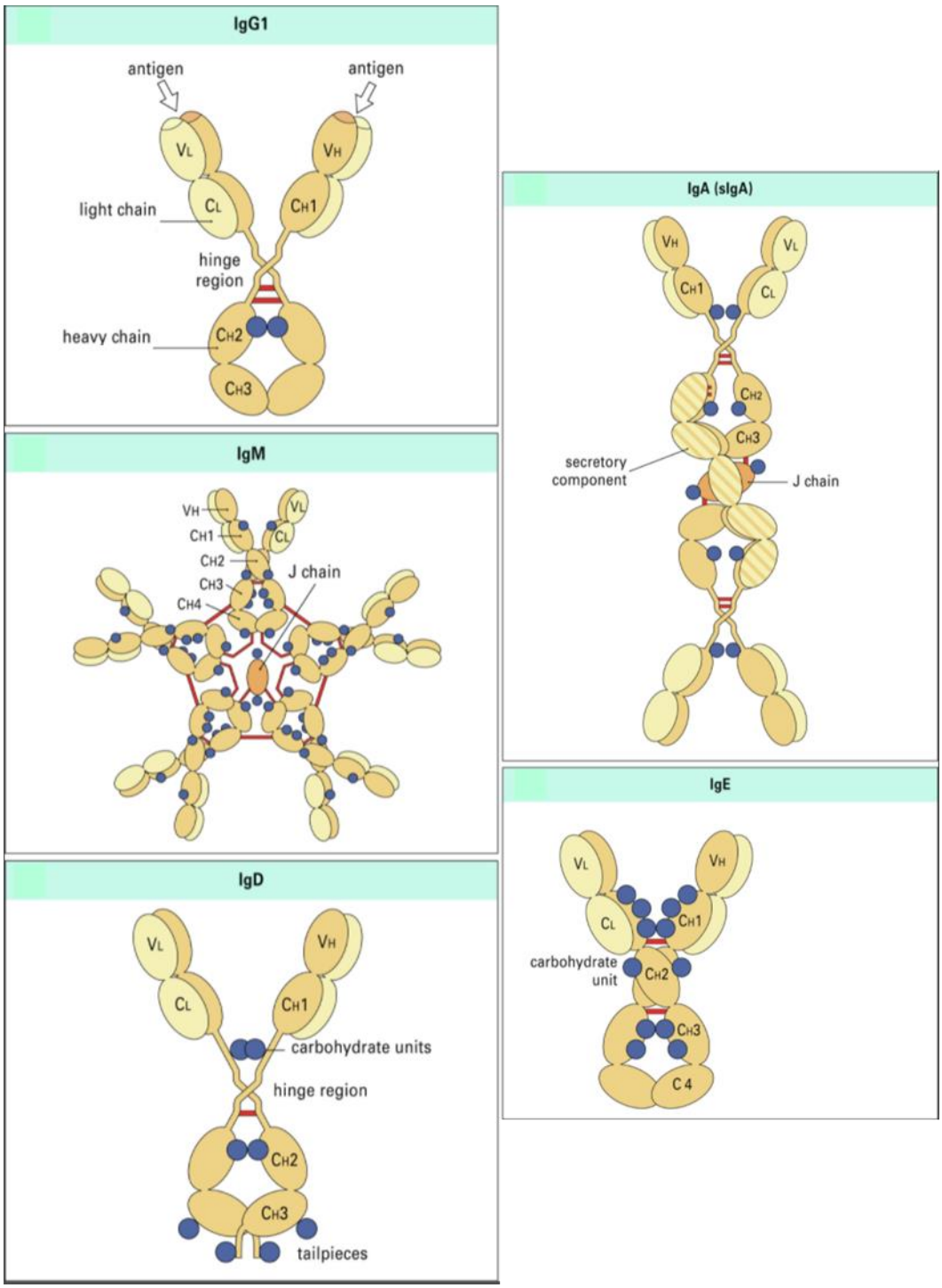


Figure 1.10. Classes of Immunoglobulin

The basic structure of an antibody as seen with IgG is characterized by 2 heavy chains and 2 light chains. The N terminal end of antibodies are characterized by sequence variability in the light and heavy chains (VL and VH respectively) and are where the antigen binding sites are located. The rest of the molecule is a relatively constant structure. The constant region of the light chain in this figure

is termed CL. The constant regions of the heavy chain are divided into 3 regions: CH1, CH2, and CH3. Flexibility in the hinge regions allows the antigen binding sites to act independently. Carbohydrate moieties are shown in blue and disulfide bonds in red. Disulfide bonds between adjacent constant heavy chain regions give the IgM is characteristic pentamer form. IgA is often found its secretory form as a dimer with a secretory component. Figure adapted from Male et al [361].

1.6.2 Role of antibodies in infection

Antibodies protect against pathogens in 3 ways: opsonization, complement activation, and neutralization. Opsonization is the coating of the pathogen with antibodies, then phagocytes recognize the constant part of the antibodies and engulf the particle and destroy it. Complement can be activated by pathogens without the presence of antibodies, however, antibodies bound to pathogens can more strongly activate complement. Lastly, antibodies bound to pathogens can neutralize them by blocking access to cells [362]. Antibodies that bind to proteins on the outer coat of the virus that prevent interaction with cellular receptors are often those with neutralizing capabilities. [362,363]. The class of antibody determines its functional properties and effector mechanisms. IgM, which forms a pentamer is the first class of antibody expressed and functions mainly through opsonization and complement activation. IgM generally have low affinity but due to their pentameric form they can have multiple interactions with antigens making them efficient in their functions. IgD is found circulating at low levels in serum, its function has not been fully elucidated yet, but it is thought to play a role in mucosal immunity [364]. IgG is the predominant antibody found in serum and is important for neutralization of viruses and toxins, as well as, activating complement and

opsonization. IgA, often found in a dimer, is important for neutralizing pathogens at the mucosal surfaces, where they are the predominant immunoglobulin. IgE has the lowest concentration in serum and is involved in the response to parasitic worms and allergic reactions [365].

In addition to the important role antibodies play in eliminating pathogens there are some circumstances in which antibodies can be detrimental. Antibody dependent enhancement (ADE) is when viral disease is more severe due to the presence of antibody through previous infection, vaccination, or antibody treatment [366]. This phenomenon has been mostly studied in relation to dengue virus (DENV), a member of the *flavivirus* genus [367,368], but also been indicated for respiratory syncytial, Ebola, and influenza viruses [369–371]. A predicted mechanism by which ADE occurs for DENV is that non-neutralizing antibodies from previous infections bind the virus and enhance uptake into Fcγ receptor cells [372].

Activated B cells and T cells can remain long after the elimination of a pathogen. These cells can reactivate much quicker than naïve lymphocytes when they encounter the same antigen for a second time, mounting a rapid, larger immune response to provide protective immunity [373]. This is called immunological memory, which is the basis of vaccination. Vaccination is the deliberate activation of the immune system by exposing it to antigens so that immunological memory will protect against natural infection. Efficacy of viral vaccination is often evaluated on the ability to induce functional antibodies, such as neutralizing antibodies to the virus [374]. Additionally, antibodies can persist in serum for months, years, or even a lifetime after the initial infection. Thus, serological studies can be used to detect a current infection or past infection and assess the immune status in an individual.

1.6.3 Serological Assays

There are many tools for diagnosing viral infections. These tests are often optimized in individual labs specifically for the pathogen in question. Direct detection of viral genetic material by reverse transcription polymerase chain reaction is a useful diagnostic in acute infections that is utilized for many viruses. However, viremia is often transient and can be cleared before the onset of symptoms, as is the case with JEV [274]. Therefore, detection of antibodies that persist for weeks, months, or even years after the clearance of virus is useful for diagnostics as well as tracking the epidemiological spread of a virus, which is critical for surveillance of emerging or re-emerging viruses. Additionally, assays that can detect neutralizing antibodies (NAbs) are important for evaluating vaccine candidates.

Enzyme linked immunosorbent assay (ELISA) employs a plate coated with viral antigens to capture and detect antibodies in a sample [375]. This technique is a useful tool for diagnostics, particularly after clearance of viremia and surveillance of seroconversion. It is especially advantageous for distinguishing antibodies raised to natural infection or to vaccination based on the composition of the vaccine. For example, a recently developed foot-and-mouth disease vaccine has mutations in two of the non-structural proteins 3B and 3D, thus screening for antibodies to these mutated regions allows for differentiation of vaccinated versus naturally infected animals [376]. An even more recent example is SARS-CoV-2 where all the vaccines authorised in Europe and USA are targeting the surface protein spike. A serological assay against the nucleoprotein can be used to identify a past infection regardless to the vaccination status [377]. ELISAs can

also be utilized in high throughput formats. However, this technique detects all antibodies and cannot distinguish NAbs from non-neutralizing antibodies.

For viruses such as influenza and others that induce hemagglutination, or agglutination of red blood cells, the hemagglutination inhibition (HI) assay is a tool used to detect antibodies. This is a fast, cost effective and sensitive tool that uses red blood cells to detect antibodies able to inhibit hemagglutination in the presence of virus. Drawbacks with this assay include that there can be nonspecific hemagglutination inhibitors in samples that need to be inactivated prior to use which can otherwise lead to false positives and high interlaboratory variability. Additionally, for influenza, this assay measures the ability of antibodies to bind to the hemagglutinin head which may not in fact neutralize the virus [378,379]. Furthermore, it does not detect antibodies to the hemagglutinin stem region or neuraminidase which can having neutralizing capabilities [374].

Plaque reduction neutralization test (PRNT) is a widely used technique for detecting neutralizing antibodies and is considered the gold standard for many viral pathogens [381–384]. This technique involves mixing dilutions of antibody containing sample with live virus and infecting a monolayer of permissive cells with this mixture. The titre of NAb is determined by the dilution that reduces the cytopathic effects (plaques) of the viruses on the cells by a certain amount, usually 50%. This technique is very sensitive and specific for detecting NAbs which is why it is widely used in evaluating vaccine effectiveness. However, PRNT necessitates the use of live virus which can require the use of a biosafety level 3 or 4 laboratories. Additionally, this is a laborious technique that requires a long incubation time and skilled personnel which makes it difficult to use for high throughput screening of NAbs.

1.6.4 Pseudotype Virus Neutralization assay (PVNA)

The use of pseudotyped viruses have addressed some of the above-mentioned shortcomings of serological assays. Particularly as it pertains to detecting neutralizing antibodies in low containment laboratories and the ability for high throughput screening. PVNA are often done in 96-well plates but can be scaled up to 384-well plates. PV are incubated with different dilutions of sera before being added to susceptible target cells. The PVs deliver their genome to the target cells and the expression of the reporter expressed is proportional to the infection events. Cells infected with PV in the presence of sera that result in a reduction of the reporter signal is attributed to neutralizing antibodies in the sample. An advantage of this system is that a relatively small amount of sample is needed, in some instances less than 10 µl [385]. PVs are versatile, allowing for incorporation of many different types of envelope proteins, which allows the production and inclusion of control viruses into assays. This is also a time efficient assay as it can be performed in as little as 24 hours. Not only is the PVNA a valuable tool for high containment viruses, it is also useful for viruses that cannot replicate in cell culture or newly emerging viruses for which cell culture systems have not been established.

Chapter 2:

Materials and Methods

2.1 Materials

Table 2.1. Bacterial Strains

Strain	Source	Identifier
NEB 5-alpha Competent *E. coli*	New England Biolabs	Cat#2987H
NEB Stable Competent *E. coli*	New England Biolabs	Cat#3040H
JM 109 Competent *E. coli*	Promega	Cat#L2005
CopyCutter™ EPI400™ Chemically Competent E. coli	Lucigen	Cat#C400CH10

Table 2.2 Cell Lines

Cell Lines	Source	Identifier
Human: HEK 293T	ATCC	Cat# CRL-11268
Human: HEK 293T CD81 KO	[386]	
Human: HEK 293T+ CLDN1	This report	
Human: HEK 293T+ CLDN6	This report	
Human: HEK 293T+ CLDN9	This report	
Human: HEK 293T + GFP	This report	
Human: HEK 293T + Chimera A	This report	
Human: HEK 293T + Chimera A1	This report	
Human: HEK 293T + Chimera A2	This report	
Human: HEK 293T + Chimera A3	This report	
Human: HEK 293T + Chimera B	This report	
Human: HEK 293T + Chimera C	This report	
Human: HEK 293T + Chimera C1	This report	
Human: HEK 293T + Chimera C2	This report	
Human: HEK 293T + Chimera D	This report	
Human: Huh7.5	RRID	CVCL_7927

Human: Huh7	[387]	
Human: Huh7 CLDN1 KO cells	[387]	
Human: Huh7 CD81 KO cells	[387]	
Human: Huh7 SR-B1 KO cells	[387]	
Human: Huh7 LDLR KO cells	[387]	
Human: Huh7 OCLN KO cells	[387]	
Human: Huh7 CLDN1 KO+ CLDN1	This report	
Human: Huh7 CLDN1 KO+ CLDN6	This report	
Human: Huh7 CLDN1 KO+ CLDN9	This report	
Human: Huh7 CLDN1 KO+ CD81	This report	
Human: Huh7 CLDN1 KO+ GFP	This report	
Human: Huh7 CLDN1 KO + CLDN1 mutant	This report	
Human: Huh7 CLDN1 KO+ marmoset CLDN1	This report	
Human: Huh7 CLDN1 KO+ armadillo CLDN1	This report	
Human: Huh7 CLDN1 KO+ guinea pig CLDN1	This report	
Human: Huh7 CLDN1 KO+ rabbit CLDN1	This report	
Human: Huh7 CLDN1 KO+ Chimera A2	This report	
Human: Huh7 CLDN1 KO+ Chimera B	This report	
Human: Huh7 CLDN1 KO+ Chimera C2	This report	
Human: Huh7 CLDN1 KO+ Chimera D	This report	
Feline: CRFK	ATCC	Cat# CCL-94
Monkey: Vero	ECACC	Cat# 85020206
Hamster: BHK	ATCC	Cat# CCL-10

Table 2.3. Plasmids

Plasmid	Source	GeneBank acc. no.*	
		nucleotide	protein

pCAGGS_**GBVB**_ **E1E2**	Mattiuzzo Laboratory, NIBSC	AY243572	
pCAGGS_**GBVB**_ **E1E2**_ **OP**	This report		AAP57528.1
pCDNA3.1D_**HCV UKN1A20.8_E1E2**	[388]	EU155192	
pD607_**J6**_**E1E2**	Grove Laboratory, UCL	JQ745650	
pD603_**H77**_**E1E2**_**OP**	Grove Laboratory, UCL	KJ701242.	
pMD2.G (**VSV-G)**	[301]	M35219	
pCAGGS_**LASV-GP**	Lambe Lab, Jenner Institute	NC_004296.1	
pCAGGS_**JEV**_**prME**	Mattiuzzo Laboratory, NIBSC	JN604986.1	
p8.91 (HIV-1 *gagpol*)	[301]		
pCMVi (MoMLV *gagpol*)	[389]		
pCFCR-Luc (MLV vector with firefly luciferase gene)	Mattiuzzo Laboratory, NIBSC		
pCSFLW (HIV vector with firefly luciferase gene)	[390]		
pCSGW (HIV vector with green fluorescence protein gene)	[391]		
Pdual GFP/CLDN1	Addgene #86981		
pDual GFP/CD81	Addgene #86980		
pDual GFP (HIV vector with green fluorescence protein gene)	[392]		
pDual GFP/CLDN1 I32ME48K mutant	This report		

pDual GFP/**marmoset (*Callithrix jacchus*) CLDN1**	This report	XM_002758187	
pDual GFP/**armadillo (*Dasypus novemcinctus*) CLDN1**	This report	XM_004460834	
pDual GFP/**guinea pig (*Cavia porcellus*) CLDN1**	This report	XM_003477088	
pDual GFP/**rabbit (*Oryctolagus cuniculus*) CLDN1**	This report	NM_001089316	
pDual_GFP/ **CLDN6**	This report	NM_021195.5	
pDual_GFP/**CLDN9**	This report	NM_020982.4	
pCAG_**JEV prME** (Nakayama strain)	[339]	EF571853	
pCVM D1-nluc-rep (DENV-1 replicon system with nano luciferase)	[339]		
pCAG_D1C (DENV-1 vector core)	[339]		

* accession numbers (acc.no.) corresponding to the gene highlighted in bold

Table 2.4. Chemicals, Peptides, and Recombinant Proteins

Reagent	Source	catalogue number
FuGENE 6	Promega	E2693
FuGENE HD	Promega	E2311
Polyethylenimine, branched (PEI)	Sigma-Aldrich	407727
Polybrene	Merck	TR-1003-G
BamHI-HF®	New England Biolabs	R3136L
SalI-HF®	New England Biolabs	R3138L
NheI	New England Biolabs	R3131L
NsiI	New England Biolabs	R3127L
T4 DNA Ligase	Promega	M1801

Fluoroshield mounting media with DAPI	Sigma-Aldrich	F6057-20ML
Foetal Bovine Serum	Pan Biotech	P30-3306
Triton X-100	Sigma-Aldrich	X100-500ML
KOD hot start DNA polymerase	Sigma-Aldrich	71086
1kb DNA ladder	Promega	G5711
SYBR Safe DNA gel stain	Invitrogen	S33102
Penicillin/Streptomycin	Sigma	P4333

Table 2.5. Biological Samples

Reagent	**Source**	**Identifier**
Saguinus labiatus serum	NIBSC	schTAM 004 v2 Serum
Non WHO reference material- Serum containing JEV antibodies	NIBSC	02/182
Non WHO reference material- JEV negative control serum	NIBSC	02/184

Table 2.6. Critical Commercial Assays

Assay	**Source**	**catalogue number**
Q5® Site-Directed Mutagenesis kit	New England Biolabs	E0554S
Bright-Glo® Luciferase Assay System	Promega	E2650
Nano-Glo® Luciferase Assay System	Promega	N1130
pGEM®-T Easy Vector System II	Promega	A1380
RNeasy Plus Mini Kit	Qiagen	74134
SuperScript™ IV First-Strand Synthesis System	Invitrogen	18090050
QIAquick Gel Extraction kit	Qiagen	28704
Plasmid Mini Kit	Qiagen	12123

Plasmid Midi Kit	Qiagen	12143
Plasmid Maxi Kit	Qiagen	12163

Table 2.7. Antibodies

Antibody	**Source**	**catalogue number**
Anti-human CD81 monoclonal (1.3.3.22)	Thermofisher	MA5-13548
Rabbit anti-JEV envelope	GeneTex	GTX125867
Anti-Rabbit IgG (Alexa Fluor 647 conjugate)	Thermofisher	A-21244
Anti-beta Actin Monoclonal (BA3R), HRP-conjugated	Thermofisher	MA5-15739-HRP
rabbit recombinant anti-Claudin 1 [EPR9306]	Abcam	ab180158
Rabbit polyclonal anti-Claudin 6	Abcam	ab99226
Rabbit polyclonal anti-Claudin 9	Proteintech	16196-1-AP
Anti-Rabbit- IgG, HRP-conjugated	Abcam	ab205718

Table 2.8. Software

Software	**Version**
GraphPad Prism	8
Geneious	10.2.3
SnapGene Viewer	4.3.5
Leica LAS-AF	3.3.0
FlowJo	10

Table 2.9. Buffers and Solutions

Buffer/Solution	**Recipe**	**Source**
Luria-Bertani (LB) Broth	1% (w/v) bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1%(w/v) NaCl, pH 7.0	
Terrific Broth	2.4% (w/v) Bacto yeast extract, 1.2% (w/v) bacto-tryptone, 0.4% Glycerol	

LB agar	1% (w/v) bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bacto-agar, pH 7.0	
1x PBS	1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.4	
1X Tris-acetate-EDTA (TAE)	40 mM Tris (pH 7.8), 20 mM sodium acetate, 1 mM EDTA	
Takasuke Lysis Buffer	20mM Tris-HCl, 135mM NaCl, 1% (v/v) Triton X-100, 10%(v/v) Glycerol	
Takasuke Loading Buffer	100mM Tris-HCl, 4% (v/v) SDS, 20% (v/v) Glycerol, Few crystals of Bromophenol blue	
Blocking Buffer (immunoblotting)	PBS, 2%(w/v) milk solution,0.1% (v/v) Tween-20	
Permeabilizing blocking buffer	5%(v/v) FBS and 0.1% (v/v) triton-X100 in PBS	
Tris-EDTA (TE) buffer	10mM Tris-HCl and 1mM EDTA	
SOC media	From manufacturer	New England Biolabs
TRIS-Glycine SDS Running Buffer	From manufacturer	Invitrogen
Opti-MEM	From manufacturer	Gibco
DMEM with Glutamax	From manufacturer	Gibco
trypLE Express	From manufacturer	Gibco

2.2 Methods

2.2.1 Cell Culture

All cells (Table 2.2) were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with Glutamax (Gibco), 10% fetal bovine serum (FBS) (Pan Biotech), 100 unit/mL penicillin, 100 µg/mL streptomycin (Sigma), and 1% non-essential amino acids (Gibco). Huh7 and HCV receptor knockout cell lines were kindly supplied by Prof Yoshiharu Matsuura (Osaka University) [387]. HEK 293T CD81 KO cells were kindly supplied by Dr Joe Grove (UCL) [386].

When cells were not in culture, they were stored in liquid nitrogen. From liquid nitrogen, vials were quickly thawed in a 37^{o}C water bath, then transferred to 9 mL

of prewarmed DMEM. Cells diluted in the DMEM were centrifuged for 5 minutes at 404 RCF. Media was replaced with 15 mL of fresh DMEM and transferred to a T75 cell culture flask and placed in a 37ºC, 5% CO_2 cell culture incubator. Cells were grown to 70–90% confluence before passaging. Cells were passaged approximately 3x per week by decanting media, incubating with TrypLE Express (Gibco) for 5 minutes at 37ºC, resuspending cells in media then splitting the cells into flasks. Depending on the cell line growth rate and the next time the cells would be utilized in an assay they were split between 1:2 to 1:20.

To freeze cells, flasks were grown to approximately 90% confluence, lifted from flasks with TrypLE Express, suspended in DMEM, pelleted to remove DMEM/Tryple Express mixture. Cell pellets were then resuspended in FBS with 10% (v/v) DMSO (Sigma) and split into 1 mL aliquots in 2 mL tubes. Aliquots were then place in a cryo-freezing container (NALGENE) at -80ºC for at least 24 hours before transferring to liquid nitrogen.

2.2.2 Plasmid amplification

2.2.2.1 Transformation of competent bacteria

Aliquots (50 µL) of competent *E. coli* (Table 2.1) were removed from -80ºC and defrosted on ice. All plasmids were transformed into either NEB 5-alpha or JM 109 Competent *E. coli* with the exceptions of p8.91, which was transformed into NEB Stable *E. coli,* and pCAG_JEV_prME (Nakayama strain), which was transformed into CopyCutter™ competent *E. coli.* To perform the transformations 10–100 ng of plasmid was added to the competent cells and incubated on ice for 30 minutes. Cells where then heat shocked at 42ºC for 30 seconds and returned to incubate in ice for 5 minutes. Cells were then supplemented with 950 µl of SOC

outgrowth media (supplied by manufacturer) and incubated at 37ºC (or 30ºC for NEB stable), 250 rpm, for 1 hour. Following incubation, 150µl of the mixture was then spread onto a LB agar plate supplemented with the antibiotic corresponding to the plasmid's resistance marker and incubated at 37ºC for approximately 18 hours. All plasmids used in the project have ampicillin resistance, thus the agar plates contained 100 µg/mL of carbenicillin (Sigma-Aldrich).

2.2.2.2 Propagation of plasmids

Colonies of cells containing plasmid were picked from the LB agar plates with a pipette tip and placed in Luria Broth supplemented with 100µg/ml of carbenicillin. The volume of Luria Broth used is seen in Table 2.10 and corresponds with the intended plasmid purification method and whether it was a high copy or low copy number plasmid. The exception being transformed cells containing low copy plasmids that were to be purified by maxi prep. In that instance the colony was placed in 5mL LB with carbenicillin for approximately 18 hours. One mL of the culture was transferred to 500mL of LB with carbenicillin and incubated at 37ºC, 250 rpm. This culture was monitored until the optical density reached a value between 0.6 and 1; then 2.5mL of chloramphenicol at a concentration of 34mg/mL was added. The culture was placed back into 37ºC, 250 rpm for approximately 18 hours.

Table 2.10. Volume of bacterial culture used for plasmid preparation

PURIFICATION METHOD	HIGH COPY NUMBER PLASMID	LOW COPY NUMBER PLASMID
MINI PREP	5mL	5mL
MIDI PREP	25mL	100mL
MAXI PREP	100mL	500mL

2.2.2.3 Purification of plasmids

The bacterial cultures were pelleted at 3000 RCF for 15 minutes. The plasmids where then purified from the bacterial cells with either a QIAGEN plasmid mini, midi, or maxi purification kit according to the manufacturer's instructions. Plasmid concentration and ratio A_{260}/A_{280} was determined with a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific). Plasmids were stored at -20ºC.

To determine if the scale of the plasmid preparation affected the yield of pseudotyped viruses produced, I prepared retroviral PVs using HCV E1E2 (low titre PV) and VSV-G (high titre PV) with all the plasmids prepared with either mini, midi, or maxi prep (Figure 2.1). Little difference was observed for the low titre PV; however up to 9-fold difference in infectivity was observed between mini and midi or maxi preps for the high titre PV, at the higher dilution points. Nevertheless, as the yield of high titre PV produced with the plasmids purified with the mini scale were still within an acceptable working range, in this thesis plasmids were produced by all 3 preparation methods.

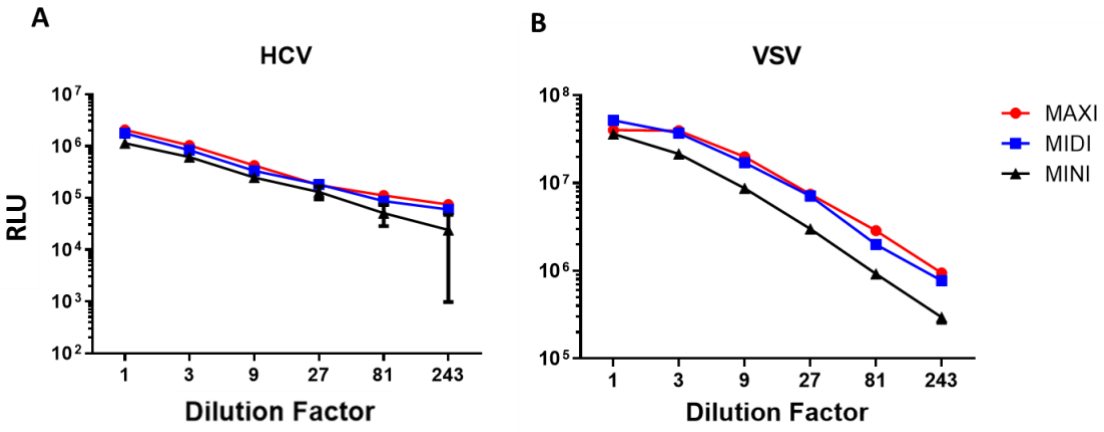


Figure 2.1. Effects of the scale of plasmid preparation on PV titre

(A) *HCV PV and* ***(B)*** *VSV PV with MLV cores and luciferase reporter gene were created with the plasmid encoding the structural and enzymatic (gag-pol) genes*

of MLV, pCMVi, the transfer plasmid carrying the luciferase reporter gene, pCFCR-Luc, and the envelope plasmid. All plasmids were purified by either Maxi kit (Red), Midi kit (blue), or Mini kit (black). PVs were titrated on Huh7.5 cells and infectivity is expressed as luciferase activity in relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate.

2.2.3 Cloning

2.2.3.1 Synthesized Genes

Genes (Table 2.11) were synthesised by GeneWiz (Leipzig, Germany). Restriction sites were added to the sequence for cloning into the expression vector indicated.

Table 2.11. Synthesized genes

Gene	**5' Cloning site**	**3' Cloning site**	**Expression vector**
GBV-B E1E2_OP	NehI	NsiI	pCAGGS
Marmoset CLDN1	BamHI	SalI	pDUAL
Armadillo CLDN1	BamHI	SalI	pDUAL
Rabbit CLDN1	BamHI	SalI	pDUAL
Guinea pig CLDN1	BamHI	SalI	pDUAL

2.2.3.2 cDNA PCR amplification of genes

To clone human CLDN6 and CLDN9 genes, RNA from 7 million Huh7 cells was extracted with RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was then synthesized from 5µg of the RNA with Superscript IV transcriptase kit (Invitrogen) according to manufacturer's instructions.

CLDN6_F, CLDN6_R, CLDN9_F, and CLDN9_R primers (Table 2.12) were used with Taq polymerase as indicated in Table 2.13 to amplify the genes from the cDNA. Primers were designed to add a BamHI restriction site at the 5′ and a SalI at the 3′ end of each gene for subcloning into pDUAL_GFP (highlighted in Table 2.12). The PCR was performed using 2µg of cDNA and the cycling conditions seen in Table 2.14 in a Veriti thermal cycler (Applied Biosystems). To ease the subcloning, 3µl PCR products containing the gene of interest were initially subcloned into the pGEM-Teasy vector system (Promega) according to the manufacturer's instructions.

Table 2.12. Oligonucleotides for Amplifying Genes from cDNA

Gene	Forward Primer (5'➞ 3')	Reverse Primer (5'➞ 3')
CLDN9	AAT TAG GAT CCG CCA TGG CTT CGA CCG GCT TAG A	GCG GTC GAC TCA CAC GTA GTC CCT CTT
CLDN6	AAT TAG GAT CCG CCA TGG CCT CTG CCG GAA TGC A	GCG GCC GTC GAC TCA GAC GTA ATT CTT GGT AGG GTA

*Highlighted regions are sequences used to introduce restriction sites

Table 2.13. PCR mix for amplifying genes from cDNA

Component	Volume (µL)
$MgCl_2$ (25mM)	3
PCR Buffer (10x)	5
dNTPs	8
F_primer (10µM)	2.5
R_primer (10µM)	3
Polymerase	0.25

Template	2
H_2O	26.75
Total	50

Table 2.14. PCR cycling conditions for amplifying genes from cDNA

Step	**Temperature**	**Time**
Initial denaturation	95°C	10 minutes
35 cycles of:		
Denaturation	94°C	30 seconds
Annealing	65°C	30 seconds
Extension	72°C	1 minute
Final Extension	72°C	5 minutes

2.2.3.3 Restriction enzyme digestion

All enzymatic restrictions were conducted on 2 µg of plasmid using an excess of each of the restriction enzymes (20 units/µL) in CutSmart digestion buffer in a final volume of 20 µL (Table 2.15). The reactions were conducted for 2 hours at 37°C in a water bath.

Table 2.15. Restriction Enzyme Digestion Mix

Plasmid	X µl (2µg)
5’ restriction enzyme	2.5µl
3’ restriction enzyme	2.5µl
CutSmart Digestion buffer 10x (New England Biolabs)	2µl
dH_2O	X µl
Total	20µl

2.2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments by size. Agarose gels were produced by dissolving 1% w/v ratio of agarose powder (Invitrogen) in 1x TAE by heating the mixture until transparent. Gels were poured into a cast and set at room temperature and then submerged in 1x TAE. The DNA

samples were then mixed with SYBR Safe DNA gel stain (Invitrogen) and loaded into the 1% agarose gel along with a 1kb ladder (Promega). Gels were run at 120 volts for approximately 45 minutes with a BioRad powerpack 200. Nucleic acid suspended in the agarose gel was visualized using a UltraBright LED Transilluminator (Applied Biological Materials). Where the visualized band contained nucleic acid that was to be ligated, bands corresponding to the size of the gene and vector of interest were excised from the gel using a scalpel. The DNA was purified from the agarose gel using QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. The resulting purified DNA concentration and A_{260}/A_{280} ratio was determined with a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific).

2.2.3.5 Ligation

Ligation of the digested products was performed by combining the reagents in Table 2.16 and incubating overnight at 4°C. The volume of vector backbone added is that which amounts to 100ng and gene insert volume was determined by a 3:1 molar ratio of insert to vector.

Table 2.16. Ligation Mix

	Ligation	**-ve control**
Vector	Xµl (100ng)	Xµl (100ng)
Insert	Xµl	0µl
10x Ligation buffer (NEB)	3µl	3µl
T4 ligase (NEB)	1µl	1µl
dH_2O	Xµl	Xµl
Total	30µl	30µl

Following overnight ligation, the samples were placed in a heat block for 10 minutes at 75°C. To amplify to the ligation products, 5µL of the ligation mix containing the plasmids was then transformed, plated out, and purified using a plasmid mini kit as described above. To confirm the successful ligation, plasmids underwent diagnostic enzymatic digestion, followed by Sanger sequencing performed by Eurofins, see section 2.2.6.

2.2.4 Site directed mutagenesis

Human CLDN1 cloned into pDual expression vector was kindly supplied by Dr Joe Grove, University College London. Primers in Table 2.17 were designed to introduce the mutations I32F and E48K into the CLDN1 sequence or modify chimeric proteins to the desired sequence. The Q5® Site-Directed mutagenesis kit (NEB) was used according to the manufacturer's instructions with these primers and PCR cycling conditions seen in Table 2.18 with the annealing temperatures seen in Table 2.17 to create the desired sequence. DpnI-digested sequences were used to transform chemically competent *E. coli*.

Table 2.17. Mutagenesis Oligonucleotides and annealing temperatures

Mutation	**Forward Primer (5' > 3')**	**Reverse Primer (5' > 3')**	**Annealing Temperature**
CLDN1 I32M	CCA GTG GAG GAT GTA CTC CTA TGC C	GGC AGG GCA GTG CTG ACG	69ºC
CLDN1 E48K	TGG GCG GTC ACG ATG TTG	GGC CAT GTA CAA GGG GCT GTG GA	69ºC
Chimera A > A1	GTG TGC TGG ACG GCG CAC GCC ATC	AGG GAT TAA AAT AGC CAG ACC TGC AAG AAA TAT CGC	71ºC

Chimera A > A2	GTG CTG GAC GGC GCA CGC CAT C	ACA GGA ACT AAA ATA GCC AGA CCT GCA AGA AAT ATC G	71ºC
Chimera A > A3	ACA GCA TGG ACG GCG CAC GCC ATC	GGC AAC TAA AAT AGC CAG ACC TGC AAG	72ºC
Chimera C > C1	GCC ACA GCA TGG TAT GGC AAT AGA ATC G	AAC GAG CAC CAG GAT GCC GGC	69ºC
Chimera C > C2	GCA ACA GCA TGG TAT GGC	GAT GAG CAC CAG GAT GCC	66ºC
Chimera C > C3	TGG TAT GGC AAT AGA ATC GTT C	GCA CAC AGG GAT GAG CAC	64ºC

Table 2.18. Mutagenesis PCR cycling conditions

Step	**Temperature**	**Time**
Initial denaturation	98°C	30 seconds
25 cycles of:		
Denaturation	98°C	10 seconds
Annealing	Table 2.17	20 seconds
Extension	72°C	5.5 minutes
Final Extension	72°C	2 minutes

2.2.5 Gene splicing by overlap PCR extension

Chimeric proteins containing the sequences of CLDN1 and CLDN9 proteins were spliced together using PCR driven overlap extension as described in [393]. Briefly, to splice CLDN1 and CLDN9 together, two PCRs are performed using primers A and B, and C and D. Internal primers B and C (Figure 2.2) were designed to

introduce an overlapping sequence of 10-12 nucleotides to each protein fragment that spans the junction where the proteins will be spliced together. In a second PCR the overlapping sequences created by the first PCRs are annealed together to join the sequences of two different proteins and primers A and D (Figure 2.2) will amplify the hybridized product.

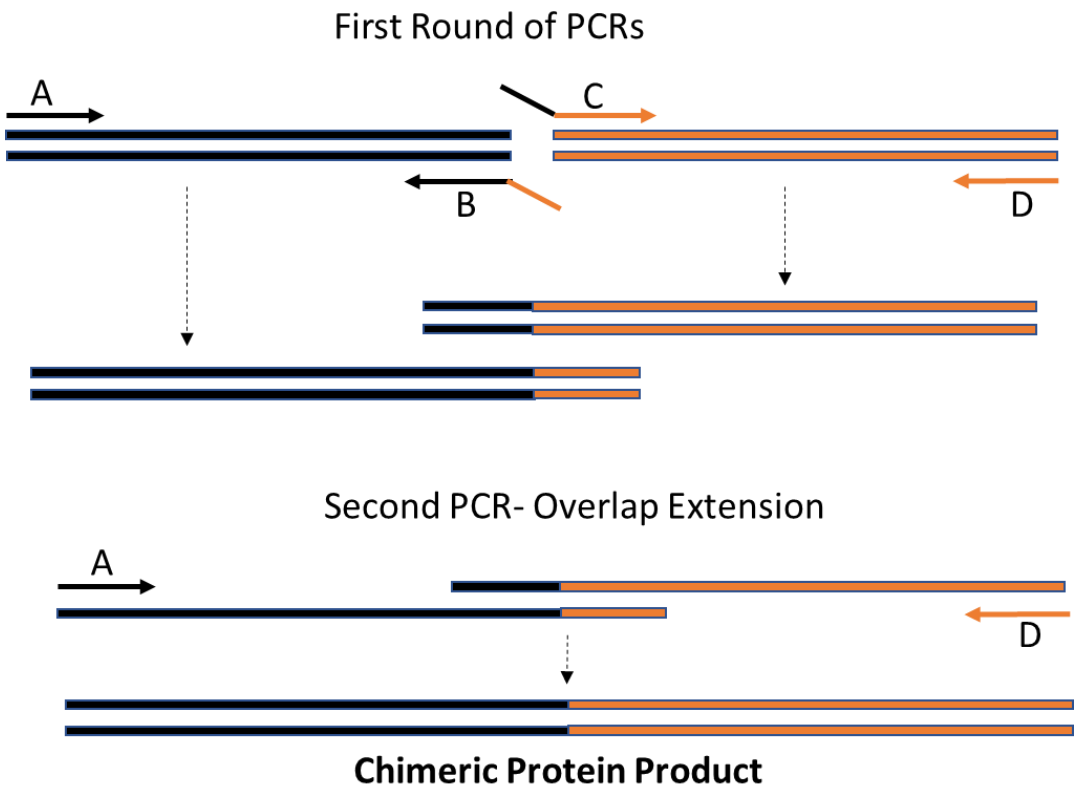


Figure 2.2. Chimeric protein production via overlap extension PCR

Diagram of the PCR steps involved in PCR overlap extension to splice genes together. Internal primers B and C used with flanking primers A and D introduce an overlapping sequence to the PCR products which are then used to anneal the two products and primers A and D amplify the hybridized product.

Primers A and D contain the restriction sites, BAMHI and SalI respectively, for the subcloning of the chimeric protein into pDUAL-GFP plasmid. The PCRs were performed with KOD hot start DNA polymerase using primers and annealing

temperatures listed in Table 2.19 in a mix described in Table 2.20, and the cycling conditions seen in Table 2.21.

Table 2.19. Oligonucleotides, templates, and annealing temperature for chimeric protein production by overlap PCR extension

Construct A	Template	Primer A	Primer B	Annealing Temperature
	CLDN1	AAA AAG GAT CCG CCA TGG CCA ACG CGG GG	ATA CCA TGC TGT GCA CAC AGG GAT	62°C
		Primer C	**Primer D**	
	CLDN9	ATC CCT GTG TGC ACA GCA TGG TAT	GCG GTC GAC TCA CAC GTA GTC CCT CTT	62°C

Construct B	Template	Primer A	Primer B	Annealing Temperature
	CLDN9	AAT TAG GAT CCG CCA TGG CTT CGA CCG GCT TAG A	ACC ATC AAG GCA CGT GCG GCC	64°C
		Primer C	**Primer D**	
	CLDN1	AGG CCG CAC GTG CCT TGA TGG T	GCC GTC GAC TCA CAC GTA GTC TTT CCC	60°C

Construct C	Template	Primer A	Primer B	Annealing Temperature
	CLDN9	AAT TAG GAT CCG CCA TGG CTT CGA CCG GCT TAG A	ATA CCA TGC TGT GCA CAC AGG GAT	62°C
		Primer C	**Primer D**	
	CLDN1	ATC CCT GTG TGC ACA GCA TGG TAT	GCC GTC GAC TCA CAC GTA GTC TTT CCC	60°C

Construct D	Template	Primer A	Primer B	Annealing Temperature
	CLDN1	AAA AAG GAT CCG CCA	GAC ACA GAG GGC ACG GGT TGC TTG	66°C

		TGG CCA ACG CGG GG		
		Primer C	**Primer D**	
	CLDN9	CAA GCA ACC CGT GCC CTC TGT GTC	GCG GTC GAC TCA CAC GTA GTC CCT CTT	66°C

Table 2.20. Overlap extension PCR mix

Component	**Volume**
$MgCl_2$	2µL
PCR Buffer (10x)	5µL
dNTPs	5µL
F_primer (5pmol/µL)	3µL
R_primer (5pmol/µL)	3µL
Polymerase	1µL
Template (300ng)	1µL
H_2O	30µL
Total	50µL

Table 2.21. Overlap extension PCR cycling conditions

Step	**Temperature**	**Time**
Initial denaturation	94°C	2 minutes
25 cycles of:		
Denaturation	94°C	15 seconds
Annealing	Table 2.19	30 seconds
Extension	68°C	1 minute
Final Extension	72°C	5 minutes

2.2.6 Sequence confirmation of plasmid vectors

All gene sequences created and/or cloned were confirmed by Sanger sequencing using the sequencing primers in Table 2.22.

Table 2.22. Oligonucleotides for sequencing

Vector backbone	**Forward Primer (5' ➡ 3')**	**Reverse Primer (5' ➡3')**
pDual-GFP	GAA TTA ACC AAT CAG CCT GCT TCT C	CCA GAG GTT GAT TAT CGA TAA GC
pCAGGS	TTC TCC ATC TCC AGC CTC GGG	CCC ATA TGT CCT TCC GAG TGA

2.2.7 Pseudotyped virus production

2.2.7.1 Retroviral core Pseudotypes

To produce lentiviral or gammaretroviral pseudotyped virus, 4 million HEK 293T cells were seeded in a 10cm dish to reach 50–70% confluence. Approximately 24 hours after the cells were seeded, a DNA plasmid mix was prepared containing 1µg of plasmid encoding either MLV or HIV structural and enzymatic proteins, pCMVi or p8.91 respectively, 1µg of viral envelope protein expression plasmid and 1.5µg of transfer vector plasmid expressing a luciferase or GFP reporter gene and made up to 15µl with TE buffer. Separately, 18µl of FuGENE HD (Promega) transfection reagent was added to 200µl of prewarmed Opti-MEM and incubated at room temperature for 5 minutes. The DNA plasmid mix was added to the FuGENE HD mix and incubated at room temperature for 20 minutes. The cell media was replaced with 10mL of fresh media just before the transfection mix was added to the cells in a dropwise manner. Cells were then incubated at 37^{o}C/ 5% CO_2 for 24 hours. Cell supernatant was then replaced with the

equivalent volume of fresh media. Approximately 48 hours after transfection, supernatant was collected and filtered through 0.45µm cellulose acetate membrane (BioWhittacker). PVs contained in the supernatant were concentrated by either 1240 RCF, overnight at 4°C, or 103,586 RCF, for 2 hours at 4°C.

2.2.7.2 Vesicular stomatitis virus core pseudotypes

VSV core particles were produced as previously described [394]. Briefly, 3 million HEK 293T cells were seeded 24 hours before transfection in 10cm dishes. For the transfection 12 µg of the envelope protein of interest was added to 200 µL of Opti-MEM and then added to a mixture of 60 µL of 1mg/mL PEI in 200µl of Opti-MEM, then incubated for 20 minutes at room temperature while frequently mixing. Media on the cells was replaced with 8 mL of complete media and transfection mix was added dropwise. Cells were then incubated at 37ºC/5% CO_2 for 24 hours. The supernatant of the transfected cells was replaced with 5 mL of complete media containing Cocal GP-luciferase rVSV at a multiplicity of infection (MOI) of 0.5. Cells were incubated at 37°C for 1 hour then media was removed, cells were washed 3 times with PBS and then media was replaced with 8 mL of complete media. After 24 hours, supernatant was collected and filtered through 0.45 µm cellulose acetate membrane.

2.2.7.3 In trans pseudotype production

For in trans PV production, bald particles were produced with an HIV core as described above, but without the plasmid expressing a viral envelope in the DNA mixture. Env-containing vesicles were produced by transfecting HEK 293T cells with 3 µg of JEV or VSV envelope expressing plasmid with 15 µL of FuGENE6 as described above. Bald core particles and Env-containing vesicles contained in

supernatant were harvested 48 hours after transfection, filtered through 0.45 µm cellulose acetate membrane and were mixed together at 1:3 (v:v) ratio, respectively. They were then incubated at 37°C for 1–2 hours, with or without the presence of 8 µg/mL of polybrene. The mixture was then concentrated 10-fold at 103,586 RCF, for 2 hours at 4°C.

2.2.7.4 Dengue core pseudotypes

HEK293T cells were seeded at 4 million cells in a 10cm dish. After 24 hours 2.5 µg of DENV core replicon plasmid, 1.25 µg of DENV core plasmid, and 1.25 µg of envelope plasmid were added to 1 mL of Opti-MEM, then mixed with 25 µL of PEI (1 mg/mL). The mixture was then incubated at room temperature for 15 minutes. Media on the cells were changed and the plasmid mixture was added to the cells in a dropwise manner. After 48 hours of incubation at 37^{o}C/ 5% CO_2 the media was replaced with 13 mL of media with 10 mM of HEPES. The supernatant was collected 24 hours later and filtered through 0.2 µm cellulose acetate membrane (BioWhittacker).

2.2.8 Infectivity assays

2.2.8.1 Luciferase Reporter

To test functionality of PVs with a luciferase reporter, target cells were seeded at 15,000 cells per well in a 96-well plate and infected with 100 µL of 3-fold serial dilutions (unless indicated otherwise) of the supernatant containing PVs, with a final concentration of 4 µg/mL of polybrene. Plates were then spin-inoculated at 1240 RCF for 30 min, at 20°C. Cells were lysed 72 hours after transduction of target cells by adding 100 µl of 1:1 (v:v) mixture of phenol red free DMEM and

Bright-Glo™ substrate and analysed for luminescence on a Glomax Navigator (Promega).

2.2.8.2 Nanoluciferase reporter

Target cells were seeded at 10,000 cells per well in a 96-well plate. Cells were infected 24 hours later cells with 100µL of 3-fold serial dilutions of the supernatant containing PVs. The cell-virus mixture was incubated at 37°C for 5 hours, then the media was removed and replaced with 100 µL of complete DMEM. After 72 hours media was removed from cells and lysed by adding 100 µl of 1:1 (v:v) mixture of phenol red free DMEM and NanoGlo substrate and incubated at room temperature for 2–3 minutes. Luminescence was then analysed on a Glomax Navigator (Promega).

2.2.8.3 GFP Reporter

For PVs expressing a GFP reporter gene, target cells were seeded 10^5 per well in a 24-well plate and transduced with 200 µL of supernatant containing PVs, with a final concentration of 4 µg/mL of polybrene and incubated at 37°C for 72 hours. Cells were then detached from plate with trypsin, fixed with 4% (w/v) paraformaldehyde in PBS, and analysed for GFP on a BD FACSCANTO™ II (Becton Dickinson) using FlowJo software (FlowJo, LLC).

The titre of the GFP-PVs was determined by the following equation:

titre (transduction unit/mL) = number of cells seeded * %GFP of positive cells * dilution factor / volume of transduction (mL)

2.2.9 Neutralization assay

Target cells were seeded 24 hours prior to infection at 15,000 cells per well in a 96-well plate. Prior to infection 25 µl of 2-fold serum dilutions were added to 100 µL of the PV and incubated for 1 hr at 37°C. Next, 100 µL of PV/serum mixture was then added to target cells with a final concentration of 4 µg/ml of polybrene, spin inoculated at 1240RCF for 30 min at 20°C and then incubated at 37°C for 72 hours. Cells were then lysed by adding 100 µL of 1:1 (v:v) mixture of phenol red free DMEM and Bright-Glo™ and analysed for luciferase activity. Percentage of neutralization at each dilution compared to untreated virus and the half maximal inhibitory concentration (IC50) was determined in GraphPad Prism software, using a log[inhibitor] vs normalise response, variable slope curve.

2.2.10 Receptor inhibition assays

Huh7.5 cells were plated in 96 well plates at 15,000 cells per well 24 hours prior to assay. Increasing concentrations of CD81 antibodies in 25 µL of media were added to the cells and the antibody-cell mixture was incubated at 4°C for 1 hour. Then 50 µL of PV was added to the mixture in the presence of 4 µg/mL of polybrene. The mixture was spin inoculated at 1240 RCF for 30 min at 20°C before the media on the cells was replaced with complete DMEM and incubated at 37°C for 72 hours. Cells were then lysed by adding 100 µL of 1:1 (v:v) mixture of phenol red free DMEM and Bright-Glo™ and analysed for luciferase activity. Percentage of neutralization at each dilution compared to untreated cells was determined in GraphPad Prism software, using a log[inhibitor] vs normalise response, variable slope curve.

2.2.11 Receptor complementation

The pDUAL-GFP plasmids are lentiviral transfer vectors that express GFP and another protein of interest from different promoters. These were used to create

VSV-G pseudotyped lentiviral vectors that express GFP and the protein of interest as described in section 2.2.7.1. Infectivity and titres of the vectors were determined by GFP expression as described in section 2.2.8.3. 1 million Huh7 CLDN1 KO or HEK 293T cells were plated in 6 well plates and approximately 2 hours later were transduced with these VSV-G pseudotyped vectors at an MOI of approximately 1, in the presence of 8 µg/ml of polybrene, to create stable cell lines expressing the protein of interest.

2.2.12 Immunoblotting

Cells were detached from flasks using trypsin, resuspended in media, and pelleted to remove trypsin and media. Cells were then resuspended in lysis buffer on ice for 5 minutes as previously described [395], then centrifuged at 16,060 RCF for 5 min and supernatant collected for analysis. Samples were boiled for 5 min, and proteins were separated by SDS-PAGE in a 4–20% tris-glycine gel (Thermo Fisher). Proteins were transferred to a PVDF membrane (Thermo Fisher) with an iBlot™ 2 Dry blotting system (Thermo Fisher). The membranes were blocked with a PBS containing 2% (w/v) non-fat milk and 0.1%(v/v) Tween-20, for 1 hr at room temperature or overnight at 4^{o}C. Membranes were then probed with a primary antibody to the protein of interest for 1 hour at room temperature. Table 2.23 shows the antibody dilutions used. After 3 5-minute washes with washing buffer (PBS with 0.1%(v/v) Tween-20, HRP-conjugated secondary antibodies were then added to the membranes to detect primary antibodies. Membranes were washed another 3 times with washing buffer and finally once with PBS for 5 minutes each. Chemiluminescence signal was then measured in a ChemiDoc™ MP Gel Imaging system (Bio Rad) or D-DiGit Gel scanner (LI-COR).

Table 2.23. Antibodies for Immunoblotting

Antibody	Dilution
Anti-CLDN1	1:1000
Anti-CLDN6	1:1000
Anti-CLDN9	1:300
Anti-β Actin- HRP conjugate	1:10000
Anti-Rabbit-HRP conjugate	1:10000

2.2.13 Confocal Microscopy

HEK 293T cells were grown to approximately 90% confluence on poly-L-lysine coverslips, then transfected with pCAGGS_JEV_prME, using Fugene6 according to the manufacturer's instructions. After 24 hours cells were fixed with 100% methanol at -20°C for 15 min. Cells were then incubated with permeabilizing blocking buffer: 5%(v/v) FBS and 0.1%(v/v) triton-X100 in PBS. JEV envelope was visualised by immunostaining using a rabbit anti-JEV E protein antibody diluted 1:100 in permeabilizing blocking buffer, at 2 hours room temperature. Cells were washed 3 times with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG at a dilution of 1:200 in permeabilizing buffer for 1 hour at room temperature. After 3 washes with PBS, cover slips were mounted on a microscope slide with FluoroShield with DAPI. Images were captured in Leica SP8 Confocal Laser Scanning Microscope and analysed using Leica LAS-AF software.

Chapter 3:

Production of GB Virus B pseudotyped virus to investigate serological responses in the tamarin animal model

3.1 Introduction

Globally, there are approximately 58 million people chronically infected with HCV [19]. The mechanisms behind how HCV persists into chronic infection are not fully understood yet. Neutralizing antibodies (NAbs) are indicated in playing an important role in viral clearance. Infections that are cleared in the acute phase are often associated with early appearance of NAbs whereas chronic infections are associated with low or absent NAbs during the acute phase of infection [66–69]. This is however not that clear cut as patients with agammaglobulinemia, an inheritable disorder characterized by very low levels of antibodies, who had no HCV specific antibodies could also spontaneously clear the virus [74]. Thus, more research into the role NAbs play in viral clearance is necessary.

Animal models, like GBV-B in new world monkeys, are useful tools for studying the immune responses. Such as in evaluating the role of NAbs in viral clearance, which may lead to key indicators of immune responses needed for viral clearance. This could inform HCV vaccine design. GBV-B, like HCV, is associated with hepatitis and is primarily found in the liver of the infected host [167,186]. Unlike HCV, GBV-B generally causes an acute infection, which is usually cleared within 6 months [167,168,188,189]. However, there are some reported cases of persistent viremia and liver pathologies similar to those associated with chronic HCV infection [182,190–192]. GBV-B cannot replicate in the immortalized cells tested thus far, which is a stumbling block to developing assays to detect and characterize the role of NAbs in viral clearance and/or persistence [396]. The use of pseudotyped viruses (PV) offers an alternative method for characterising NAbs.

Retroviruses, such as MLV or HIV, and the rhabdovirus VSV are the most common viruses employed for pseudotyping [286,326]; these viruses bud from the plasma membrane incorporating on their surface many types of protein/glycoprotein present in the cell membrane [394,397]. PVs often contain a reporter gene (*e.g.* GFP or firefly luciferase) instead of structural proteins which eases the detection of infectivity. The reporter gene is expressed after infection of target cells; retroviral PVs integrate the reporter gene into the host genome, followed by transcription and translation of the gene and reporter genes delivered by VSV PVs are expressed in the cytoplasm upon entry [286,394]. Reporter gene expression is an indicator of successful cell entry, thus reduction of expression in presence of sera can be attributed to the presence of NAbs.

HCV and GBV-B bud from the ER acquiring ER membrane containing the glycoproteins as their envelope. Assembled virions in the ER use cellular machinery to move along the secretory pathway to exit the cell through exocytosis [14,15]. Therefore, hepacivirus envelope proteins are rarely presented on the cell membrane; this makes generating pseudotyped retroviral or VSV particles a challenge. However, HCV E1E2 glycoproteins are well documented to make pseudotypes with retroviral and VSV cores [121,122,398]. HCV PV are usually low titre and for many isolates there is no detectible titre at all [284]. Establishment of these systems often requires extensive optimization to yield titres high enough for a functional assay development. There is only one published report of a hepacivirus pseudotyped virus other than HCV, which was a GBV-B PV which yielded low infectivity [399]. In this chapter I describe the production and optimization of GBV-B pseudotypes to develop a neutralization assay for screening experimentally infected tamarin sera for neutralizing antibodies to GBV-B envelope proteins.

3.2 Results

3.2.1 Development of GBV-B PV retroviral core systems

Production of GBV-B pseudotyped virus was, in the first instance, attempted with MLV and HIV core systems, in which the viral particles formed with structural and enzymatic proteins of these viruses harbour the envelope proteins of GBV-B and carry a genome that encodes a luciferase reporter gene (Figure 3.1A,B). The genes in the GBV-B envelope protein plasmid were subcloned from cDNA that was created from the viral RNA isolated from the tamarins experimentally infected with GBV-B at the National Institute of Biological Standards and Control [196]. The sequence contains the last 60 nucleotides of the core protein, which contains the signal peptide, in addition to the E1E2 genes. Hepatocytes were most likely to contain all the entry factors needed because GBV-B is a hepatotropic virus. Therefore, Huh7.5 cells, a cell line shown to be susceptible to HCV PV infection, were chosen as the target cell line in the experimental design [400]. Both these core systems have been employed for HCV PVs, therefore HCV PVs serve as the positive control for the systems [284]. The HCV envelope proteins are from HCV isolate UKN1A20.8. Both the HIV and MLV systems produced HCV PV as expected. However, GBV-B PV with infectivity significantly higher than the Env-less particles was not detected in either system.

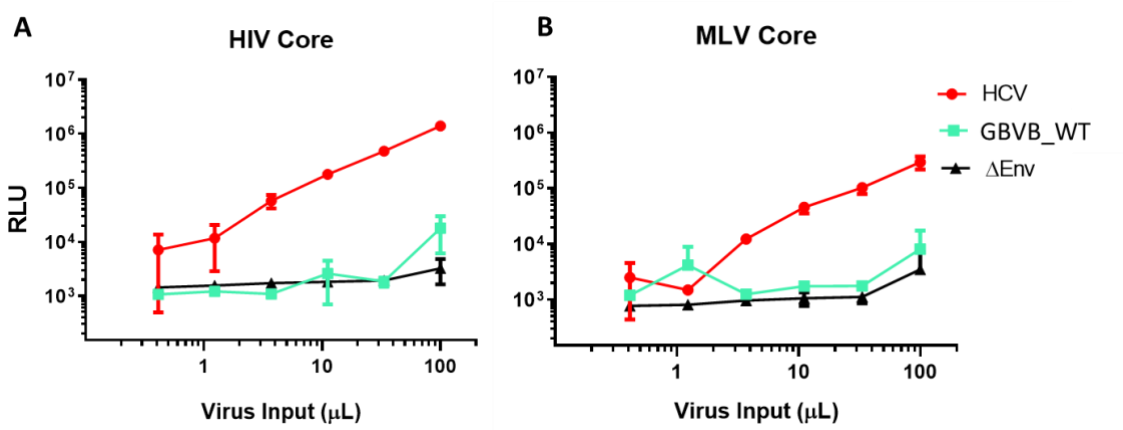


Figure 3.1. Production of HCV pseudotypes on retroviral cores

HCV-UKN1A20.8, GBVB_WT (wild type sequence) and ΔENV (the negative control without any envelope protein) PVs were produced on a retroviral vector, HIV ***(A)*** *or MLV* ***(B)****, with a luciferase reporter gene. Producer cell supernatant containing particles were titrated on Huh 7.5 cells. Infectivity is expressed as luciferase activity in relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate. These are representative of 3 independent experiments.*

To confirm the virus produced was HCV PV, an inhibition assay using a monoclonal antibody against the known HCV receptor, CD81 [346], was performed using the MLV core HCV PV ($HCV_{(MLV)}$). A dose dependent reduction in infectivity of the $HCV_{(MLV)}$ was observed, as expected, with no impact on $VSV_{(MLV)}$, a virus known not to utilize CD81 (Figure 3.2A). HEK 293T (HEK) cells do not express claudin proteins, which are necessary for HCV infection, thus it is expected they are not susceptible to HCV PV infection [34]. Therefore, the $HCV_{(MLV)}$ was titrated on HEK cells to confirm lack of infectivity (Figure 3.2B), but the HEKS were permissive for $VSV_{(MLV)}$ infection, as expected.

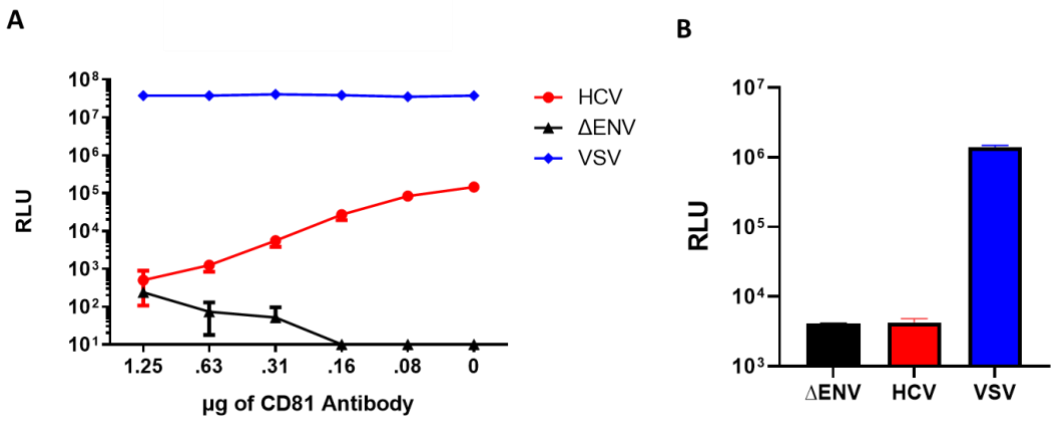


Figure 3.2. Confirmation of HCV$_{(MLV)}$ PV

HCV-UKN1A20.8 $_{(MLV)}$, ΔENV$_{(MLV)}$, and VSV$_{(MLV)}$, a virus known to infect a wide variety of cell lines and not utilize CD81 as a receptor, were used to infect ***(A)*** *Huh7.5 with decreasing concentrations of anti-CD81 antibody or on* ***(B)*** *HEK293T cells. Infectivity is expressed as luciferase activity in relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate. These are representative of 2 independent experiments.*

HCV envelope proteins produce low titre PV in comparison to many plasma membrane budding viruses, such as VSV (Figure 3.2A). I hypothesized that this may also be the case for a GBV-B PV, as GBV-B and HCV have similar lifecycles with internal acquisition of their membrane from the ER [179]. Optimizing the codon sequence to the preferred codons of the producer cells improves protein expression, often increasing PV titres [326]. Therefore, I investigated if using GBV-B E1E2 genes codon optimized for human expression had an impact on PV production (Figure 3.3). The codon optimized DNA sequence of GBVB E1E2 was synthesized (GENEWIZ) and cloned into a pCAGGS expression vector. The use of the codon optimized GBVB E1E2 resulted in the production of GBVB PV with a MLV core (GBVB$_{(MLV)}$) with similar infectivity to that of HCV$_{(MLV)}$ (Figure 3.3A).

The codon optimization failed to produce $GBVB_{(HIV)}$ with infectivity higher than the Env-less particles (Figure 3.3B). From this point onward all GBV-B PV were produced with a codon optimized sequence for E1E2 and will be termed $GBVB_{(Core)}$.

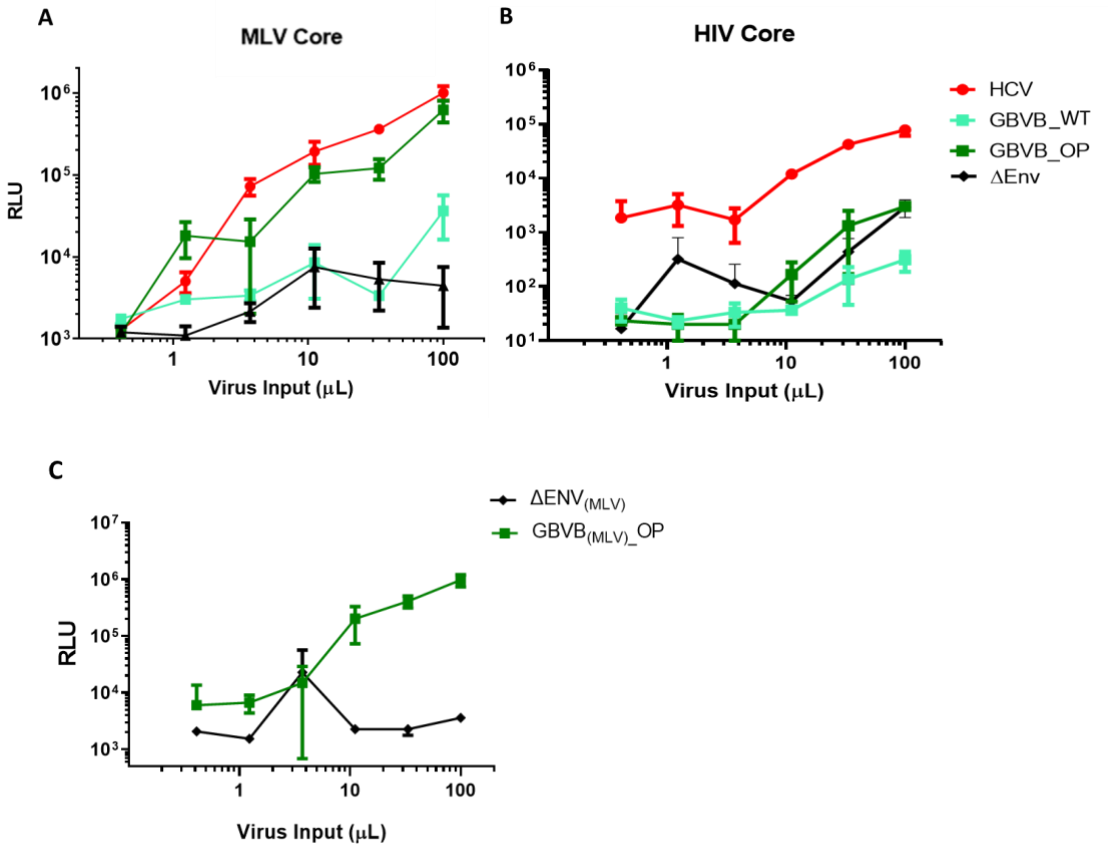


Figure 3.3. Use of codon optimized GBV-B envelope plasmid results in MLV PV formation

HCV-UKN1A20.8 (red), GBVB_WT (light green), GBVB_OP (dark green), which was produced using a plasmid with GBV-B E1E2 sequences codon optimized for human expression, and ΔENV (black) pseudotyped viruses were produced on the indicated vector ***(A,B)*** *with a luciferase reporter gene. Producer cell supernatant containing particles were titrated on Huh 7.5 cells.* ***(C)*** *Cells transduced with GBVB_OP and ΔENV MLV core PVs were kept in culture for 2 weeks and reanalysed for luciferase activity. Infectivity is expressed as luciferase activity in*

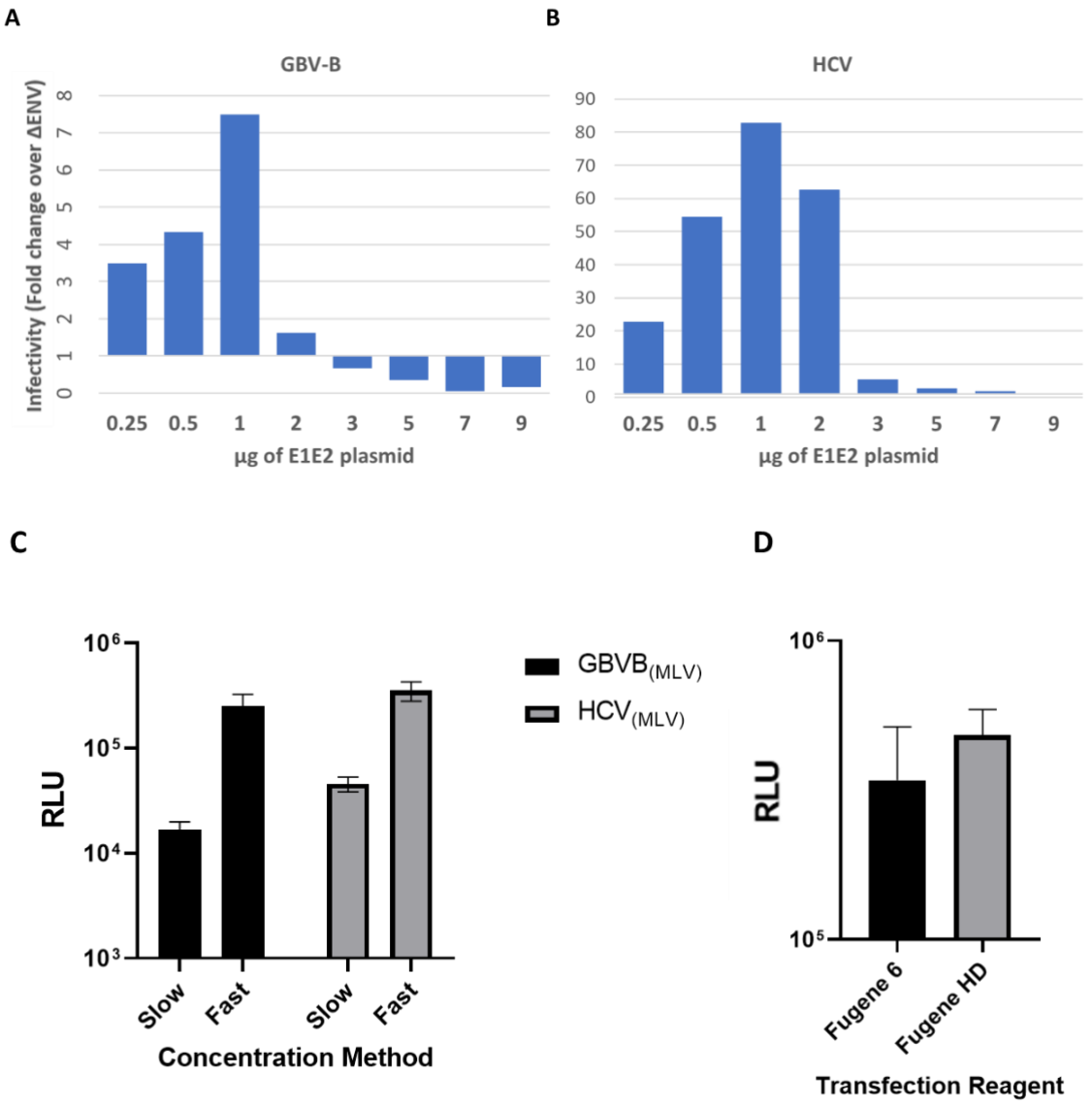
relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate.

When producing PV with a luciferase reporter gene, luciferase protein is produced in the PV-producer cells and can be present in the supernatant containing the PV. Therefore, there is the possibility that luciferase activity seen from target cells after infection may be extracellular luciferase adhering to the membrane of the target cells. To verify that the luminescence signal observed in $GBVB_{(MLV)}$ infection was due to PV entry into the target cells, cells transduced with $GBVB_{(MLV)}$ or $\Delta ENV_{(MLV)}$ vectors were kept in culture for two weeks. During that time the cells appeared to have normal growth with a doubling time of approximately 24 hours. They were passaged and provided fresh media every 2–3 days for a total of 5 times. After two weeks the target cells still produced luciferase at a similar level to that of 3 days after transduction (Figure 3.3C). This confirms that the luciferase output from target cells is largely if not all due to integration of the reporter into the target cells and not from extracellular luciferase in the PV preparation adhering to the cell surface.

3.2.2 Optimization of retroviral GBV-B PV

As GBV-B envelope proteins, similarly to HCV, produce low titre PV of approximately 100-fold higher than background, a number of methods to optimize production and titre were undertaken (Figure 3.4). Using a constant quantity of MLV packaging plasmid (1 µg) and luciferase reporter plasmid (1.5 µg), different amounts of GBV-B E1E2 plasmid were added to the transfection mix to determine which achieved the highest titre (Figure 3.4A). In the current scale of production

using 4 million HEK 293T cells in a 10cm dish, the use of 1 µg of envelope plasmid produced the highest viral transduction titre for both $GBVB_{(MLV)}$ and $HCV_{(MLV)}$. At amounts higher than 2 µg of transfected GBV-B E1E2 plasmid, infectivity falls below background; this is not seen in HCV E1E2 transfection (Figure 3.4B), possibly suggesting that GBV-B glycoproteins may be more toxic to producer the cells.



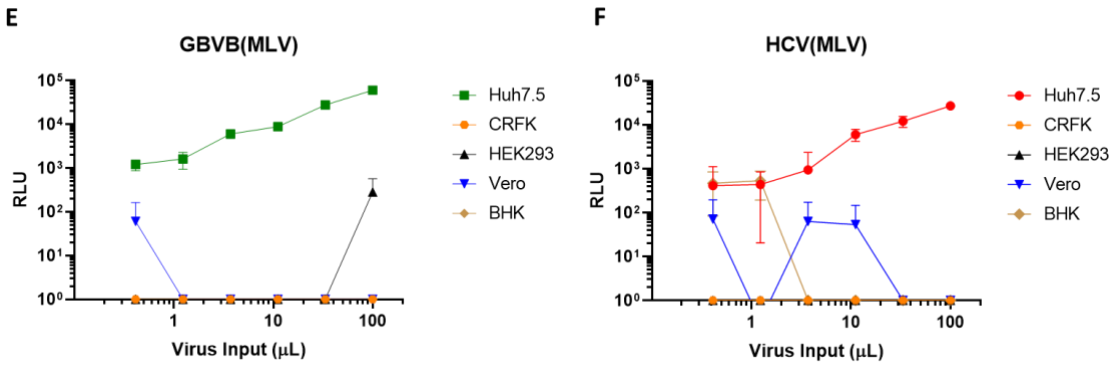


Figure 3.4. Optimization of GBVB$_{(MLV)}$ production

GBVB$_{(MLV)}$ ***(A)*** *and HCV-UKN1A20.8$_{(MLV)}$* ***(B)*** *were produced with constant amounts of MLV core (1µg) and luciferase reporter plasmids (1.5µg) and the indicated amount of GBV-B or HCV E1E2 plasmid to determine optimal plasmid ratio for PV production. GBVB$_{(MLV)}$ and HCV$_{(MLV)}$-containing supernatants were titrated on Huh7.5 cells and infectivity is displayed as fold change over negative control, a vector with no envelope protein.* ***(C)*** *HCV-UKN1A20.8$_{(MLV)}$ and GBVB$_{(MLV)}$ were concentrated 10 times by two methods: 1240 RCF for 16 hours (slow) or at 103,586 RCF for 2 hours (fast).* ***(D)*** *GBVB$_{(MLV)}$ were produced using the indicated transfection reagent and titrated on Huh7.5 cells.* ***(E)*** *GBVB$_{(MLV)}$ and* ***(F)*** *HCV-UKN1A20.8$_{(MLV)}$ expressing luciferase reporter gene were titrated on the indicated cell lines. Infectivity is displayed as relative light units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate.*

Next, I investigated what method of concentration was the most effective for GBVB$_{(MLV)}$. As some viral glycoproteins, such as those of some retroviruses [401], cannot withstand the forces of ultra-centrifugation and shear off, I investigated both ultra-centrifugation at 103,586 RCF over a short period and a slow concentration method by centrifugation of 1240 RCF for 16 hours. After centrifugation, the supernatant was removed, and the pellet resuspended in a

tenth of the original volume. For both $GBVB_{(MLV)}$ and $HCV_{(MLV)}$, ultracentrifugation for 2 hours was more than twice as efficient as the slow method (Figure 3.4C). The slow method of concentration resulted in a recovery rate of less than 50%. Infectivity for $GBVB_{(MLV)}$ increased by 4.8-fold for the slow method and the 11.6-fold for the fast method. Similarly, $HCV_{(MLV)}$ infectivity increased by 3.1 and 8.7-fold with the slow and fast methods of concentration, respectively.

FuGENE6 was chosen over other transfection reagents, such as the more cost effective PEI, because it has been shown to have very low toxicity in the producer cells, has high transfection efficiency, and minimal alteration of cellular gene expression [402]. FuGENE HD has also been reported as a high efficiency transfection reagent with minimal induction of endogenous genes [403]. Therefore, I investigated which of these two reagents yielded the highest titre $GBVB_{(MLV)}$. Although not statistically significantly, FuGENE HD produced a $GBVB_{(MLV)}$ with 1.4-fold higher infectivity than FuGENE6 (Figure 3.4D). Therefore, FuGENE HD was chosen as the transfection reagent for subsequent experiments.

As explained above, hepatocytes were chosen initially as the target cell line as GBV-B is a hepatotropic virus; however, as there were no known entry factors for GBV-B, 4 additional cell lines (Vero, CRFK, BHK and HEK293T) were investigated as potential target cell lines for GBV-B PV assays. Of the cell lines tested only the Huh7.5 were permissive to $GBVB_{(MLV)}$ (Figure 3.4E), and as expected, $HCV_{(MLV)}$, in a dose dependent manner (Figure 3.4F).

A recent study showed that production of HCV PV using CD81 knockout (KO) HEK producer cells afforded higher titres than the parental HEK cell line [386]. These CD81 KO cells were kindly supplied by Dr J Grove (University College

London) and used to investigate if they improved the titre of $GBVB_{(MLV)}$ (Figure 3.5). The infectivity of $GBVB_{(MLV)}$ produced in the CD81 KO cells was 2.85x higher than those produced in the parental HEKs (Figure 3.5A). As positive control, $HCV_{(MLV)}$ using 3 different HCV E1E2 isolates were also produced in the CD81 KO cells. As previously reported, PVs for the two HCV strains, J6 and H77 produced in the CD81-KO cells have higher infectivity, 1.52 and 6.5-fold higher, respectively. Patient isolate UKN120.8, however, had slightly lower infectivity with a decrease of 11.5%, which was consistent with small number of patient isolates produced in the CD81 KOs that yielded lower infectivity [386]. As I could not previously produce an HIV core GBV-B PV, I sought to determine if the higher infectivity I observed with the $GBVB_{(MLV)}$ using the CD81 KO cells would translate into a $GBVB_{(HIV)}$ detectable over background. However, the CD81 KO cells failed to yield a detectable $GBVB_{(HIV)}$ (Figure 3.5B).

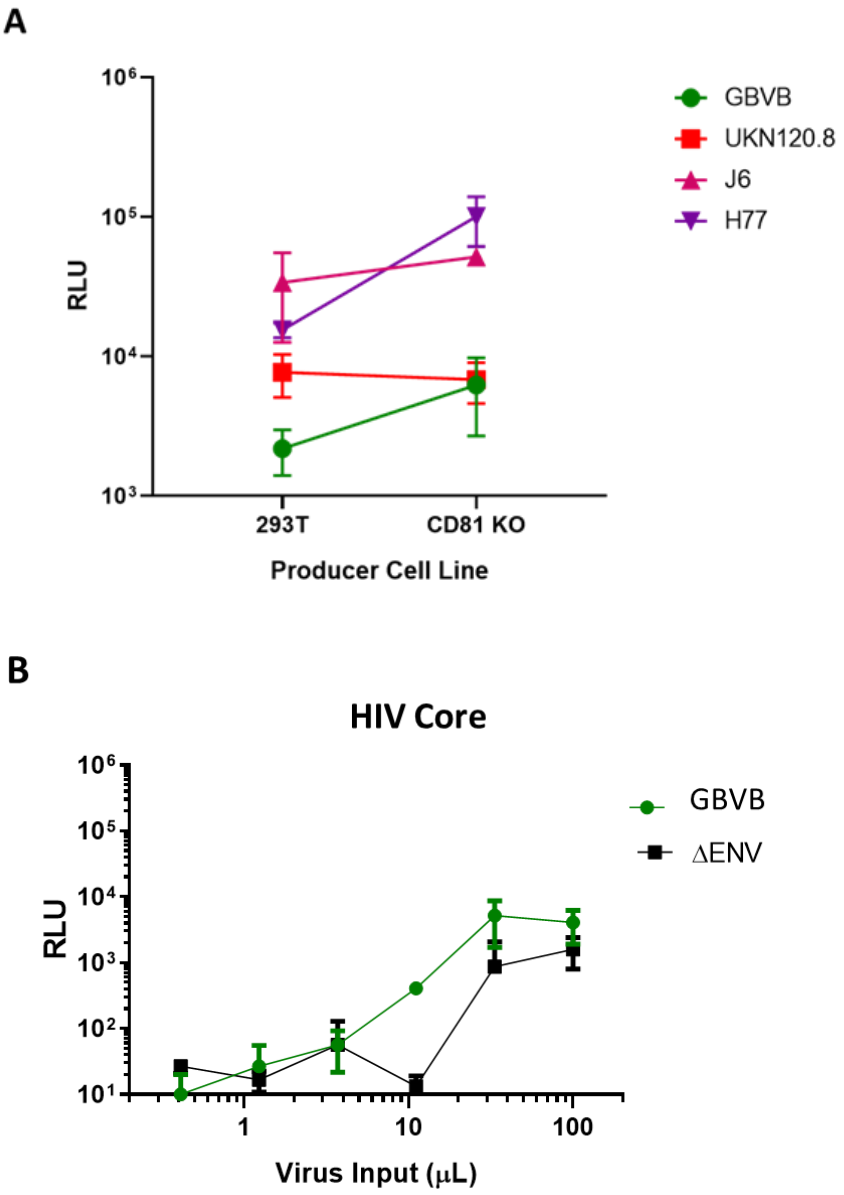


Figure 3.5 Effects of CD81 KO cells on GBVB$_{(MLV)}$ production

(A) *The indicated PV were produced in HEK293T parental cells or HEK293T CD81 KO cells with an MLV core and luciferase reporter and titrated onto Huh7.5 cells.* ***(B)*** *GBVB and ΔENV PV were produced in CD81 KO cells with an HIV core and luciferase reporter and titrated onto Huh7.5 cells. Infectivity is expressed as luciferase activity in relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate.*

3.2.3 Development of GBV-B PV with VSV core system

In parallel with developing the GBV-B PV system with a retroviral core I attempted to produce a GBV-B PV with a VSV core ($GBVB_{(VSV)}$). The VSV core system can have many benefits over the traditional retroviral cores, including potentially higher titre PVs. As $GBVB_{(MLV)}$ is a relatively low titre producing PV it would be advantageous to produce a higher titre PV with a VSV core. VSV PV are produced by transfecting producer cells with a plasmid that contains the viral envelope proteins of interest, then 24 hours later infecting those cells with rVSV at an MOI of .5, then harvest the PV containing supernatant 24 hours later. rVSV has been engineered to have the gene encoding its envelope protein deleted and replaced with a reporter gene, in this thesis it is firefly luciferase. GBV-B and HCV PVs with VSV core produced infectivity that was only approximately two times higher than background (Figure 3.6A). In an attempt to reduce the background I decided to produce the $GBVB_{(VSV)}$ by using less rVSV than was used in the initial experiment; starting with an MOI of .3 and reducing down to .01 with a constant amount of GBVB E1E2 plasmid (Figure 3.6B). At each MOI tested, infectivity was below that of background, indicating that possibly the GBVB E1E2 proteins may be toxic to the producer cells. To mitigate toxicity to the cells, should the glycoproteins be toxic to the cells, $GBVB_{(VSV)}$ was produced with a constant MOI and varying amounts of GBVB E1E2 plasmid (Figure 3.6C). An optimized protocol for rVSV PV production in our lab for multiple viruses uses 3 million HEK cells and 12 µg of viral envelope plasmid. As seen in Figure 3.4A higher amounts of GBVB E1E2 plasmid resulted in PV that fell below background levels, which is also observed in Figure 3.6B. Therefore, with the constant MOI of 0.5 of rVSV, I used a range of GBVB E1E2 plasmid starting with 1 µg up to a maximum of 12 µg. However, none of the conditions produced a PV with significant infectivity

over the ΔENV control (Figure 3.6C). With further optimization the VSV system could potentially be used to produce higher titre pseudotyped virus. However, due to time constraints, for the purpose of this project all GBV-B PV from this point were produced using the MLV core.

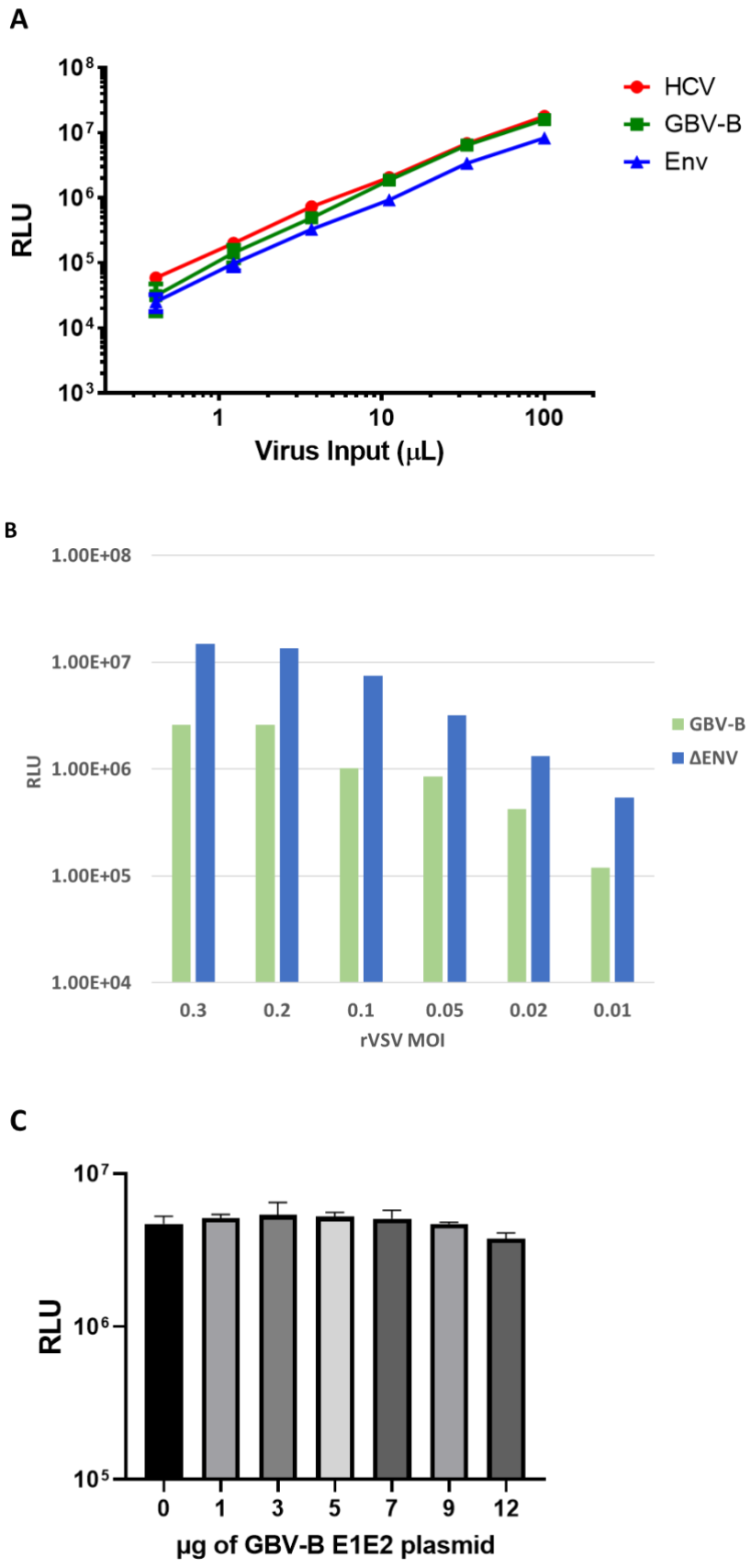


Figure 3.6. Production of GBVB and HCV pseudotyped viruses with VSV cores

(A) *VSV core-based HCV- UKN1A20.8, GBV-B, and ∆ENV PV were produced using a rVSV MOI of 0.5. Producer cell supernatants containing PV were titrated on Huh 7.5 cells.* ***(B)*** *GBVB and ∆ENV PV with a VSV core were produced using the indicated rVSV MOI. Huh7.5 cells were infected with 100µl of undiluted PV.* ***(C)*** *GBVB with a VSV core were produced with a rVSV MOI of 0.5 and the indicated mass of GBVB E1E2 plasmid. Producer cell supernatants containing PV were titrated on Huh 7.5 cells and infectivity is expressed as luciferase activity in relative luminescence units (RLU). RLU values are mean +/- standard deviation of one experiment in triplicate.*

3.2.4 Neutralization assay development to investigate Tamarin sera

A critical aspect of characterizing the new world monkey/GBV-B animal model for HCV is to determine the role neutralizing antibodies play in the clearance of the virus. At the National Institute for Biological Standards and Control (Potters Bar, UK), I was given access to serum samples from tamarins (*Saguinus labiatus*) that had been experimentally infected with GBVB. Viral load information was collected at multiple timepoints for these animals for over a year as well as after re-challenge with the virus [196]. Using the $GBVB_{(MLV)}$ I developed a neutralization assay to determine if the sera samples contained antibodies that specifically neutralized $GBVB_{(MLV)}$. The pseudotyped virus neutralization assay (PVNA) was performed with 100 µl of $GBVB_{(MLV)}$ in the presence of tamarin sera in a 2-fold dilution series with a startling dilution of 1:20. Where sample volume allowed, a PVNA was also performed with $VSV_{(MLV)}$, as an irrelevant virus, to demonstrate specificity to GBVB E1E2. Figure 3.7 shows the viral load of one animal (V2)

experimentally infected with GBV-B with green arrows above time points indicating where neutralizing antibodies were found in the serum sample and black arrows where the presence of NAbs could not be determined. Additionally, samples collected at days 378, 382, and 399, which are not shown in Figure 3.7, as there was no viral load data for that time point, were also tested.

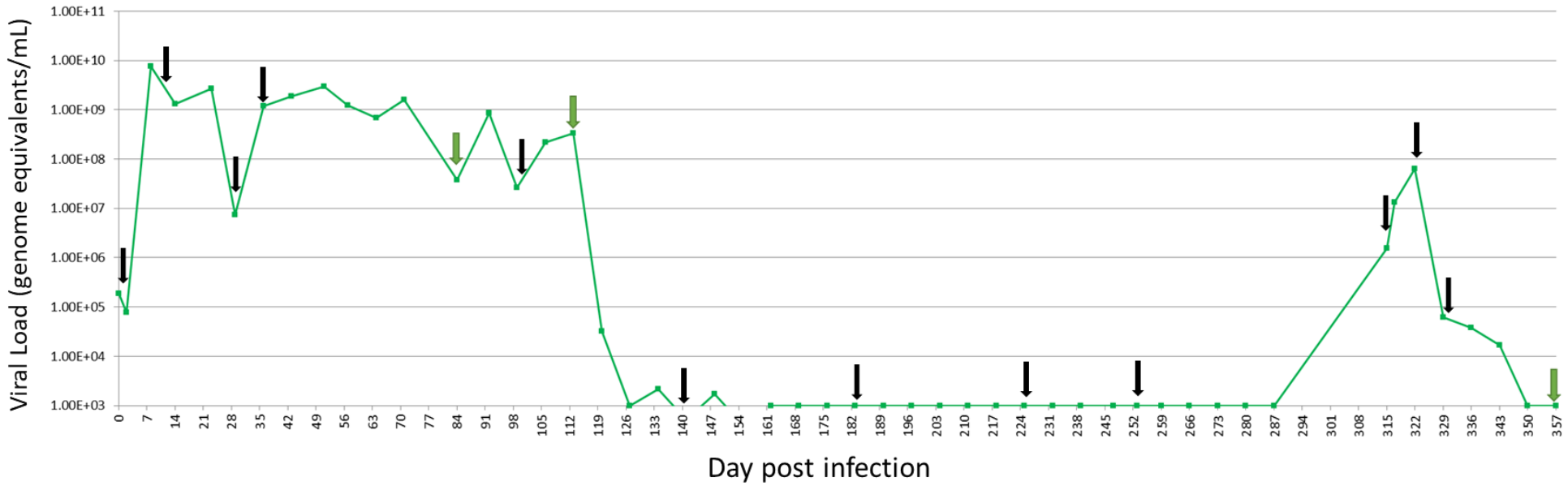
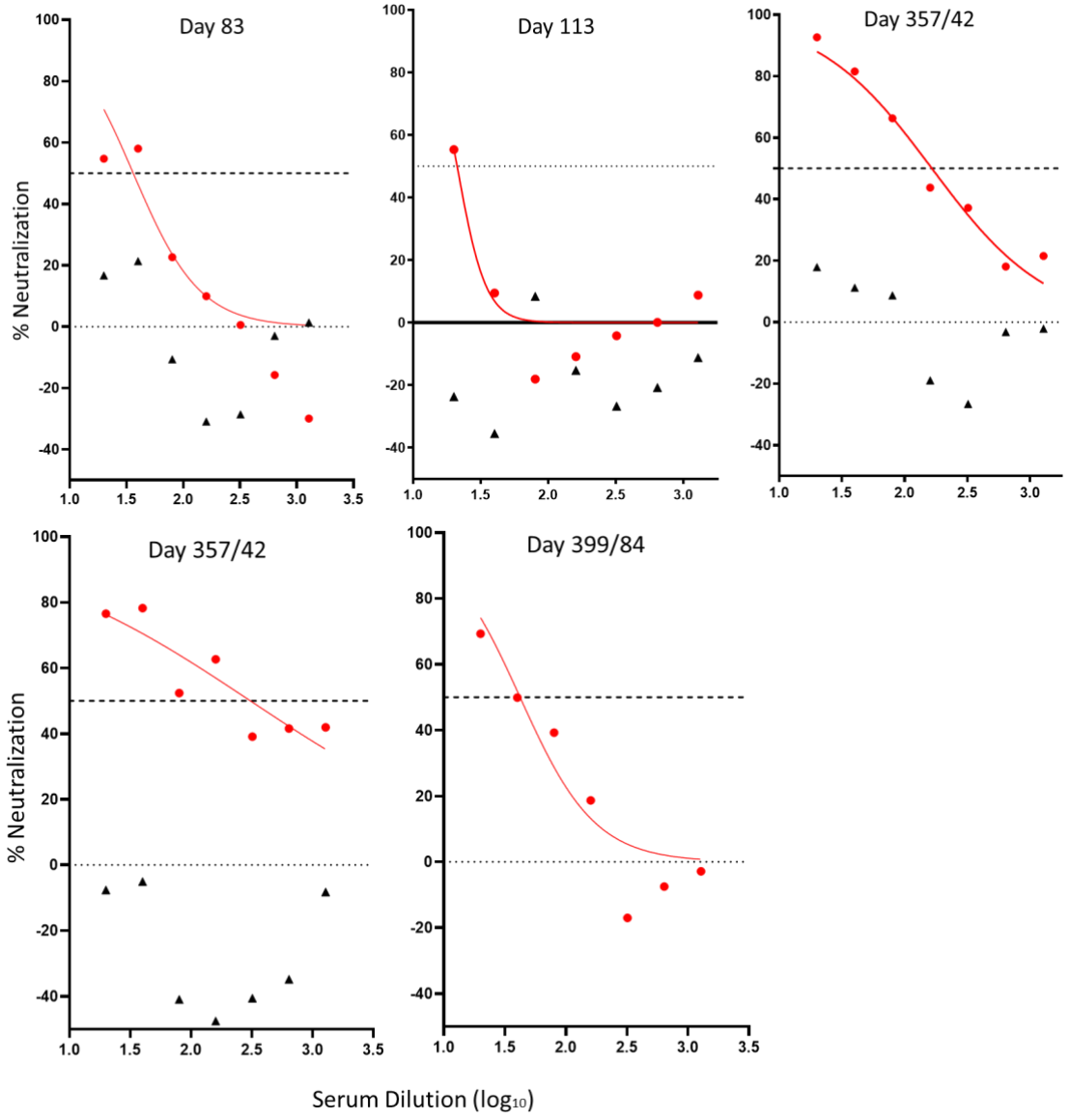


Figure 3.7. GBVB viral loads of infected tamarin V2

Tamarin V2 was inoculated with 10^7 genome equivalents of GB Virus B at day 0 and rechallenged at day 315. Viral load in genome equivalents/mL at the indicated time points is shown. Green arrows are indicating the time points in which specific neutralizing antibodies to $GBVB_{(MLV)}$ were detected and black arrows are at time points tested but no neutralization was observed. This viral load data was taken from the Dale et al., 2020 [196].

Figure 3.8 shows the dose dependent neutralization of $GBVB_{(MLV)}$, but not $VSV_{(MLV)}$, in the presence of decreasing amounts of infected tamarin serum. Day 357 had enough sample to be tested twice (Figure 3.8). Both samples show specific neutralization of $GBVB_{(MLV)}$. The difference in the observed neutralization curve could be possibly due to the sample being stored at $4^{o}C$ for several months between the respective PV neutralization assays, which could have led to degradation of the antibodies. As these samples were surplus to other studies, most samples had very limited volume. Therefore, sample day 399 was tested against $GBVB_{(MLV)}$, but sample size did not allow testing against the irrelevant virus. Therefore, the neutralization observed cannot be confirmed to be specific to $GBVB_{(MLV)}$.

A


Day 83
Day 113
Day 357/42
Day 357/42
Day 399/84
% Neutralization
% Neutralization
Serum Dilution (log10)

B

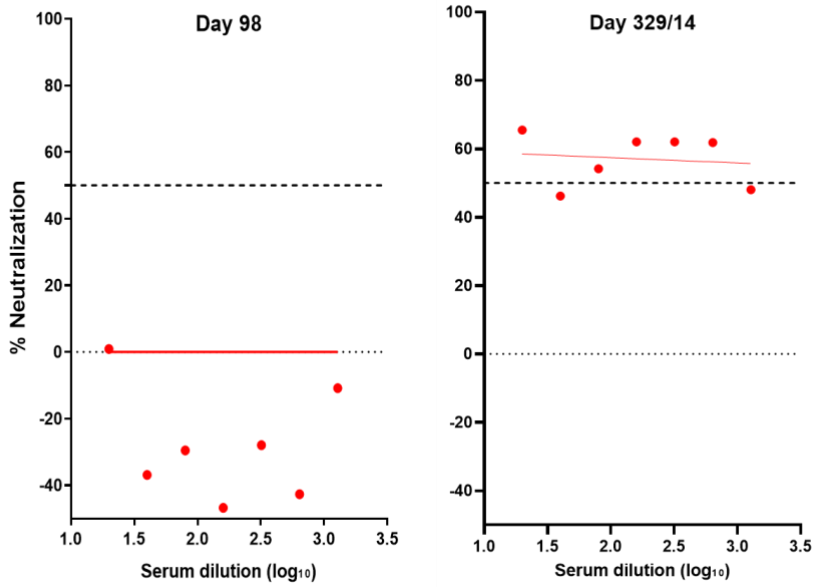

Day 98
Day 329/14
% Neutralization
Serum dilution (log10)
Serum dilution (log10)

Figure 3.8. Neutralization of GBVB$_{(MLV)}$ with sera from infected tamarin V2

Neutralizing antibody assay was performed by infecting Huh7.5 cells with GBVB$_{(MLV)}$ (Red) and VSV$_{(MLV)}$ (Black) after incubation with decreasing amounts of serum from a tamarin experimentally infected with GBV-B. Input RLU of VSV$_{(MLV)}$ was normalized to match the RLU of GBVB$_{(MLV)}$. (A) Samples show neutralization of GBVB. (B) Examples of samples that do not show specific neutralization of GBVB.

The samples shown here are those in which the neutralization curve fit for the assay obtained an R^2 value of greater than 0.6. Samples that did not show specific neutralization of GBVB$_{(MLV)}$ or where the R^2 value was below 0.6 were deemed negative. Figure 3.8B shows 2 examples of negative samples- Day 98 did not show any GBVB$_{(MLV)}$ neutralization and Day 329 shows neutralization at all points but not in a dose dependant manner; therefore, the neutralization observed is likely attributed to nonspecific inhibitors in the sample. The lack of neutralizing antibodies found in the 12 negative samples (Figure 3.7 black arrows) at time of the PVNA do not necessarily mean they were not there at the time of collection. As these samples have been used in multiple studies over many years, they have undergone multiple rounds of freeze/thaw, different storage conditions, and possible other unknown factors such as dilution. There is a possibility that antibodies that were once present have since degraded. Therefore, a negative result may not be reflective of neutralizing antibodies in newly obtained samples. The ability of the PVNA with the GBVB$_{(MLV)}$ to detect neutralizing antibodies specifically to GBVB E1E2 and produce an IC_{50} titre is proof of concept that this system can be used to evaluate the role of neutralizing antibodies in the role of GBV-B clearance in infected tamarins. Table 3.1 shows

the IC50 titres of serum samples in Figure 3.8. The titre for day 357 is the mean of the two PVNA.

Table 3.1. GBVB Infected Tamarin V2 Sera Titre

Day Post Infection	IC_{50} Titre
83	30
113	21
357	237
399	42

3.3 Discussion

Although GBV-B has been used as a model in new world monkeys for acute HCV infection, until now, there has not been a neutralization assay developed to investigate the role of neutralizing antibodies in virus clearance. This development was largely hampered by the fact that GBV-B does not replicate in immortalized cells [184]. To circumvent this issue here I developed a retroviral pseudotyped virus harbouring the full-length GBV-B E1E2 glycoproteins.

Both HIV and MLV core failed to produce GBV-B PVs with wildtype E1E2 sequences above background levels, differently from HCV PVs. Codon optimization of the gene of interest is known to increase protein production [326,404] and is a commonly used technique in generating PVs [399,405]. Therefore, the GBV-B E1E2 genes were codon optimized for human expression and resulted in successful PV generation for $GBVB_{(MLV)}$ but not $GBVB_{(HIV)}$ PV. This is consistent with the one published study of a GBV-B pseudotype that reported production of a MLV but not HIV core GBV-B PV with a codon optimized E1E2 sequence [399]. The ability of GBV-B glycoproteins to pseudotype MLV but not a HIV cores was not entirely surprising as glycoproteins of some HCV isolates have been shown to pseudotype MLV cores but not HIV cores [284]. It is not known why this occurs, however the slight differences in the assembly of the core virus may influence whether it colocalizes with the glycoproteins. Similarly, genetic differences in glycoproteins may also affect cellular location.

GBVB E1E2 produced low titre PV, therefore several parameters in the production were evaluated to increase the yield. Some have speculated that HCV PV production is achieved through overexpression of the envelope proteins that move to the plasma membrane by ‘leaking’ from the ER which allows for PV

production [122]. Therefore, I hypothesized that increasing GBV-B E1E2 envelope plasmid input into the producer cells may result in this 'leaking', which would enable higher titre PV production. However, increasing the amount of GBVB E1E2 expression plasmid in the transfection did not correlate with higher PV titre. The initial amount, 1 µg, produced the highest titre PV for both GBVB and HCV. A caveat to this experiment is that there was no confirmation that increased plasmid input results in increased protein production. This was hampered by the lack of commercially available antibodies to GBV-B E1E2 proteins. Alternatively, expression of the MLV core proteins or the luciferase-coding vector RNA could have been analysed at different GBV-B envelope plasmid concentrations to determine if this had effects on the production of the core particles. Contrary to the 'leaking' theory, others have demonstrated that it may be more likely that HCV PV assembly occurs intracellularly rather than at the membrane [313]. Indeed, retroviral Gag protein is known to assemble intracellularly in some cell types and HCV has been shown to colocalize with Gag not at the plasma membrane but in late endosomes [313,317,318]. Furthermore, it was shown that the glycosylation patterns of the HCV glycoproteins found at the cell surface was different from that of the glycoproteins on infectious lentiviral particles [321]. Therefore, it is entirely possible, as GBV-B shares a similar lifecycle to HCV, that GBV-B PV may assemble intracellularly and that increasing 'leaking' to the cell surface does not enhance PV production.

Further optimization of the protocol for the production of $GBVB_{(MLV)}$ determined that concentration with ultracentrifugation for 2 hours was more than twice as efficient than a slow concentration over 16 hours. This indicates that unlike some lab adapted HIV strains [401], GBV-B (and HCV) envelope proteins can withstand ultracentrifugation forces. Additionally, while not significant, $GBVB_{(MLV)}$ PV

produced with FuGENE HD had higher infectivity than those produced with Fugene6. It may be worth investigating other transfection reagents to determine the effect on PV titre. As GBV-B is a hepatotropic virus in new world monkeys, Huh7.5, a hepatocyte derived carcinoma cell line that are susceptible to HCV infection were the natural choice as a target cell line. However, the natural host of GBV-B is unknown and other members of *Flaviviridae* such as JEV are known to infect multiple cell lines originating from different species and cell types [222]. Similar, to HCV, dose dependent $GBVB_{(MLV)}$ infection was only observed in the Huh7.5 cells. $GBVB_{(MLV)}$ and $HCV_{(MLV)}$ infectivity was observed in some other cell lines, however infection did not titrate in a dose dependant manner. Thus, these points of infectivity for both GBVB and HCV could be attributed to nonspecific uptake of the viral particles, which is documented in the literature for both MLV and HIV vectors [406]. Lastly, producing $GBVB_{(MLV)}$ in CD81 knockout cells produced particles with an average infectivity of 2.85 times higher than parental HEKs. This is similar to what has been seen with HCV PVs produced in these cells, however the mechanism by which this occurs is unknown [386]. Although higher infectivity was seen with the MLV core, the use of these producer cells failed to yield a $GBVB_{(HIV)}$ PV with infectivity detectible above background. Due to the unknown mechanism by which the CD81 KO cells yield higher titres it is difficult to speculate why they failed to produce $GBVB_{(HIV)}$. However, if there is a mismatch in cellular location between the envelope proteins and the HIV core components, this is likely still the case in these cells.

I also attempted to create $GBVB_{(VSV)}$ PV. Initial attempts with the VSV core yielded $HCV_{(VSV)}$ and $GBV\text{-}B_{(VSV)}$ with infectivity approximately 2 times higher than background. While this was not sufficient for neutralization assay development, it was encouraging and prompted further investigation. Neither

varying the amount of GBV-B E1E2 plasmid nor reducing the amount of rVSV put into the system were successful in creating higher titre $GBVB_{(VSV)}$ and resulted in titres lowers than the env-less background levels. One explanation for this is that the GBVB glycoproteins may be toxic to the cells, which has been seen for Ebola virus glycoproteins [407,408]. Alternatively, competition for cellular machinery to produce the envelope protein may lead to a reduction in VSV core being produced. The inability of HCV glycoproteins to efficiently pseudotype the VSV core is contradictory to studies that indicate otherwise [398]. However, this might be down to the use of glycoproteins of different strains; as mentioned above, some isolate glycoproteins will pseudotype HIV and some will not. Further optimization could potentially result in $GBVB_{(VSV)}$, however as the $GBVB_{(MLV)}$ system has been optimized that system was used going forward.

Following the evaluation of the conditions examined, in the GBV-B neutralisation assays, PVs were produced in CD81 KO cells, with MLV cores, using a ratio of 1:1:1.5 (Env, gag pol, transfer vector) and concentrated by ultracentrifugation. The $GBVB_{(MLV)}$ PV was successfully used to perform neutralization assays with sera from a tamarin experimentally infected with GBV-B. The results of these assays showed specific neutralization of $GBVB_{(MLV)}$ but not an irrelevant virus, $VSV_{(MLV)}$. From the neutralization curves, the IC50 of the samples could be determined. This is proof of concept that the $GBVB_{(MLV)}$ is an appropriate system for serological investigations. To date, the antibodies detected in experimentally infected animals have not been against the glycoproteins, but to core and non-structural proteins [182,186,188,199,200]. As PV only contain the glycoproteins of GBV-B, the results presented in this chapter show that antibodies to E1E2 are raised during infection and likely play a role in the clearance of the virus at least in some cases. The sera used for these neutralization assays were from a study

that tracked viral loads within the animal over an extended period of time. With this newly developed GBV-B PV neutralization assay at each of these time points sera can be analysed in studies like that one to determine if the presence of neutralizing antibodies has an effect on viral load and pathology seen in the animals. Further to that, with the sequences of isolated viruses at different time points, new PV can be generated to investigate if mutations acquired over the course of infections are rendering neutralizing antibodies less effective, particularly in the few cases that have established chronic infection. In HCV infection, the high mutation in the glycoproteins leads to genetically and antigenically diverse quasispecies that aid in immune evasion [96,409]. This results in NAbs lagging behind the current circulating viruses [95]. However, as GBV-B infection is rarely associated with chronic infection, it would be interesting to determine if an early, strong NAb response plays a role in the rapid clearance of the virus and whether this response is delayed in cases where persistent infections are observed. Elucidating the immune responses that lead to clearance of GBV-B in the acute phase of infection and how these responses are different to what is observed in HCV infection may give further clues as to why the majority of HCV infection lead to chronic infections while GBV-B does not.

Chapter 4:

Claudin 1 is shared entry factor between GB virus B and HCV

4.1 Introduction

The hepacivirus genus includes Hepatitis C virus (HCV), a significant human pathogen associated with nearly 300,000 deaths a year [19]. GB virus B (GBV-B) was the second hepacivirus described and long remained the only HCV homolog known until another animal hepacivirus was isolated from the nasal swab of a dog with a respiratory illness in 2011 [159]. Since then, exploration of other animals as potential hosts has uncovered hepaciviruses in a diverse range of species [160,171,173–177]. Studies on these related viruses, including GBV-B, have highlighted the broad hepacivirus host range and offered some clues about the origins of HCV [3]. Indeed, characterization of the life cycle of these viruses and the identification of other hepaciviruses could be the key to understanding their cross-species transmission and zoonotic potential. Furthermore, animal models of hepaciviral disease could be important surrogate models for HCV disease pathology, and for screening the efficacy of vaccine candidates [170].

The hepatotropic nature of most mammalian hepaciviruses described to date suggests these viruses (or common ancestors) have undergone a high degree of evolutionary adaptation to the liver. Indeed, the 5′ untranslated regions of most mammalian hepacivirus sequences contain putative binding sites for microRNA-122, which is liver-specific [3]. From this, it can logically be inferred that hepaciviruses may have evolved to co-opt identical/similar cellular host factors for efficient propagation in the liver. Therefore, the specific molecular interactions of HCV with host factors may be conserved in other hepaciviruses, for which there are currently no full-length replicative *in vitro* systems with replication competent viruses.

As viruses are obligatory parasites, they need to enter host cells to replicate. To this end, HCV has evolved a complex mechanism for cell entry, involving at least four host factors: CD81, SR-B1, claudin-1 (CLDN1), and occludin (OCLN) [31–33,410]. Many other factors have been implicated in HCV cell entry as well; however, the exact mechanisms by which all these factors contribute to HCV entry are not yet known. More information on HCV cell entry is covered in section 1.2.3.

Currently, no entry factors have been identified for any hepaciviruses other than HCV. *In vitro* studies to rectify this are handicapped by the lack of replication-competent cell culture viruses. However, the discovery of said entry factors could help understanding of mechanistic details surrounding HCV entry, thus potentially informing vaccine and/or therapeutics design. On a more ambitious note, these studies could uncover a novel mechanism of lipid bilayer fusion. Indeed, hepaciviral E1E2 are genetically and structurally predicted to belong to a novel class of membrane fusion protein, outside of the three described so far [411].

In this chapter I have used the $GBVB_{(MLV)}$ system established in Chapter 3 to study GBV-B cell entry. HCV PVs were crucial in the identification of CLDN1 and OCLN as HCV entry factors [121,122,412]. I employed a receptor knock-out cell-line screen to characterize GBV-B entry and found that CLDN1 is a necessary host factor for GBV-B. Subsequent characterisation found GBV-B interacts with CLDN1 in a different mode from that of HCV.

4.2 Results

4.2.1 Claudin-1 mediates GBV-B entry in human cells

To further characterise GBV-B as animal model for HCV, GBV-B entry mechanisms were investigated using the developed $GBVB_{(MLV)}$. Due to GBV-B's close relation to HCV, I sought to determine if GBV-B utilized any of the known HCV receptors in human cells. To understand whether the HCV receptor CD81 also has a role in GBV-B infection, effects of an anti-CD81 antibody were evaluated in an infectivity assay using $GBVB_{(MLV)}$ (Figure 4.1). As expected, there was a dose-dependent reduction in infectivity for $HCV_{(MLV)}$ as the concentration of antibody increased. However, there was no effect seen on $GBVB_{(MLV)}$ or for an irrelevant MLV-pseudotyped with VSV-G protein, indicating that GBV-B does not utilize CD81 as a receptor.

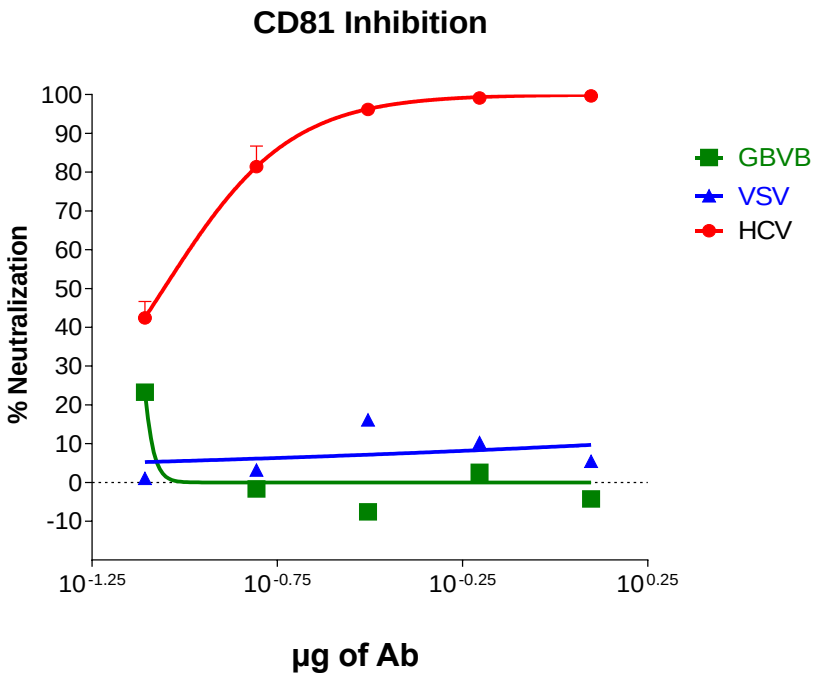


Figure 4.1. Effect of anti-CD81 antibody of viral entry

$HCV_{(MLV)}$-UKN1A20.8, $GBVB_{(MLV)}$, and $VSV_{(MLV)}$ were used to infect Huh7.5 with increasing concentrations of anti-CD81. Neutralization is expressed as a

percentage in the reduction in infection of PVs with anti-CD81 Ab as compared to infection without the presence of the Ab.

The evaluation of the roles of other known HCV receptors could not be conducted in the same manner due to the lack of commercially available antibodies; as an alternative, I looked at GBV-B entry in a panel of HCV receptor knockout (KO) Huh7 cell lines (Figure 4.2); these cells were kindly donated by Yoshiharu Matsuura, Osaka University [387]. Only CLDN1 KO cells showed significantly less $GBVB_{(MLV)}$ cell entry than the parental Huh7 cells. No effect was observed in CD81, OCLN, LDLR, or SR-B1 KO cell lines (Figure 4.2). CLDN1 KO did not significantly impact $HCV_{(MLV)}$ entry; this is because E1E2 used for the pseudotyping were derived from a HCV isolate (UKN1A20.8) which is able to utilize CLDN6 and CLDN9 in addition to CLDN1 [36]. $HCV_{(MLV)}$ had a significant decrease in cell entry for SR-B1, CD81, and OCLN KO cells, but not LDLR KO which was expected as LDLR is used redundantly with SR-B1 [387]. $VSV_{(MLV)}$ was not significantly affected in any cell line other than OCLN KO; albeit this is an interesting observation, as OCLN KO had no impact on GBV-B entry, the reasons behind this inhibition were not further investigated.

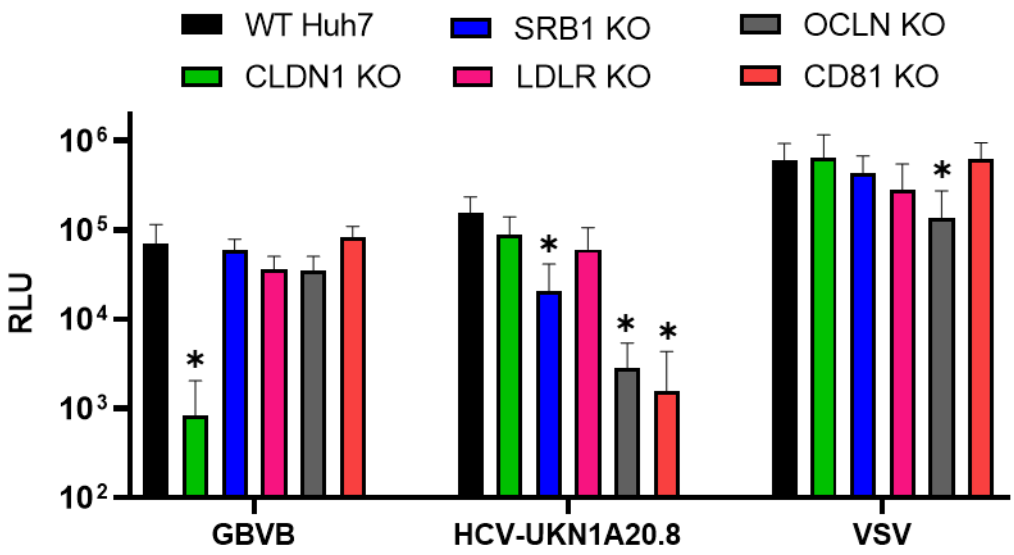


Figure 4.2. Claudin-1 is essential for GBV-B cell entry

*Huh7 cells in which CLDN1, SR-B1, LDLR, OCLN, or CD81 were knocked out were challenged with GBVB$_{(MLV)}$, HCV$_{(MLV)}$-UKN1A20.8, or VSV$_{(MLV)}$. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate *t-test p=<0.01.*

To further confirm the role of CLDN1 in GBV-B entry, CLDN1 expression in KO cells was reconstituted to determine if this restored susceptibility to GBVB$_{(MLV)}$. Lentiviral vectors with VSV glycoproteins, expressing GFP and either CLDN1 or an irrelevant protein (CD81) were produced, and titre based on GFP expression in Huh7 cells was determined by flow cytometry. CLDN1 KOs were then infected with lentiviral vector at a MOI of approximately 1, resulting in expression of CLDN1 that is higher than parental Huh7 cells (Figure 4.3A). Exogenous expression of CLDN1 restores susceptibility of the KO cells to GBVB$_{(MLV)}$ and HCV$_{(MLV)}$-J6, a CLDN1 dependent strain of HCV, to wildtype levels (Figure 4.3B). This result further supports the notion that CLDN1 is necessary for GBV-B entry.

Expression of CLDN1 above endogenous levels did not enhance entry, similarly to what has been observed for HCV entry [34]. $HCV_{(MLV)}$ carrying isolate H77-derived E1E2 could similarly infect CLDN1 KO and wildtype cells, consistent with previous findings that this isolate can use CLDN6 or CLDN9 as well as CLDN1 for entry. The expression of an irrelevant protein, CD81, in the KO cells had no effect on any PV entry.

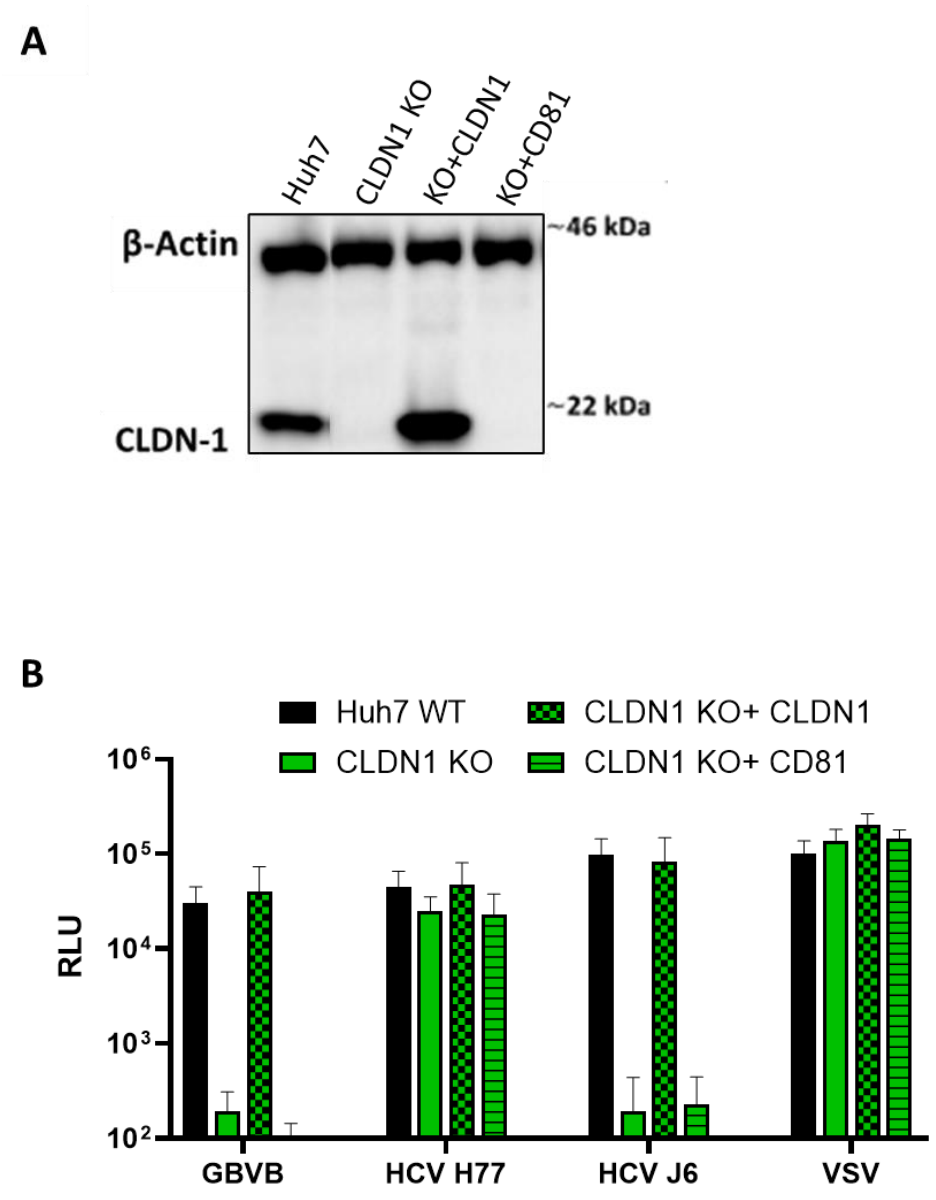


Figure 4.3. Reconstitution of Claudin-1 in knockout cells restores $GBVB_{(MLV)}$ entry

(A) *CLDN1 expression in modified Huh7 cells was assessed by immunoblotting using an anti-CLDN1 antibody. Protein input was verified using an anti-β-actin antibody. Samples were run with a protein size marker; size in kDa is indicated to the right of the blot. *This was performed by Mphatso Kalemera of Joe Grove's laboratory, UCL* ***(B)*** *Huh7 cells, Huh7 CLDN1 KO cells transduced to express CLDN1 or CD81 were challenged with the indicated PVs. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate.*

4.2.2 Interaction of GBV-B with CLDN1 differs from HCV

Claudin-1 is a small protein (211 amino acids) located at the cell surface with 4 transmembrane domains and 2 extracellular loops, with the N and C-termini both intracellular [413] (Figure 4.4A). Previous work has demonstrated that amino acid residues at positions 32 and 48 in the first extracellular loop (EL1) of CLDN1 are crucial for interactions with HCV particles [34]. Therefore, I sought to determine whether the interaction between GBV-B and CLDN1 was similar to that of HCV. Through site-directed mutagenesis, I generated a lentiviral vector to express a double mutant CLDN1 that is defunct for HCV entry, which has substitutions of isoleucine to methionine at position 32 (I32M) and glutamic acid to lysine at position 48 (E48K) in EL1 (Figure 4.4B). I32M/E48K CLDN1 was then introduced into KO cells through lentiviral transduction and confirmed by western blot (Figure 4.4C). The double mutant, as expected, reduced $HCV_{(MLV)}$-J6 entry to background levels and had no impact on the irrelevant $VSV_{(MLV)}$ (Figure 4.4D). There was no effect seen on $GBVB_{(MLV)}$ entry (Figure 4.4D), indicating these residues are not essential for GBVB interaction with CLDN1. The cell line expressing an irrelevant protein (GFP) did not affect infectivity of any virus.

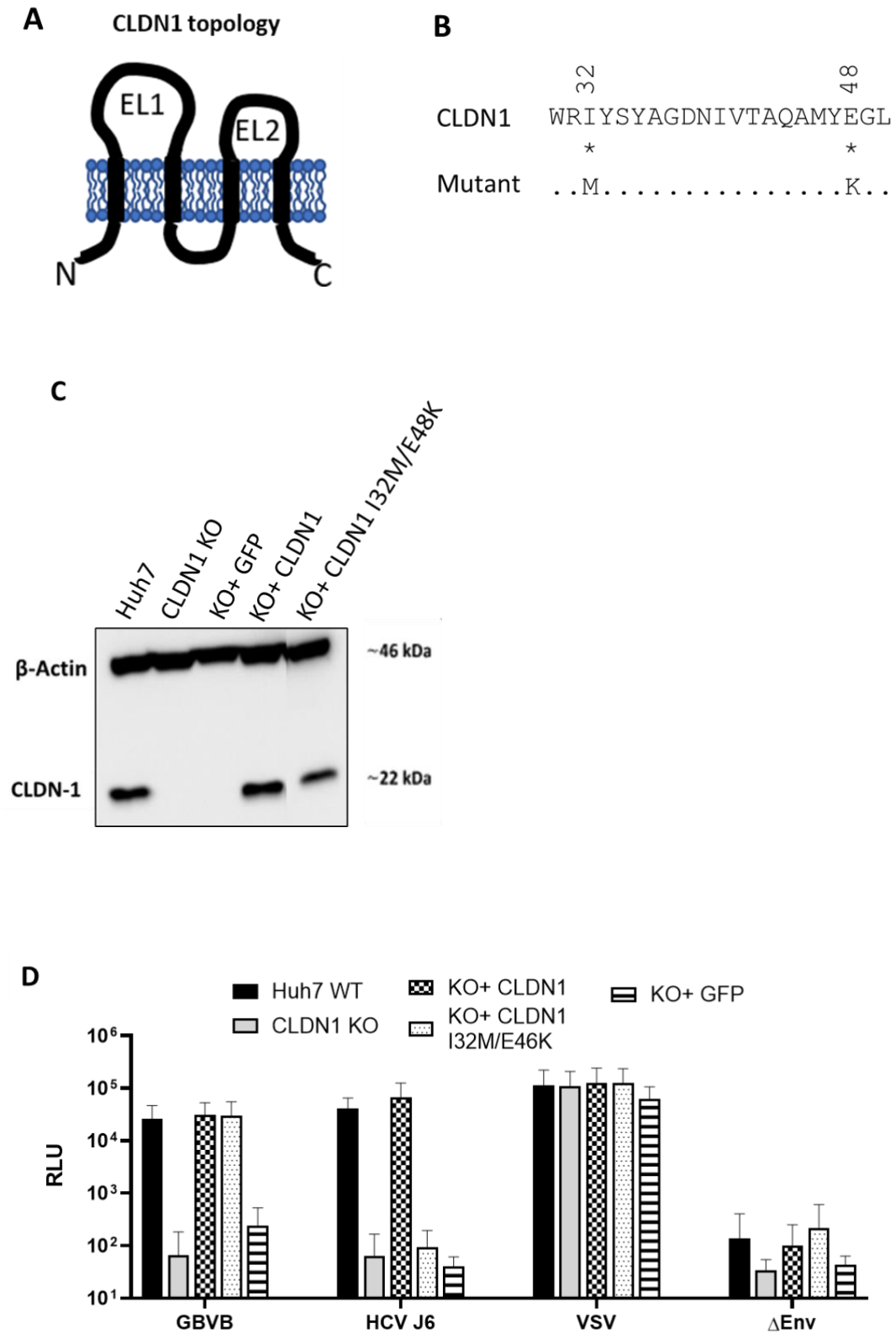


Figure 4.4. GBV-B interaction with Claudin-1 differs from that of HCV

(A) *Schematic of the topology of CLDN1.* ***(B)*** *Alignment of CLDN1 amino acids 30–50 in extracellular loop 1 with the mutant created by site directed mutagenesis*

to introduce I32M and E48K. Identical amino acids are represented by a full stop and numbering represents the amino acid position in the full length CLDN1. ***(C)*** *CLDN1 expression in modified Huh7 cells was assessed by immunoblotting using an anti-CLDN1 antibody. Protein input was verified using an anti-actin antibody. Samples were run with a protein size marker; size in kDa is indicated to the right of the blot.* ***(D)*** *Huh7 CLDN1 KO cells were transduced to express CLDN1, the double mutant, or GFP only and challenged with MLV-based PV harbouring the indicated envelope proteins. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate.*

HEK293T cells do not endogenously express CLDN1 [34], but upon exogenous expression of CLDN1, HEKs become highly permissive to HCV, indicating that all other entry factors are present on HEKs for HCV cell entry. To determine if all cellular factors needed were also present for $GBVB_{(MLV)}$ entry, CLDN1 was transduced into HEKs using a lentiviral vector (Figure 4.5A) and modified cells tested for permissivity to $GBVB_{(MLV)}$ infection. The exogenous expression of CLDN1 conferred susceptibility to HEK cells to $HCV_{(MLV)}$-J6, whereas, no infection was detected for $GBVB_{(MLV)}$ (Figure 4.5B). This observation suggests that there is at least one other entry factor, present in Huh7 but not HEKs, that is essential for GBV-B entry. Alternatively, there may be unknown inhibitory factors in HEK cells specific to GBV-B and not HCV.

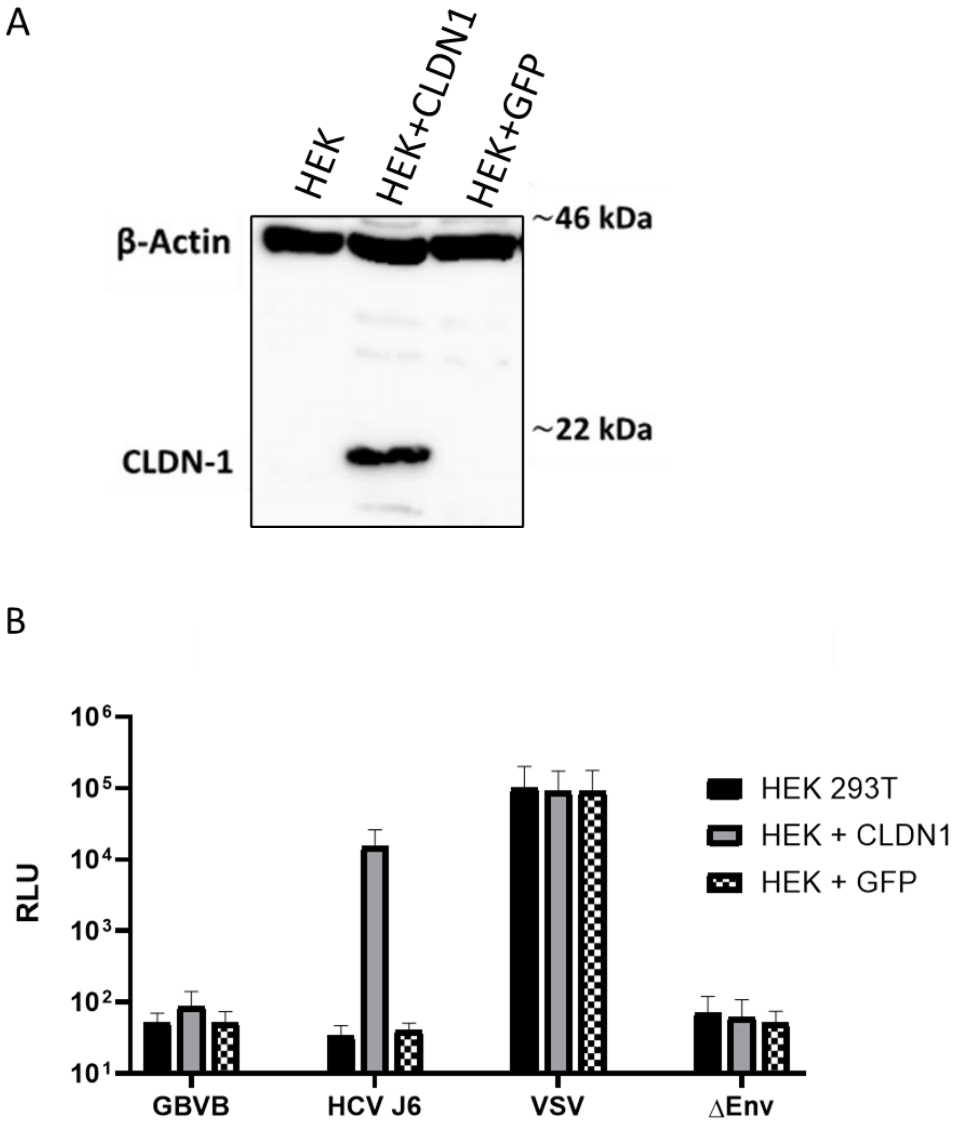


Figure 4.5. Introduction of CLDN1 into HEK 293T cells does not confer susceptibility to GBVB$_{(MLV)}$

(A) *CLDN1 expression in modified HEK 293T cells was assessed by immunoblotting using an anti-CLDN1 antibody. Protein input was verified using an anti-β-actin antibody. Samples were run with a protein size marker; size in kDa is indicated to the right of the blot.* ***(B)*** *HEK 293T cells were transduced to express CLDN1 or an irrelevant protein (GFP) and challenged with MLV-based PV harbouring GBVB, HCV, or VSV envelope proteins. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate.*

4.2.3 Claudin-1 is not a host range determent for GBV-B

To determine whether CLDN1 is a determinant for species specificity, CLDN1 KO cells expressing CLDN1 from different mammalian species were tested for susceptibility to $GBVB_{(MLV)}$. These CLDN1 proteins were selected based on their probability of supporting, GBV-B entry, or not. *Callithrix jacchus* (marmoset) CLDN1 was chosen as GBV-B is able to experimentally infect marmosets [164,187], thus should confer susceptibility to $GBVB_{(MLV)}$. *Dasypus novemcinctus* (armadillo), *Orvctolagus cuniculuc* (rabbit), and *Cavia porcellus* (guinea pig) CLDN1 were chosen based on their distance from human CLDN1 on the phylogenetic tree comprised of mammalian CLDN1 (Figure 4.6), as the farthest and therefore least likely to confer susceptibility to GBV-B.

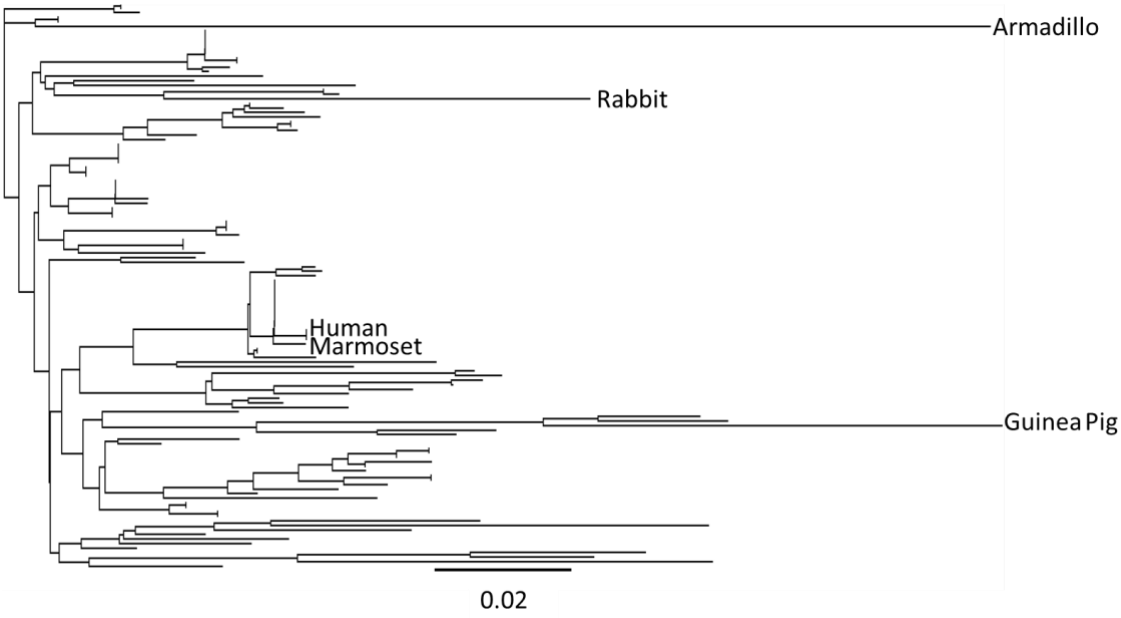


Figure 4.6. Phylogenetic tree of mammalian Claudin-1

Phylogenetic tree of the ClustalW alignment of mammalian CLDN1 amino acid sequences deposited in GenBank, created with Geneious software using the Jukes-Cantor distance model and neighbour joining method. Scale corresponds to amino acid substitutions per site.

Mammalian CLDN1 genes were introduced into CLDN1 KO cells via lentiviral vector transduction and then tested in a $GBVB_{(MLV)}$ infection assay. All the tested mammalian CLDN1 conferred susceptibility to GBV-B (Figure 4.7A) indicating that CLDN1 on its own is not a determinant for host range. It has been reported that CLDN1 is also not a species specificity determinant for HCV, as murine CLDN1 confers susceptibility to HCV in HEK cells [410]. CLDN1 KO cells expressing armadillo and guinea pig CLDN1 are not permissive to $HCV_{(MLV)}$-J6, while rabbit and marmoset CLDN1 does allow infection (Figure 4.7A). This is likely, at least in part, due to residue differences in amino acids 32 or 48 of armadillo and guinea pig CLDN1, which are essential for CLDN1 use by HCV (Figure 4.7B). $VSV_{(MLV)}$ and $\Delta ENV_{(MLV)}$ were not affected by the expression of the various mammalian CLDN1. Confirmation of mammalian CLDN1 expression was attempted by western blot using a human CLDN1 antibody. The human CLDN1 antibody could detect marmoset and rabbit CLDN1 but could not detect guinea pig or armadillo CLDN1 (Figure 4.7C). There are no commercially available antibodies to guinea pig or armadillo CLDN1, so it was not possible to detect these proteins by western blot. However, their presence can be inferred by the restoration of infection by $GBVB_{(MLV)}$ to CLDN1 KO cells (Figure 4.7A).

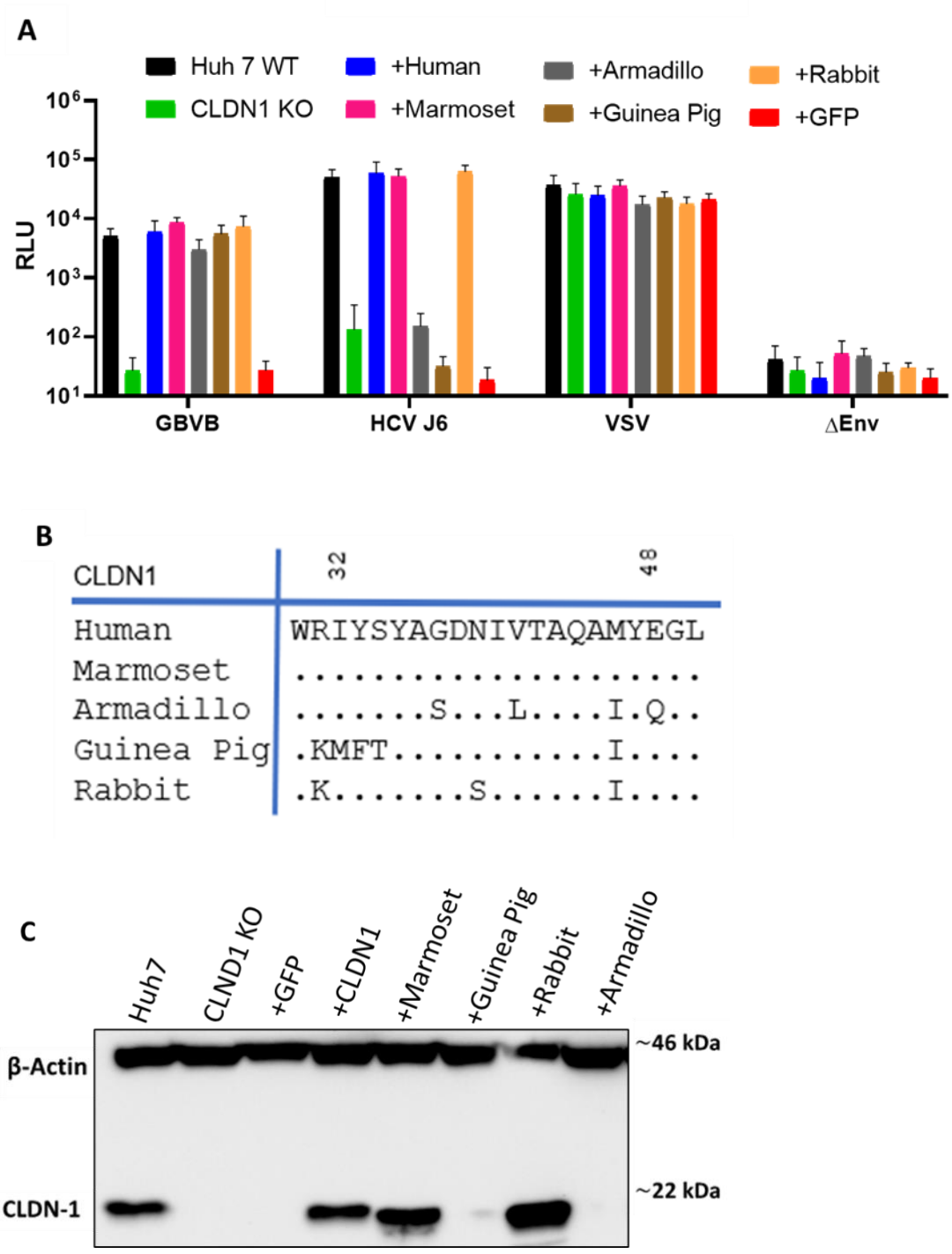


Figure 4.7. Use of mammalian CLDN1 by GBVB$_{(MLV)}$

(A) *Huh7 CLDN1 KO cells transduced with lentiviral vector to express the indicated mammalian CLDN1 were challenged with HCV$_{(MLV)}$-J6, GBVB$_{(MLV)}$, VSV$_{(MLV)}$ and ΔENV$_{(MLV)}$. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate.* ***(B)*** *Alignment of human CLDN1 amino acids 30–50 in extracellular loop 1 with the same regions of the selected mammalian CLDN1. Identical amino acids are represented by a*

full stop and numbering represents the amino acid position in the full length human CLDN1. ***(C)*** *CLDN1 expression in modified Huh7 cells was assessed by immunoblotting using an anti-CLDN1 antibody. Protein input was verified using an anti-β-actin antibody. Protein size in kDa is indicated to the right of the blot.*

4.2.4 Characterization of GBV-B E1E2 interaction with CLDN1

I next sought to determine what region of CLDN1 is important for GBV-B interaction through domain swapping studies. As no mammalian homologs of CLDN1 were identified as a suitable non-permissive candidate for domain swapping, human CLDN6 and CLDN9 were selected; this choice was driven by the observation that CLDN1-independent HCV strains can enter CLDN1 KO cells (Figure 4.2) which indicates that CLDN6 and/or CLDN9 are expressed in Huh7 cells, but $GBVB_{(MLV)}$ is unable to utilize them for cell entry. CLDN6 and CLDN9 genes were cloned by RT-PCR from Huh7 cells using specific primers (Table 2.12); the sequences were confirmed by Sanger sequencing and subcloned into the same pDual lentiviral vector previously used to introduce CLDN1 into KO cells. VSV-pseudotyped particles containing CLDN6 or 9 genes were used to transduce CLDN1 KO cells. Only the addition of CLDN1, not overexpression of CLDN6 or CLDN9 in KO cells conferred susceptibility to $GBV\text{-}B_{(MLV)}$ and CLDN1-dependent $HCV_{(MLV)}$-J6 (Figure 4.8A). No effect was seen for $HCV_{(MLV)}$-H77 or $VSV_{(MLV)}$. To confirm that CLDN6 and CLDN9 were both conformationally correct and functional, transduced HEK cells were infected with $HCV_{(MLV)}$-H77, which is known to utilize CLDN6 and CLDN9 for cell entry [414]. Expression of both CLDN6 and CLDN9 rendered HEK cells susceptible to $HCV_{(MLV)}$-H77 but had no effect on $HCV_{(MLV)}$-J6 or $VSV_{(MLV)}$ (Figure 4.8B) confirming correct protein conformation and localization. CLDN6 expression in HEK 293Ts was confirmed by western blot

using a human CLDN6 antibody (Figure 4.8C). At the time these experiments were preformed multiple CLDN9 antibodies were tested but none were suitable. Subsequently I have obtained a suitable CLDN9 antibody as seen below but due to time restraints, detection of CLDN9 by western blot was not done. However, its presence can be inferred by the HEKs becoming permissive to $HCV_{(MLV)}$-H77 infection (Figure 4.8B).

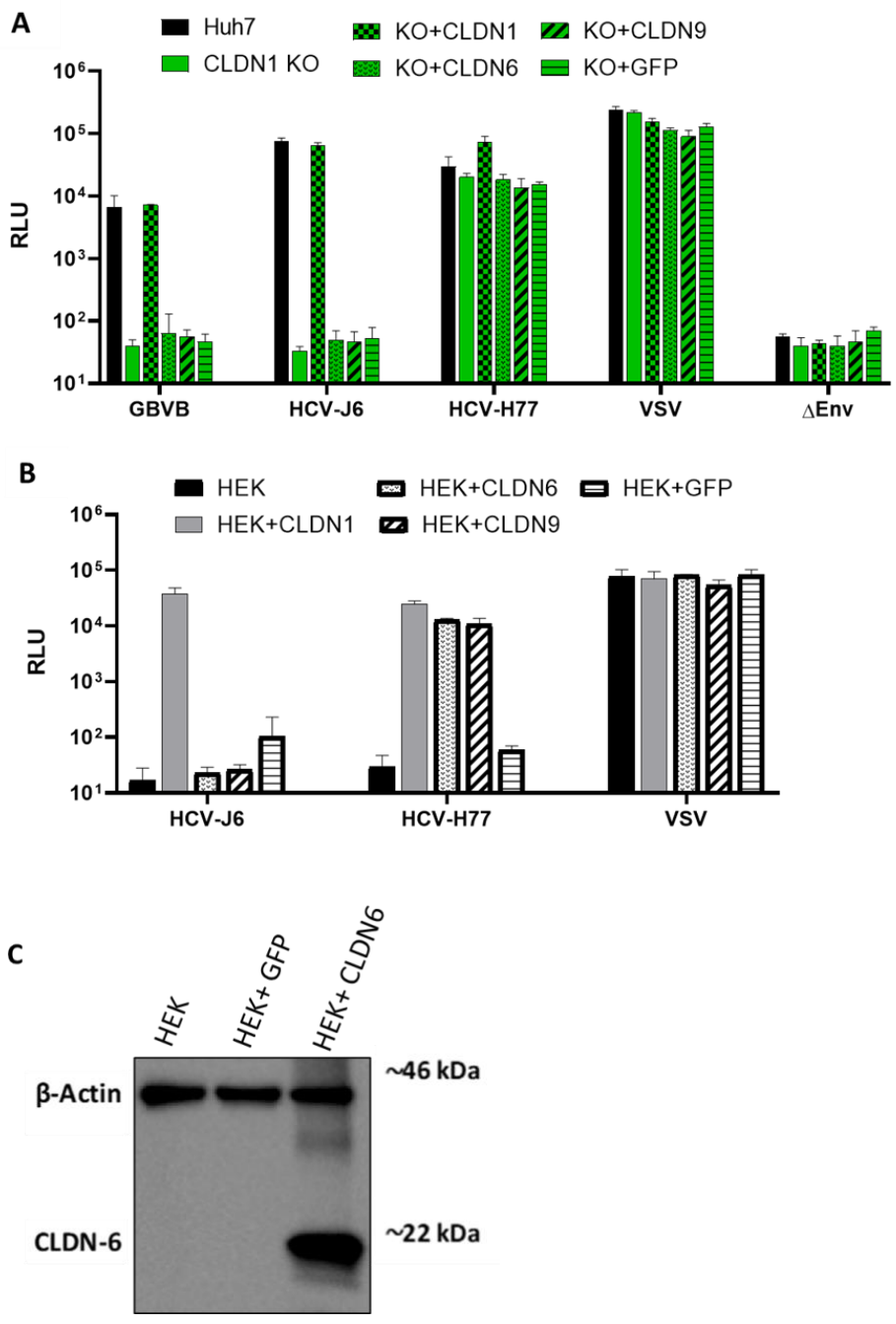


Figure 4.8. CLDN6 and CLDN9 do not support $GBVB_{(MLV)}$ entry

Huh7 CLDN1 KO **(A)** or HEK 293T cells **(B)** transduced to express CLDN6 or CLDN9 proteins were challenged with PV harbouring glycoproteins from the indicated virus. Infectivity is expressed as mean RLU values +/- standard deviation of two experiments run in triplicate. **(C)** *CLDN6 expression in modified HEK 293T cells was assessed by immunoblotting using an anti-CLDN6 antibody. Protein input was verified using an anti-β-actin antibody. Protein size in kDa is indicated to the right of the blot.*

CLDN9 was chosen for domain swapping experiments as it has slightly higher homology to CLDN1 at the amino acid level than CLDN6, 45% versus 43%. An overlapping PCR strategy was employed to generate CLDN1/CLDN9 chimeras with their extracellular loops (EL1 and EL2)reciprocally swapped as seen in Figure 4.9. The resulting products were chimeric proteins with the following amino acids: A (CLDN1 1-138/CLDN9 141-217), B (CLDN9 1-81/CLDN1 82-211), C (CLDN9 1-133/CLDN1 138-211), and D (CLDN1 1-81/CLDN9 82-217).

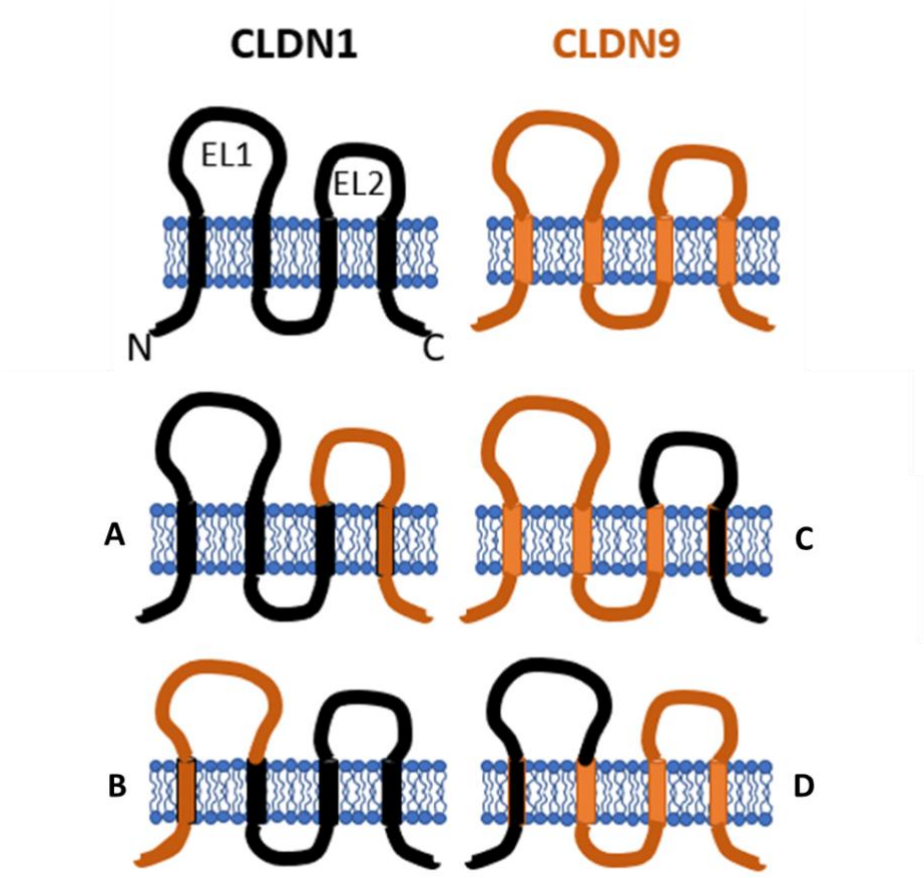


Figure 4.9. Topology of CLDN1/CLDN9 chimeric proteins

Claudin-1 (black) and Claudin-9 (orange) topology with the indicated regions of each proteins swapped with the respective region of the other CLDN to produce chimeric proteins to determine region of CLDN1 important for GBVB cell entry.

To control for expression and correct presentation of the chimeric CLDN, HEK cells were transduced using lentiviral vectors encoding the chimeric CLDN1/9 and $HCV_{(MLV)}$ derived from the H77 isolate was used in an infectivity assay. I hypothesized that as H77 can utilize both CLDN1 and CLDN9 to gain entry into cells, that it would be able to use the chimeric proteins as well if expressed in the correct conformation. $HCV_{(MLV)}$-H77, together with a $\Delta ENV_{(MLV)}$ as the negative control, and $VSV_{(MLV)}$ as a control to ensure the general infectibility of the cell were not altered, were titrated on HEK cells expressing the CLDN1/9 chimera and tested for susceptibility (Figure 4.10). Chimeras B and D were permissive to

HCV$_{(MLV)}$-H77 above background, confirming these chimeras are functional as entry factors. However, HCV$_{(MLV)}$-H77 infectivity on HEK cells was the same as the ΔENV$_{(MLV)}$ for chimeras A and C (dotted line, Figure 4.10), indicating they are either not expressed properly or are non-permissive. VSV$_{(MLV)}$ infectivity was not altered by the chimeric protein expression.

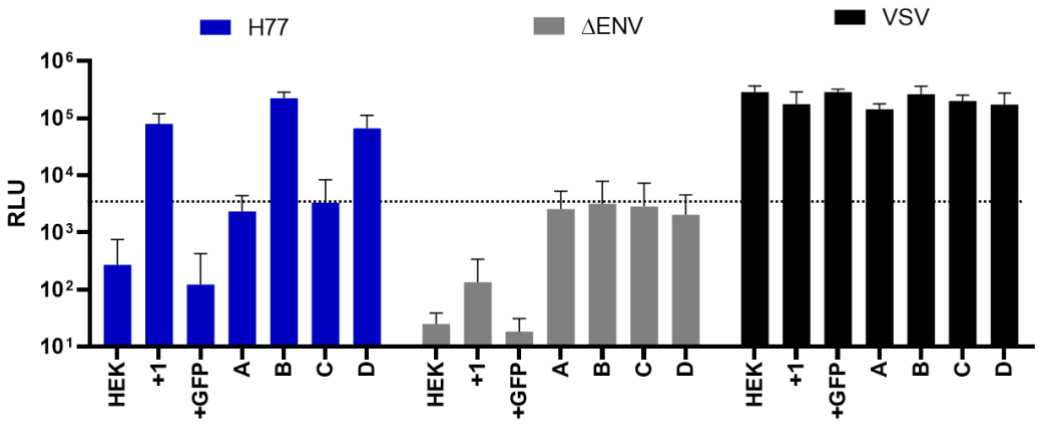


Figure 4.10. Chimeric CLDN1/9 B and D support HCV$_{(MLV)}$-H77 entry

HEK 293T cells were transduced with lentiviral vectors to express CLDN1, GFP or the indicated chimeric CLDN1/9 and were challenged with HCV$_{(MLV)}$-H77, VSV$_{(MLV)}$, and ΔENV$_{(MLV)}$. Infectivity is expressed as mean RLU values +/- standard deviation of two independent experiments run in triplicate.

As chimera B and D, which contain the same EL regions as C and A respectively, were permissive to HCV$_{(MLV)}$-H77, I speculated that chimeric proteins A and C were mostly likely not displayed properly rather than non-permissive to HCV$_{(MLV)}$-H77. Initially, I determined which points in the proteins to splice together using the UniProt website that uses sequence analysis to predict the start and end of each of the EL regions and spliced the proteins together at these predicted regions [415,416]. Chimera B and D had EL1 (and upstream) replaced with the other respective EL1. The EL1 regions of CLDN1 and CLDN9 aligned well and

were predicted to be the same size and start at the same amino acid location; therefore, when spliced together the size of the protein did not change. However, the EL2 regions were predicted to be different sizes starting at different points (red line Figure 4.11A). Therefore, when spliced together it created chimeras of altered size and may have resulted in improper folding.

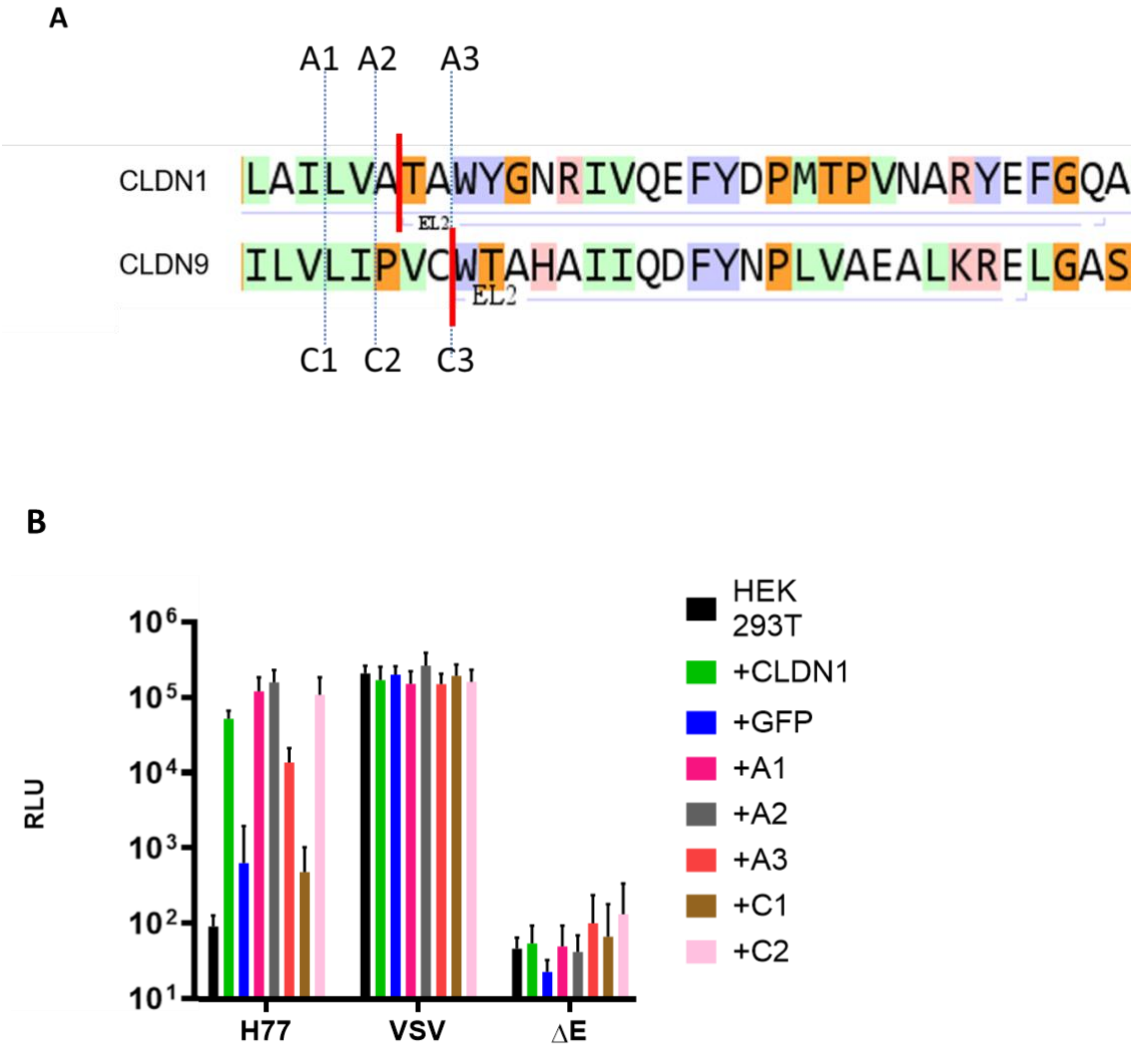


Figure 4.11. Production of Functional A and C Chimeric CLDN1/9

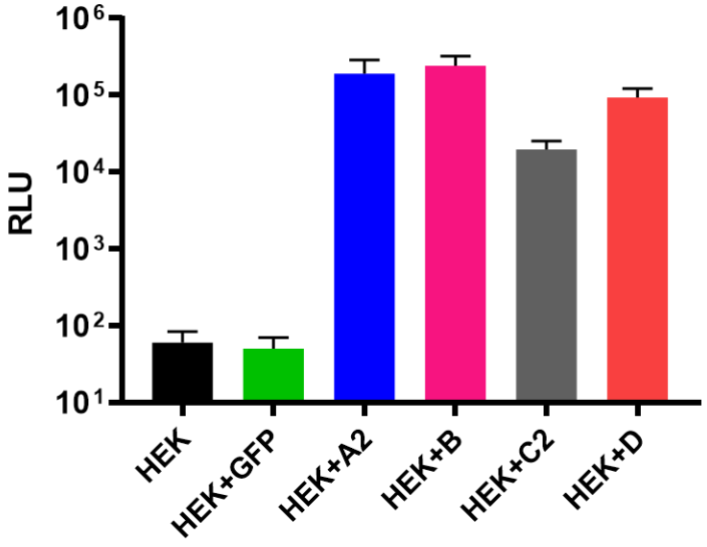
(A) *Alignment of CLDN1 and CLDN9 amino acids with extracellular loop 2 annotated. Dotted lines indicate where the indicated chimera was spliced together. Red lines indicate where the proteins were spliced together with the initial overlap extension PCR.* ***(B)*** *HEK 293T cells were transduced with lentiviral vectors to express the indicated chimeric CLDN1/9 and were challenged with*

$HCV_{(MLV)}$-H77, $VSV_{(MLV)}$, and $\Delta ENV_{(MLV)}$. Infectivity is expressed as mean RLU values +/- standard deviation of two independent experiments run in triplicate.

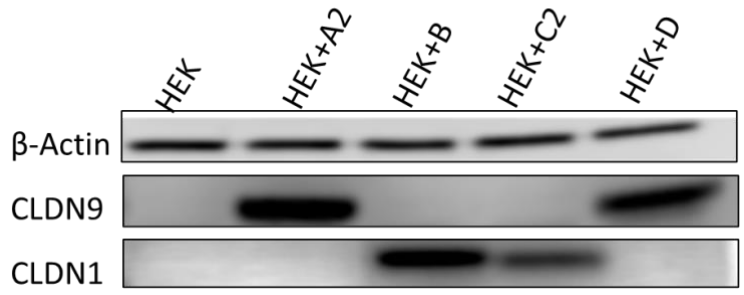
In an attempt to create permissive A and C chimeric proteins I decided to splice them together based on alignment at the regions denoted with the dotted line in Figure 4.11A. This created three candidates for each chimeric protein. Site directed mutagenesis was used to the alter the existing chimeric proteins to create the desired amino acid sequence. All three A chimeras (A1, A2, and A3) became permissive to H77 infection (Figure 4.11B). A3, however, did not re-instate infectivity to the level of the unaltered CLDN1, whereas A1 and A2 did. I could not establish a C3 protein with the correct sequence. Therefore, in the interest of time, I continued with only C1 and C2. C1 failed to establish infectivity over the level of the negative control (HEK expressing GFP only) and C2 restored infection to levels of unaltered CLDN1 (Figure 4.11B).

A2 was chosen over A1 to continue with as it is the complement/reverse chimera to the only permissive chimeric C protein, C2. Figure 4.12A shows the complete panel of Chimeric CLDN1/9 proteins in HEKs, which have conferred susceptibility to $HCV_{(MLV)}$-H77 while there is no detectable infection in the parental cells and the transduction control (expressing GFP only). This indicates that all chimeras are now conformationally correct. Chimeric CLDN expression in HEKs was confirmed by western blot by using a human CLDN1 and human CLDN9 antibodies (Figure 4.12B). The anti CLDN1 and 9 antibodies appear to recognize a region in the C-terminus of their respective protein; therefore, Chimera A and D are detected by anti-CLDN9 antibodies and C and B are detected by anti-CLDN1 antibodies.

A


RLU
10^6
10^5
10^4
10^3
10^2
10^1
HEK
HEK+GFP
HEK+A2
HEK+B
HEK+C2
HEK+D

B


HEK
HEK+A2
HEK+B
HEK+C2
HEK+D
β-Actin
CLDN9
CLDN1

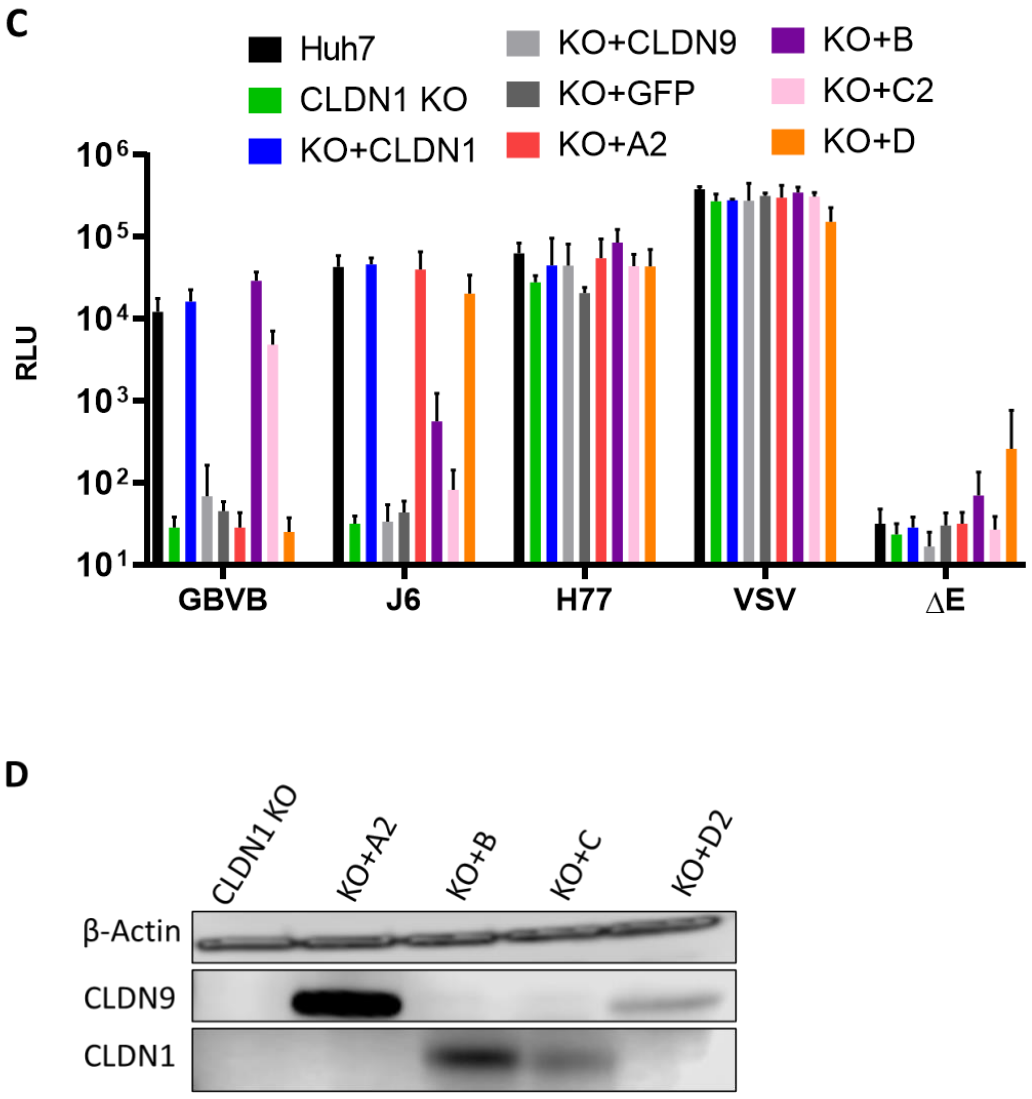


Figure 4.12 GBV-B utilizes extracellular loop 2 of Claudin-1

*HEK 293T **(A)** or Huh 7 CLDN KO **(C)** cells were transduced with lentiviral vectors to express the indicated chimeric CLDN1/9 and were challenged with HCV$_{(MLV)}$-H77 **(A)**, and the indicated virus in **(C)**. Infectivity is expressed as mean RLU values +/- standard deviation of 2 independent experiments run in triplicate. CLDN1/9 expression in modified HEK 293T **(B)** or CLDN1 KO **(D)** cells was assessed by immunoblotting using anti-CLDN1 and anti-CLDN9 antibodies. Protein input was verified using an anti-β-actin antibody.*

The panel of chimeras were then introduced into CLDN1 KO and tested for susceptibility (Figure 4.12C) and protein expression was confirmed by western blot (Figure 4.12D). GBVB$_{(MLV)}$ can infect CLDN1 with the EL1 replaced with that

of CLDN9 (CLDN1/9 B) but infection is below the limit of detection when EL2 is swapped (CLDN1/9 A2), suggesting that the EL2 of CLDN1 is critical for GBV-B entry. Similarly, when EL2 of CLDN1 is introduced to CLDN9 (CLDN1/9 C2), infection is restored. Whereas no infectivity is observed when EL1 in introduced (CLDN1/9 D), confirming EL2 is the critical region. The opposite was observed for $HCV_{(MLV)}$-J6. $HCV_{(MLV)}$-J6pp infected CLDN1/9 A2 and D which contain EL1 of CLDN1, this is expected since J6 is CLDN1 dependent and is known to utilize EL1 [410]. $HCV_{(MLV)}$-J6 could not infect the chimeras lacking EL1 of CLDN1 (CLDN1/9 B and C2). Finally, $VSV_{(MLV)}$ infectivity was not impacted, as expected, as claudin proteins are irrelevant to VSV infection.

4.3 Discussion

Much of what is known of the hepaciviral life cycle is inferred from HCV, which is by far the most extensively researched virus in the genus due to the significant disease burden it poses to humans. GBV-B was sequenced in 1995 and has since been a useful model for HCV research [187,201–203]; despite this, no receptors or entry factors had been described for it, until now. Here, I demonstrate that like HCV, CLDN1 is an entry factor that is necessary for GBV-B cell entry. When CLDN1 is knocked out of Huh7, a susceptible cell line to $GBVB_{(MLV)}$, entry is diminished and upon exogenous expression of CLDN1, susceptibility is completely restored.

In addition to HCV, dengue virus, coxsackievirus B, some reoviruses, and some adenoviruses also utilize tight junction proteins as coreceptors [417–419]. In polarized cells, the majority of CLDN1 is localized in tight junctions [410]. HCV is thought to follow a similar cell entry pathway as coxsackievirus B, where it binds a primary receptor on the luminal cell surface then migrates laterally along the plasma membrane to encounter tight junction proteins CLDN1 and OCLN [37]. However, there is a small amount of CLDN1 that localizes at the basal membranes of hepatocytes, and there is evidence to suggest that HCV interacts with this fraction of CLDN1 [420–422]. Of note, $GBVB_{(MLV)}$ were insensitive to OCLN deletion, which may further highlight a divergence in the entry pathways of HCV and GBV-B as OCLN, which exclusively localizes at the tight junctions, is indispensable for HCV infection.

Two amino acids in the extracellular loop 1 (EL1) of CLDN1, previously identified as essential for HCV entry [410], have no impact on GBV-B. These residues are responsible for the interaction of CLDN1 with CD81 to form a complex that is

indispensable for HCV entry [423]. It is not surprising that these residues are not important for GBV-B entry because CD81 does not appear to play a role in GBV-B entry into human cells. Thus inability to form this complex was expected to have no effect, further confirming CD81 independence of GBV-B entry. I have also shown that GBV-B is dependent on the EL2, or a downstream region of CLDN1 for infection, differently from HCV. Using chimeric proteins between permissive CLDN1 and nonpermissive CLDN9, $GBVB_{(MLV)}$ were able to enter only cells expressing a chimeric CLDN containing CLDN1 EL2, while CLDN1-dependent $HCV_{(MLV)}$-J6 were able to infect cells expressing chimeras with EL1 of CLDN1. Further investigation is needed to determine the exact region of EL2 that is important for interaction and whether GBV-B directly interacts with CLDN1. A study that identified CLDN1 as an essential entry factor for another member of the *Flaviviridae* family, dengue virus, indicated a direct interaction between CLDN1 EL1 and the prM protein by pulldown assays [419].

It was initially thought that CLDN1 does not directly interact with HCV particles but is important for the receptor complex formed with CD81 [423,424]. However, some studies have indicated there may be direct interaction between CLDN1 and the HCV E1E2 complex. CLDN1 has been shown to not interact with E2 alone, but with the E1E2 heterodimer [425], and mutations in E1 have been shown to shift use of CLDN1 to CLDN6 [426,427], possibly indicating a direct interaction with E1. However, this interaction is poorly understood; it is not known which domains on CLDN1 are important for interaction with the glycoproteins or if binding to CLDN1 is needed for receptor clustering. It is also currently unknown whether CLDN1 binds with E1 or E1 and E2 together in a ternary interaction.

Another discrepancy observed between $HCV_{(MLV)}$ and $GBVB_{(MLV)}$ is in the infection of HEK cells transduced to express CLDN1; these are susceptible to

$HCV_{(MLV)}$-J6 but not $GBVB_{(MLV)}$. This suggests that GBVB may utilize at least one entry factor not conserved between GBV-B and HCV that is expressed in Huh7 cells, but not in HEK293 cells. Thus, further investigation into other entry factors is needed to fully characterize GBV-B cell entry. Identification of further receptors or entry factors that are not conserved may help shed light on the physiological differences between the new world monkey animal model for GBV-B and human HCV infection. Alternatively, HEK293, but not Huh7, may have a factor restricting GBV-B entry.

A caveat to these investigations is that $GBVB_{(MLV)}$ may not be representative of authentic replicating GBV-B particles. Assuming GBV-B, like HCV, is extensively adapted to the liver, it is likely that GBV-B particles resemble low-density lipoproteins and are high in apolipoprotein content. Therefore, apolipoprotein receptors like LDL-R and SR-BI are likely to play a role in GBV-B entry as they can tether apolipoprotein-associated virions to the basolateral surface of hepatocytes. The establishment of full-length replication-competent GBV-B and subsequent ultrastructural analyses of this virus will help identify whether co-option of the LDL biogenesis pathway is a conserved feature among hepaciviruses.

GBV-B provides a potential avenue to investigate the interaction between E1E2 glycoproteins and CLDN1 without reliance on the CD81 complex, potentially helping further define the role of CLDN1 in HCV cell entry. Characterization of the entry mechanism may unveil new potential targets for therapeutics and uncover a novel mechanism of membrane fusion. Unlike the flavivirus entry protein E, hepaciviral E1E2 proteins are not grouped into class II membrane fusion proteins-indeed they fall outside the three classes of fusion proteins described so far [411]. To this end, studying GBV-B E1E2 entry may prove a more facile path than HCV

to deciphering the hepaciviral membrane fusion mechanism as it is structurally predicted to have fewer disordered segments compared to HCV. Understanding the similarities and differences between HCV and GBV-B E1E2 will likely provide mechanistic insight into the complex entry of HCV and possibly offer clues as to why it seems HCV has uniquely evolved to cause chronic disease in humans while closely related viruses in other hosts succumb to immune pressure in the acute stages of infection.

Chapter 5:

Development of pseudotyped viruses with Japanese encephalitis virus envelope proteins

5.1 Introduction

Japanese encephalitis virus (JEV), is a member of the *Flaviviridae* family, within the *Flavivirus* genus. JEV causes 68,000 cases a year with approximately 20,000 deaths. Furthermore, up to half of those who have recovered experience debilitating neurological sequelae [206]. While there are vaccines to prevent the disease there are currently no available treatments for JEV [278]. Additionally, recent decades have seen a geographical spread of the virus from subtropical parts of Asia to Australia, as well as viral RNA being detected in birds and mosquitoes in Italy, further fuel concerns for epidemics in naïve populations [274–277]. Production of PVs with the viral envelope proteins (VEP) of viruses with epidemic potential, such as JEV, can support serosurveillance studies without being hampered by the need for high containment facilities, which are often unavailable in resource limited regions.

Indeed, work with live JEV not only requires a level 3 biosafety laboratory, it is also a schedule 5 pathogen in the UK under the Anti-terrorism, Crime and Security Act 2001, as well as a being classified as a specified animal pathogen under the Specified Animal Pathogens Order 2008. Therefore, not only is access to a level 3 containment laboratory needed, but further security features and licenses are required to use replication competent JEV [428,429]. Live JEV is known to infect several cell lines [222,430]. However, Vero cells, derived from African green monkey kidney, are the most commonly used in plaque assays, the assay required for a plaque reduction neutralization assay to determine the presence of neutralizing antibodies in sera samples.

Like members of the genus *Hepacivirus*, members of the genus *Flavivirus* also have 7 non-structural proteins and 3 structural proteins. However, their structural

proteins consist of capsid (C), envelope (E), and premembrane/membrane protein (prM/M) [2]. The prM protein is cleaved by furin to from the mature M protein during transit through the Golgi [431]. Additionally, part of the life cycle of JEV is the production of subviral particles, which consist of JEV envelope proteins excreted from the ER in a lipid bilayer that follow the same trafficking within the cell as viral particles [432].

JEV has been documented in the literature to be successfully pseudotyped using retroviral or VSV cores [399,433–435]. However, while these are examples of successfully produced *flavivirus* PV, these systems suffer from low or lack of inter-laboratory reproducibility and usually low titres [336]. Standardisation of methods and protocols for generation of *Flaviviridae* PVs is needed to address these issues to enable faster development of assays essential for outbreak preparedness.

Flaviviruses bud from the ER, making traditional PV cores such as MLV, VSV, or HIV suboptimal systems for their production as these cores bud from the plasma membrane [281,394,436]. Attempts have been made to alter or replace the transmembrane domain (TMD) of some *Flaviviridae* envelope proteins with heterologous viral envelope protein TMDs to weaken ER retention or reroute them to the plasma membrane but these alterations have resulted in incorrectly folded or non-functional proteins [437–440]. Therefore, attempts to alter envelope protein location via cellular factors rather than altering the envelope proteins may be more successful. Or using a core system that will allow localization with the envelope proteins at cellular locations they normally are found during the lifecycle of the virus.

In this chapter I evaluate several systems for the production of JEV PV in an attempt to identify a suitable and reproducible system for JEV that may be applied to all flaviviruses.

5.2 Results

5.2.1 Production of JEV pseudotyped virus with traditional methods

5.2.1.1 Retroviral cores

As there are reports in the literature of JEV pseudotyping retroviral cores, I first attempted to produce JEV PV with the optimized pseudotyping system used for developing the HCV and GBV-B PVs with retroviral cores produced in HEK 293T cells. For these assays I chose CRFK cells as target cells based on previous work in the literature on the susceptibility of multiple cell lines to $JEV_{(MLV)}$ PVs where CRFKs yielded the highest titre [441]. The plasmid used here to express the JEV envelope proteins contains the last 21 amino acids of the core protein as well as full length PrM and E proteins of the SA14-14-2 JEV strain, which is used in live attenuated and inactivated vaccines [204,272]. JEV envelope failed to create PV above env-less background with either HIV or MLV cores (Figure 5.1A). However, VSV glycoproteins, were successfully pseudotyped in both systems. Another study reported the production of a $JEV_{(VSV)}$ PV with the use of Huh7 cells as the producer cells [435]. Therefore, I also attempted JEV PV production in Huh7.5 producer cells (Figure 5.1B). Again, both cores failed to produce a JEV PV above background, but successfully pseudotyped VSV G protein.

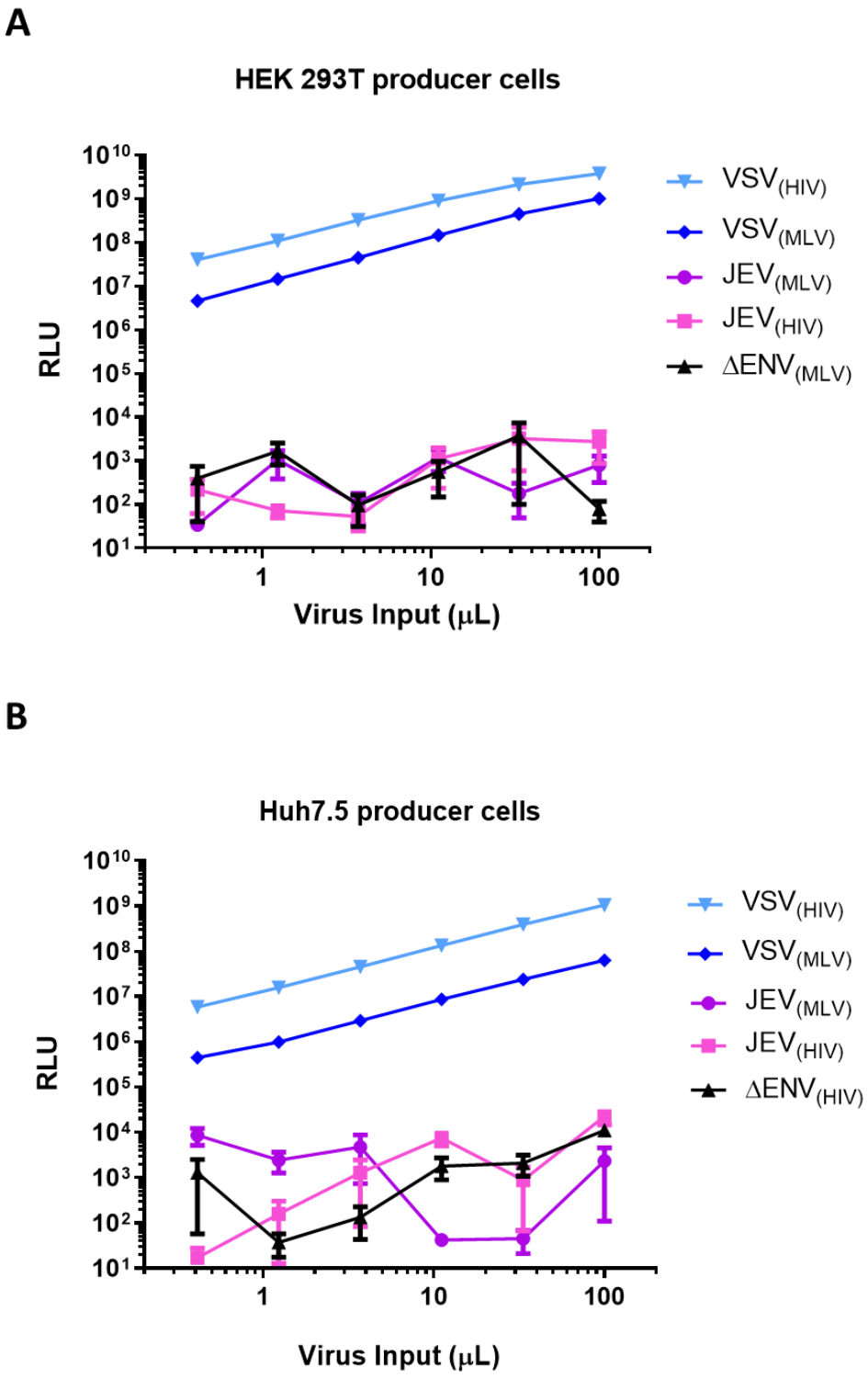


Figure 5.1. Production of JEV pseudotyped virus on retroviral cores

Serial dilutions of JEV, VSV, or ΔENV PV containing supernatants produced in ***(A)*** *HEK 293T cells or* ***(B)*** *Huh7.5 cells with either HIV or MLV core systems were used to infect CRFK cells. Infectivity was reported as mean RLU ± standard deviation of one experiment in triplicate.*

To confirm that the lack of JEV PV produced was not due to lack of envelope protein expression in the producer cells, HEK293T cells transfected with the JEV envelope plasmid were stained with DAPI, a nuclear dye, and an anti-JEV E antibody and then examined by confocal microscopy. Figure 5.2 shows specific staining of JEV E in the transfected cells but not the untransfected HEK 293T cells.

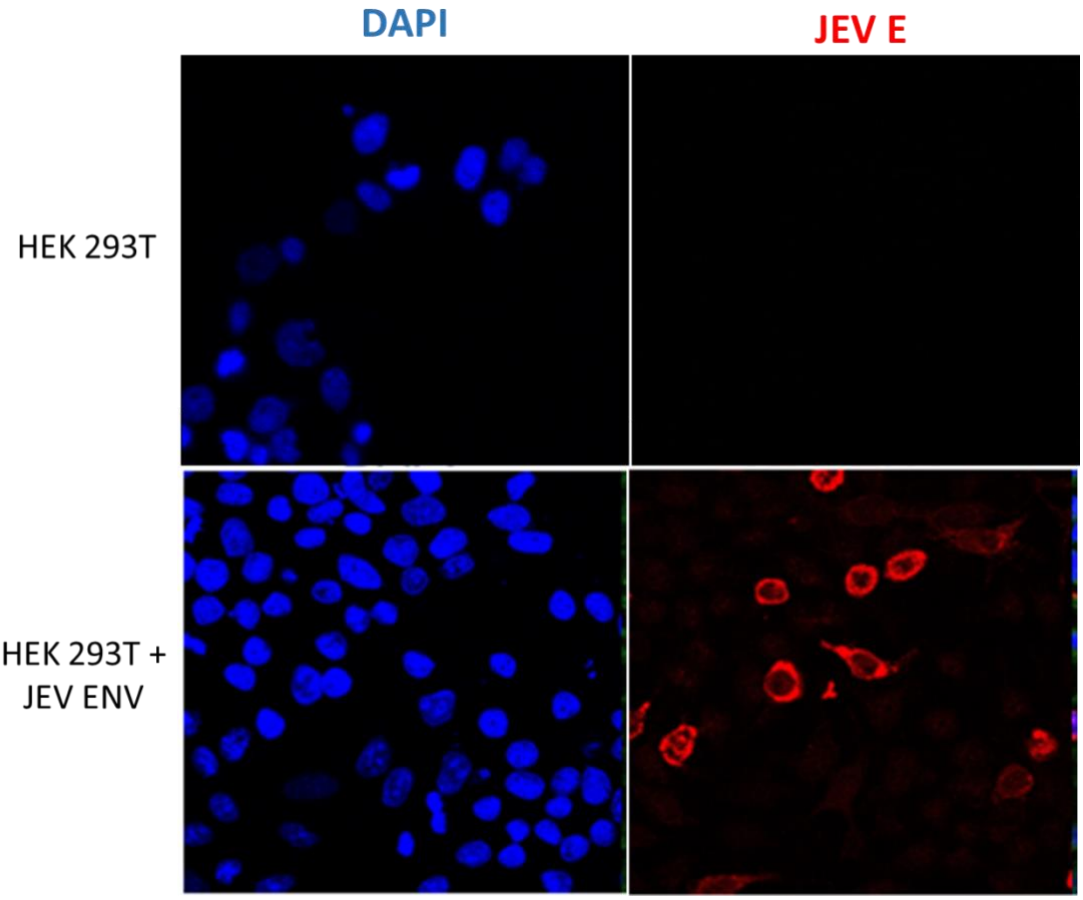


Figure 5.2 JEV envelope protein expression in producer cells

HEK 293T cells and HEK 293T cells transfected with JEV VEP with staining with DAPI (blue) and with an anti-JEV antibody (red).

5.2.1.2 VSV core

There are published reports of JEV pseudotyping VSV core [435]. Therefore, using an optimized protocol for production of VSV core PV for multiple different glycoproteins, including Rift Valley fever virus, which is also an internally budding virus, I attempted to produce a JEV PV with a VSV core (Figure 5.3). The system, however, failed to produce a $JEV_{(VSV)}$ detectible over background. The system was successful in producing a Lassa virus (LASV) pseudotype, a virus known to pseudotype VSV cores [442]. LASV envelope protein was used as the positive control in these experiments instead of VSV, seen in previous chapters, as there were biosafety concerns regarding possible recombination that may result in replicative virus if VSV-G was pseudotyped onto a VSV core.

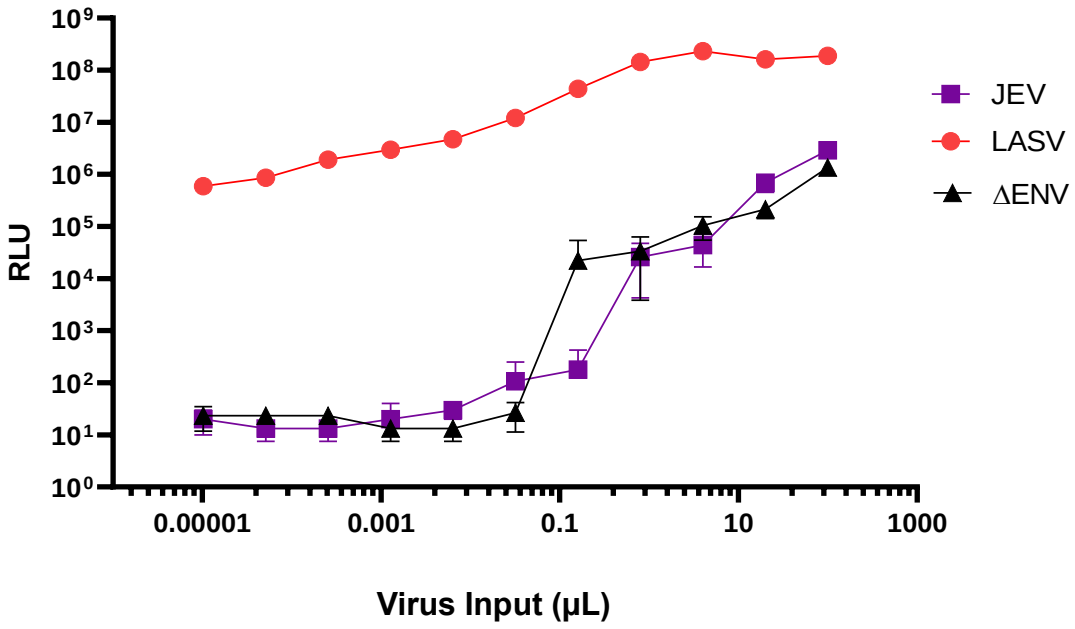


Figure 5.3. Production of JEV pseudotyped virus on VSV core

Serial dilutions of JEV, LASV, or ΔENV PV containing supernatants produced in HEK 293T cells with VSV cores were used to infect CRFK cells. Infectivity was reported as mean RLU ± standard deviation of one experiment in triplicate.

Although there are multiple reports of JEV glycoproteins being pseudotyped onto retroviral and VSV cores, I decided at this point to investigate alternative methods of pseudotyping rather than trying to further optimize these systems. This decision was driven by personal communication with multiple lab groups that have extensively attempted to replicate these systems but failed to do so.

5.2.2 JEV PV production with dengue virus core system

An alternative method for JEV PV production is to use a system that employs a core that originates from an internally budding virus. Recently, a study described the use of a DENV core with a nano luciferase (NanoLuc) reporter, that could be pseudotyped with multiple flavivirus envelopes, including JEV [339]. The constructs for the DENV replicon system were kindly donated by Dr Ryosuke Suzuki (National Institute of Infectious Diseases, Tokyo). To transfer the technique, I have used the same reagents as the published work by Dr Suzuki; this includes the prME gene from the Nakayama strain, therefore, PVs with this envelope will be termed $JEV_{NAK\_(DENV)}$, and Vero cells as target cells. The JEV PV were produced in HEK 293T cells using two different transfection reagents and conditions (Figure 5.4). The protocol typically used for retroviral PV production, with FuGENE6 and collection of virus 48 hours after transfection, failed to generate a signal above background (Figure 5.4A). I then changed the transfection reagent to PEI, moved the media change to 48 hours and collection to 72 hours after transfection (Figure 5.4B) to be in line with the published method of creating this PV; however, this also failed. Next, I attempted production again with PEI but also added 10 mM HEPES to the complete DMEM used for the media change (Figure 5.4C); as this was also in the published methods. Again,

this failed. Lastly, the protocol was adapted to add a media change with complete DMEM 6 hours after transfection, as well as the media change with HEPES at 48 hours. This small alteration in the protocol yielded $JEV_{(DENV)}$ with infectivity approximately 100-fold higher than background (Figure 5.4D). Table 5.1 summarizes the changes in the protocols with the highlighted column indicating the protocol that resulted in detectable $JEV_{NAK\_(DENV)}$.

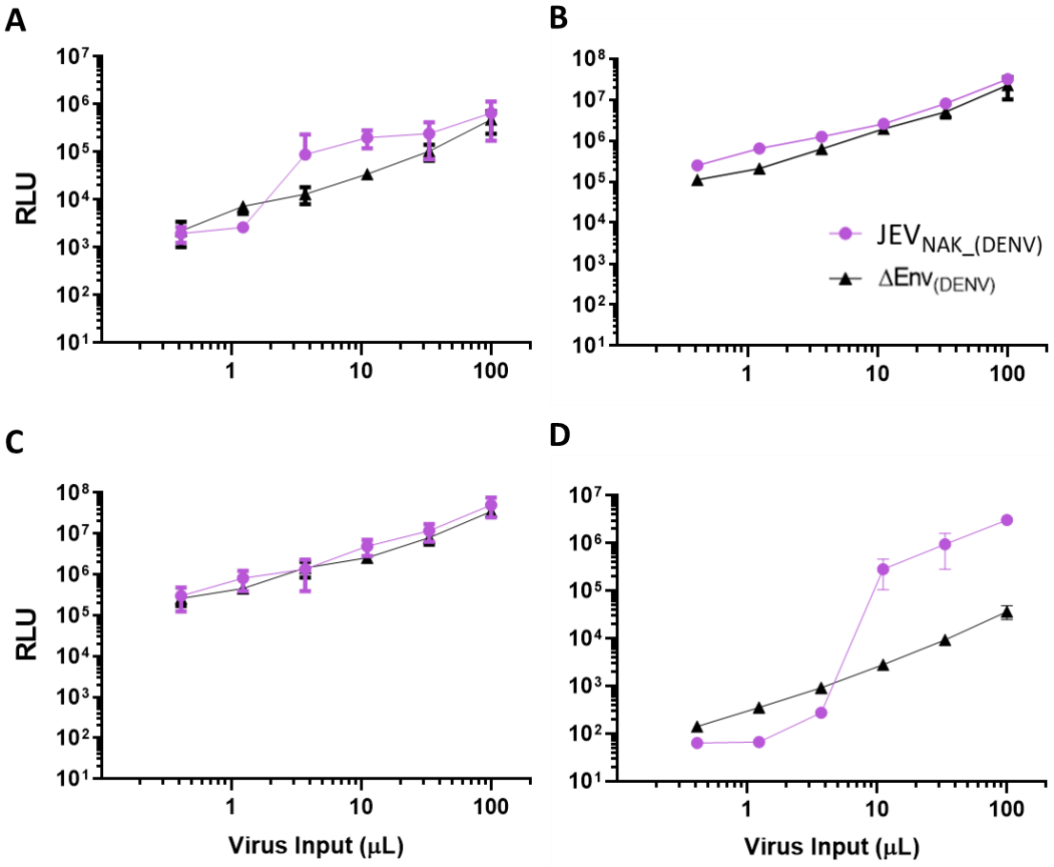


Figure 5.4. Production of JEV pseudotyped virus with DENV core

Serial dilutions of DENV core JEV_{NAK} PV-containing supernatants produced in HEK 293T cells using FuGENE6 ***(A)*** *and a media change at 24 hours post transfection, or PEI* ***(B,C)*** *and a media change 48 hours after transfection, or with 10 mM HEPES added to the media at the 48 hours change* ***(C),*** *or with the HEPES media change at 48 hours and with a media change 6 hours after*

*transfection **(D)**. Infectivity was reported as mean RLU ± standard deviation of one experiment in triplicate.*

Table 5.1. Changes in protocol to optimize JEV$_{(DENV)}$ production

	Protocol			
	A	B	C	D
Transfection Reagent	Fugene6	PEI	PEI	PEI
6-hour media change				✓
24-hour media change	✓			
48-hour media change		✓	✓	✓
HEPES in 48-hours media change			✓	✓
48-hour PV Harvest	✓			
72-hour PV Harvest		✓	✓	✓

** Protocol names correspond with the data the protocol yielded in figure 5.4 A-D*

*** Shaded protocol produced JEV$_{(DENV)}$ PV at detectible levels*

Although infectious JEV$_{NAK_(DENV)}$ were produced, the background in the system was very high, approximately 10^5 RLU at the highest virus input, therefore, to address this, 6 hours after transducing the target cells, a media change was added to remove JEV$_{NAK_(DENV)}$, that has not entered the cells. The background was approximately 100-fold lower after this transduction wash (Figure 5.5). To determine if the system could pseudotype envelope proteins from different JEV strain, JEV PV were produced using the SA14-14-2 JEV strain envelope proteins.

$JEV_{(DENV)}$ generated with the SA14-14-2 strain produced a PV with an infection signal approximately 5-fold higher (Figure 5.5).

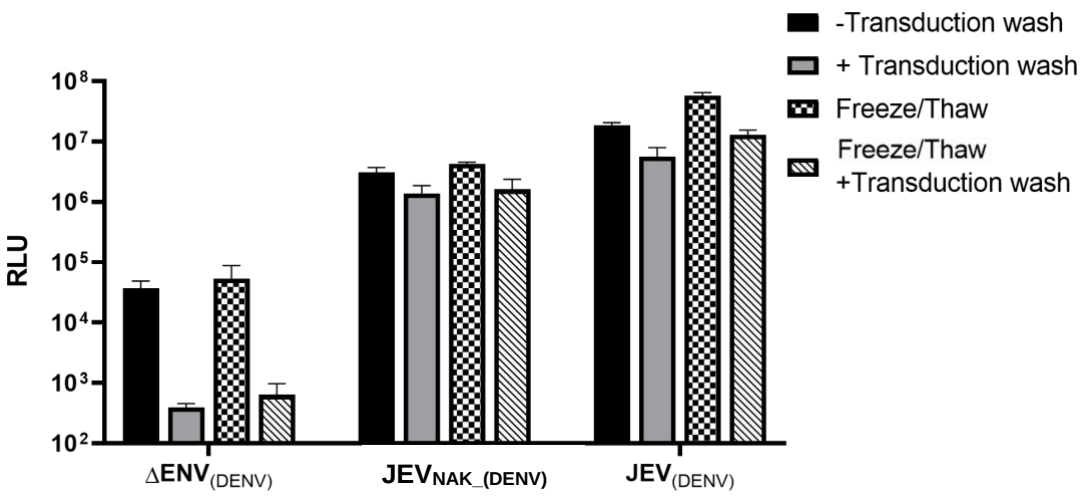


Figure 5.5. Optimization of $JEV_{(DENV)}$

$JEV_{(DENV)}$, $JEV_{(DENV)\_NAK}$, and $\Delta ENV_{(DENV)}$-containing supernatants produced in HEK 293T cells using PEI with media changes at 6 hours and 48 hours after transfection, with 10 mM HEPES added to the media at the 48-hour change then collected 24 hours later. Supernatants were either used immediately after collection or snap frozen and stored at -80°C for one week before 100 µL was titrated on Vero cells. The cells were analysed for expression of the nanoluciferase 72 hours after transduction or an additional media change was added 6 hours after transduction (transduction wash). Infectivity was reported as mean RLU ± standard deviation of one experiment in triplicate.

The ability to freeze PV for storage is advantageous for using the same stock of virus long term, which reduces variability between assays. Not all PV can withstand the freeze/thaw process. For example, $GBVB_{(MLV)}$ infectivity falls below background after one round of freeze/thaw (data not shown). Therefore, a new

batch had to be produced whenever needed. It has also been speculated that a freeze/thaw can lower background levels. To determine the effects of frozen storage on DENV core JEV PV, immediately after harvesting the PVs they were snap frozen in dry ice, then move to -80^{o}C for storage for one week. The DENV core PVs were then thawed on ice and immediately titrated onto Vero target cells and analysed for NanoLuc expression 72 hours later. The infectivity of the $JEV_{NAK\_(DENV)}$ and $JEV_{(DENV)}$ after storage at -80^{o}C was very similar to what was observed before freezing (Figure 5.5). Without the transduction wash, freeze/thaw did not ablate high background, but infectivity remained largely unaffected. Again, the removal of free virus in the media 6 hours after transduction resulted in a drastic decrease in background post freeze/thaw (Figure 5.5.).

Lastly, to confirm the presence of JEV E proteins on the $JEV_{(DENV)}$ and demonstrate its utility as a tool for serological investigations, $JEV_{(DENV)}$ produced with the SA14-14-2 strain envelope protein was used in a pseudotyped virus neutralization assay against the NIBSC JEV reference material (human serum known to have anti-JEV antibodies by plaque reduction neutralization test) and a negative human serum control. The assay was performed with a constant amount of $JEV_{(DENV)}$ (100 µL) incubated with a 2-fold series dilution of sera starting with a 1:10 dilution. $JEV_{(DENV)}$ in the presence of anti-JEV serum produced a dose dependent neutralization, reaching 100% neutralization at the 1:10 dilution point (Figure 5.6). The negative control shows a non-specific low level of neutralization, apart from the two lowest serum dilution points, which is likely due to nonspecific inhibitory factors in the serum at high concentrations.

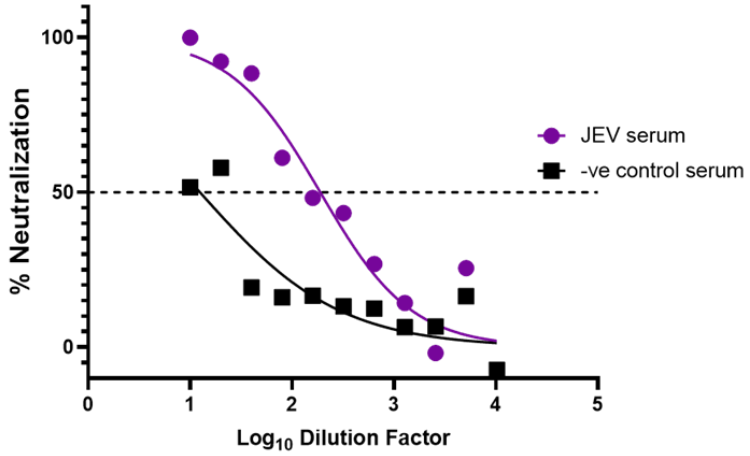


Figure 5.6. Neutralization of JEV$_{(DENV)}$

PVNA was performed by infecting Vero cells with JEV$_{(DENV)}$ after incubation with decreasing amounts of serum containing anti JEV-antibodies or a negative control human serum.

With the optimized DENV core system I wanted to determine if the system would efficiently pseudotype GBV-B, which is also a member of the *Flaviviridae* family but a different genus to both DENV and JEV. The DENV core system failed to produce a GBVB$_{(DENV)}$ with infectivity over background (Figure 5.7). I also attempted transduction after a round of freeze/thaw, however this did not lower the background to enable detection of GBVB$_{(DENV)}$ (Figure 5.7).

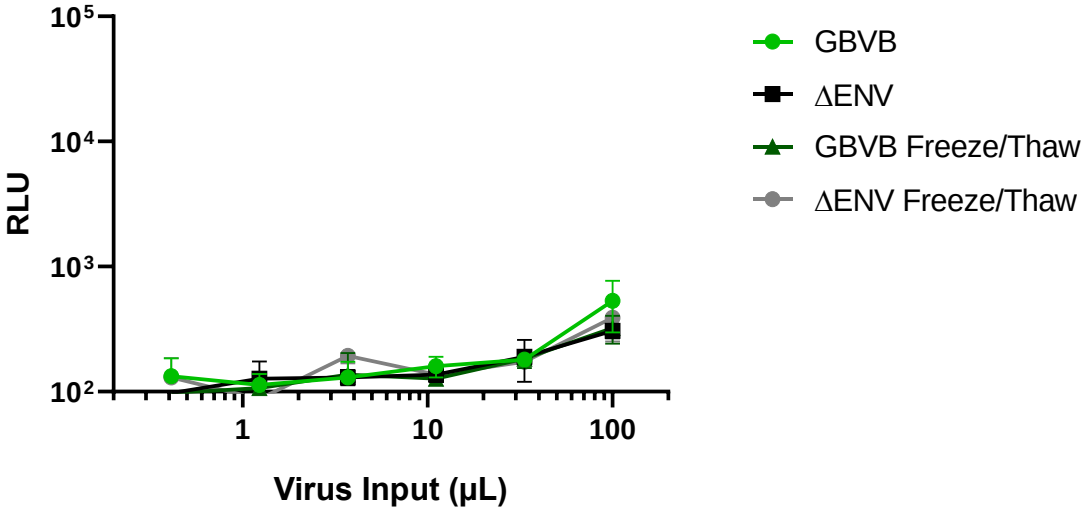


Figure 5.7. Production of GBV-B pseudotyped virus with DENV core

Serial dilutions of GBVB or ΔENV PV containing supernatants produced in HEK 293T cells with the DENV core systems were used either immediately after collection or after snap freezing and storage at -80°C for 1 week to infect Huh7 cells. Infectivity was reported as mean RLU ± standard deviation of one experiment in triplicate.

The JEV$_{(DENV)}$ PVs are functional and suitable for serological work; however, it is worth attempting to optimize multiple core systems as some systems have benefits over others. For example, the DENV core system employed above uses a NanoLuc Luciferase reporter; the substrate for which is very costly which could be inhibitory to using that method for high throughput studies. Therefore, I investigated an alternative method of pseudotyping JEV envelope proteins onto a lentiviral vector.

5.2.3 JEV$_{(HIV)}$ PV production via the *in vitro* assembly method

An alternative method for pseudotyping, which does not rely on the presence of the envelope protein on the cell membrane, consists of mixing Env-less viral particles with Env-rich vesicles (subviral particles) that bud from Env-producing cells. The bald particles and subviral particles associate to create infectious particles, for this thesis it will be termed *in vitro* assembly. This system has been described in the literature for *Vesiculovirus* glycoprotein pseudotyping [443,444], however it has not been described for any members of *Flaviviridae*. The mechanism by which the infectious particles are formed is unknown; two possibilities include total fusion of the subviral particle with the membrane or multiple subviral particles aggregating around the bald particle (Figure 5.8). In both of these forms, the envelope protein would interact with receptors on the surface of target cells and trigger endocytosis and/or membrane fusion.

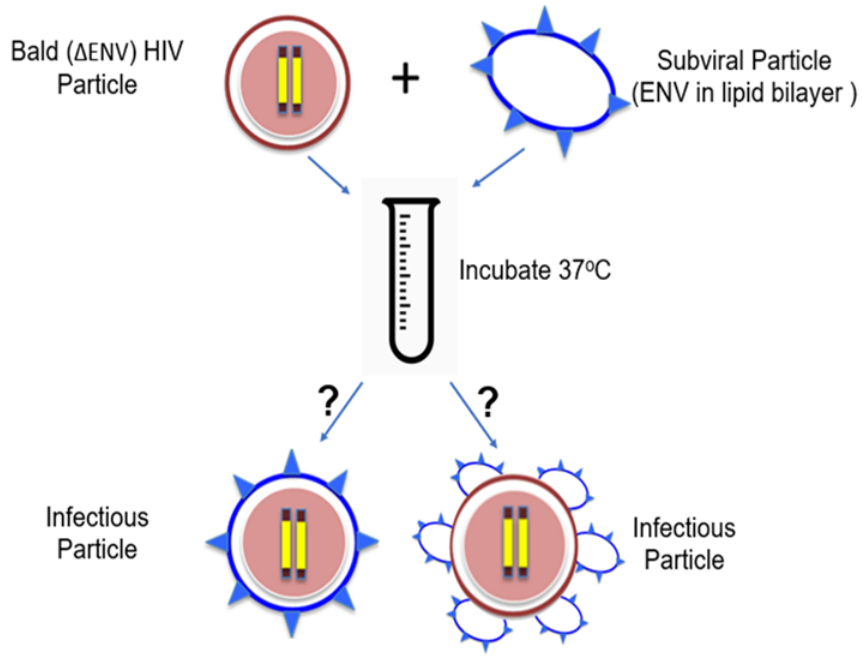


Figure 5.8. Diagram of potential infectious particle structure from in vitro assembly method of pseudotyping

Infectious particles are produced by mixing bald, ENV-less, lentiviral particles with subviral particles (envelope proteins in a lipid bilayer) and incubating at 37°C. The structure of these infectious particles is currently unknown; two potential mechanisms are the fusion of subviral particles with bald particle (bottom left) or aggregation of subviral particles around the bald particles (bottom right).

As JEV produces subviral particles as part of its natural lifecycle [445], I attempted to produce JEV PV with lentiviral cores with a GFP reporter using the *in vitro* assembly method (Figure 5.9). A 1:3 (v:v) ratio of bald virus (produced by omitting the envelope plasmid) to subviral particle (produced by transfecting producer cells with only envelope protein) were mixed and incubated for approximately 1 hour at 37ºC and then titrated onto CRFK cells. The target cells for these assays were changed from Vero cells back to CRFK cells as old-world monkey cells are known to be resistant to lentiviral infection [446]. Supernatant

from cells transfected with an empty expression vector serves as the negative control and VSV-G as the positive control. This resulted in formation of infectious lentiviral JEV PV (Figure 5.9A). The ENV-less vector created a relatively high background with a GFP positive cell population approximately one third of those for JEV and VSV PVs. To improve titres, polybrene, a cationic polymer, was added into the mixing step, to neutralize the negative charges between the membranes in the bald particles and the Env-vesicles. This did not improve the titres but it did unexpectedly reduce the background (Figure 5.9B).

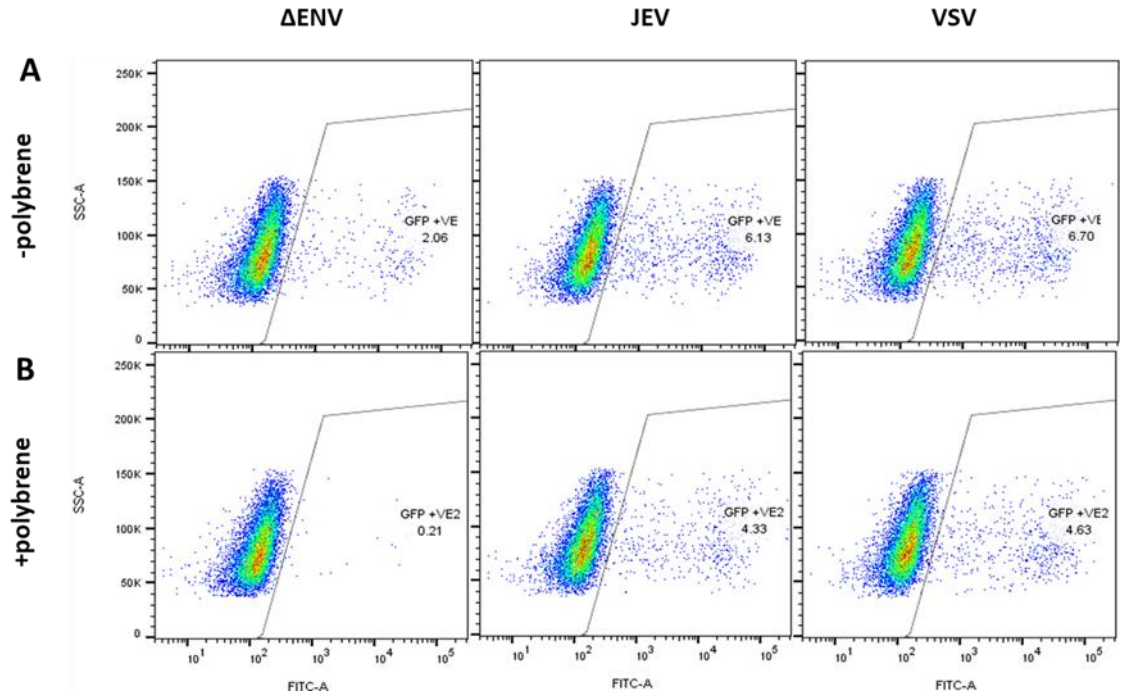


Figure 5.9. Lentiviral pseudotypes with a GFP reporter produced using in vitro assembly

*CRFK cells were transduced with 200µL of the indicated PV-containing mixture without **(A)** and with **(B)** the addition of 8 µg/mL of polybrene and 72 hours later cells were analysed using flow cytometry to determine the percentage of GFP-positive cells. Figures representative of 2 independent experiments.*

Luciferase is known to be more sensitive than GFP as a reporter as well as being preferred for high throughput applications [447,448]. Therefore, I swapped the GFP lentiviral vector for one containing the firefly luciferase gene to produce $JEV_{(HIV)}$ (Figure 5.10) via *in vitro* assembly. Without polybrene at the mixing step $JEV_{(HIV)}$ infectivity was not detectible above background (Figure 5.10A). However, the addition of polybrene considerably lowered the background levels to enable detection of $JEV_{(HIV)}$ (Figure 5.10B), similar to what was observed previously (Figure 5.9). The *in vitro* assembly method appears to overcome the obstacle of JEV envelope protein presentation at the plasma membrane for lentiviral PV production as well as offering the flexibility of incorporating different reporters.

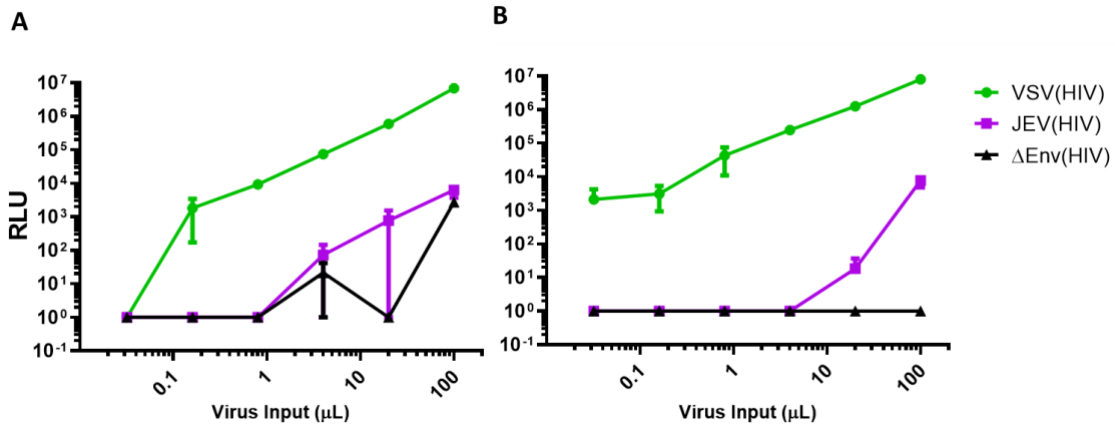


Figure 5.10. Lentiviral pseudotypes with a luciferase reporter produced using in vitro assembly

A 5-fold serial dilution of 100 µL of the indicated PV-containing mixture without ***(A)*** *or with* ***(B)*** *the addition of 8 µg/mL polybrene was titrated on CRFK cells. The target cells were analysed for luciferase activity 72 hours later. Infectivity was reported as mean RLU/mL ± standard deviation of one experiment in triplicate.*

Lastly, with the optimized protocol for $JEV_{(HIV)}$ I attempted to create an $HCV_{(HIV)}$ by *in vitro* assembly. Many HCV glycoproteins are known to pseudotype HIV cores including the one used in this experiment (isolate UKN1A20.8), as seen in chapter 3. However, this method, while successful for VSV, failed to produce an $HCV_{(HIV)}$ above background (Figure 5.11).

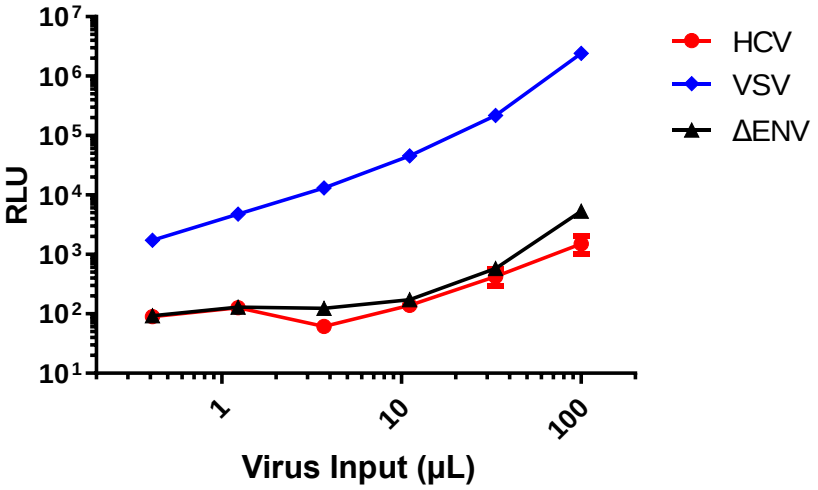


Figure 5.11 Production of $HCV_{(HIV)}$ by in vitro assembly

A 3-fold serial dilution of the indicated PV-containing mixture was titrated on Huh7.5 cells. The target cells were analysed for luciferase activity 72 hours later. Infectivity was reported as mean RLU/mL ± standard deviation of one experiment in triplicate.

5.3 Discussion

Despite vaccines being available, there continues to be major outbreaks of Japanese encephalitis virus that are erratic and unpredictable [449], and in recent decades there has been geographic spread of the virus even further highlighting it epidemic potential [274–277]. Here I show the production of JEV pseudotyped viruses using a DENV core, and a HIV core using *in vitro* assembly, for use as surrogates to live virus, which serves to increase epidemic preparedness.

Attempts to pseudotype JEV onto retroviral or VSV core by traditional methods failed. This is in disagreement with published reports of these JEV PVs [435,450]. Due to personal communication with the viral pseudotype unit (VPU- University of Kent/University of Sussex), who have expert knowledge in pseudotype production, having relayed that after extensive optimization could not produce JEV PV by these methods, I decided to investigate alternative methods.

Pseudotyping the JEV envelope onto the dengue replicon system was successful, which is in agreement with the recently published report of our collaborator Dr Suzuki (NIID, Japan) [339]. Importing the technology in our lab required multiple alterations to the protocol to produce a $JEV_{(DENV)}$ with infectivity over background. Background was greatly reduced with the addition of media changes at 5 hours transfection to remove non-transfected plasmids and 5 hours after transduction to remove uninternalized virus and free NanoLuc in the supernatant that may have been excreted from the producer cells during PV production. The removal of non-transduced virus from the target cells is not always done but it is in line with many published protocols for PV production that remove the virus approximately 3–6 hours after infection [122,281,325,451–453].

A second media change 5 hours after transfection, however, does not appear to be commonplace. The majority of published reports for PV production have one media change 16-24 hours after transfection. It was not unexpected to diverge from typical PV production protocols as this was a DENV core system as opposed to retroviral, which has incorporated the more sensitive NanoLuc luciferase reporter [454].

The $JEV_{(DENV)}$ or $JEV_{NAK\_(DENV)}$ were stable after a cycle of freeze-thawing at -80ºC which has also been shown in stability studies for retroviral PVs [455,456]. The ability to make large batches of PV and store at -80ºC is advantageous for reducing variability between experiments by using the same batch of virus over a long period. Furthermore, stability after freeze/thaw of PVs offer an advantage over wild type virus, that rapidly lose titre, as this could allow for in-field studies where infrastructure is less reliable [336]. Specific neutralization of the $JEV_{(DENV)}$ by sera collected from JEV vaccinees confirmed the PV was suitable for serological studies. This DENV core system failed to pseudotype GBV-B envelope proteins, suggesting that it may be restricted to only some members of the *Flaviviridae* family. As GBV-B and DENV are thought to have similar lifecycles [185,250], with their envelope proteins being displayed in the ER before virion assembly it is unclear as to why the DENV core failed to bud with GBV-B glycoproteins. Recent studies have shown a direct interaction between the capsid proteins of flaviviruses and their envelope proteins, and disruption of transmembrane domains of the envelope proteins results in the formation of more capsid-less subviral particles [445,457,458]. Therefore, correct assembly of the capsid may rely on specific interactions with motifs conserved amongst members of the flavivirus genus, but not in hepacivirus genus members.

While optimizing the DENV core system alternative methods to pseudotype JEV envelope proteins onto traditional cores were also investigated. Here I show for the first time that pseudotyping by *in vitro* assembly, which has been described in the literature for VSV-G [444,459], can be used to create $JEV_{(HIV)}$ PVs. The flexibility of the system allows for use with both a GFP and luciferase reporter. Due to time restrictions, the $JEV_{(HIV)}$ PV created by *in vitro* assembly were not used in neutralization assays to confirm their utility as surrogates in serological studies. It is worth noting that the results of this method were quite variable, and that it appeared that slight changes in incubation times would drastically affect the titre of the PV. Therefore, more optimization is required to define the parameters in which reliable results are generated. It well documented in the literature that different viruses have different thermal stability profiles [460–462]. Therefore, it may be the case that optimized conditions for some viral envelopes may not be optimal for others because different envelope proteins may not have the same stability at the 37^{o}C mixing step. The mechanism by which the subviral particles and the bald cores interact to create infection is unknown and thus warrants further investigation. Electron microscopy images of the particles could give clues as to what the mechanism is and elucidate whether there is total membrane fusion or subviral particles aggregating around the core. VSV can infect HEK cells [333], the cells in which the bald HIV core particles were made, therefore there may be interactions between the VSV-G and its receptors on the core particles that are facilitating this interaction. Similarly, there are many cellular factors indicated for JEV attachment and cell entry (reviewed in section 1.4.4), therefore its plausible that there may be attachment/entry factors drawing the particles into close contact with each other to allow membrane fusion. An attempt to pseudotype HCV envelope proteins via *in vitro* assembly failed; perhaps as

HEK cells are resistant to cell entry by HCV [34], protein interactions between the glycoproteins and cellular receptors are not present to overcome the repulsive forces between juxtaposed membranes. Alternatively, transfection of just HCV E1E2 may not result in release of extracellular glycoproteins. Subviral particle formation and release are well documented for flaviviruses [251,432], however it is not known if this occurs for HCV. Confirmation of HCV E1E2 in the supernatant could clarify this point.

Pseudotyped viruses are incredibly useful and safe tools. Especially for viruses like JEV that require biosafety containment level 3 labs, which are often a scarcity in endemic areas. The production of the two JEV PV here add to the tool kit for studying JEV, particularly for conducting, fast, high-throughput, low containment level serosurveillance studies to help predict and mitigate outbreaks.

Chapter 6:

General Discussion and Future Directions

Pseudotyped virus are safe and versatile tools that can be utilized for a number of applications [281,283,463]. While numerous viral envelope proteins have been pseudotyped onto the most commonly used core systems (HIV, MLV, VSV), members of the family *Flaviviridae* have been shown to be difficult, presumably at least partly due to a mismatch in location of viral assembly and budding between flaviviruses and these core systems. The aims of this project included creating pseudotyped viruses for members of this family while endeavouring to create an optimized, streamlined protocol that may be used to all *Flaviviridae* viruses and possibly other internally budding viruses. This thesis has shown the production and optimization of PVs with the envelope proteins of two viruses within this family: JEV and GBV-B, a model virus for HCV. Moreover, the utility of the GBV-B PV was further demonstrated by development of a pseudotyped virus neutralization assay and the identification of an entry factor required for GBV-B cell entry.

Here I've shown production and optimization of GBV-B pseudotyped virus with a MLV core, while the glycoproteins failed to produce detectible PV on HIV, VSV, or DENV core systems. This is in agreement with other studies that have shown that some HCV isolates fail to pseudotype HIV core but will pseudotype MLV cores [284]. As MLV and HIV are both members of *Retroviridae* and have similar lifecycles, it is not fully understood why this occurs. However small difference in assembly sites between the viruses may determine if core components colocalize with the glycoproteins, as seen with feline endogenous retrovirus RD114 PV formation [464]. Further to that, genetic variations in the envelope proteins may influence their ability to pseudotype as well by altering interactions with cellular chaperones which could alter cellular location; it is known that HCV E1E2 interaction with chaperones is dependent on the envelope glycans [465]. Indeed,

as little as one nonsynonymous nucleotide mutation in HCV glycoproteins in an isolate known to produce infectious PV can result in loss of detectible PV [284,466].

$GBVB_{(MLV)}$, but not $VSV_{(MLV)}$, is neutralized by serum from a GBV-B infected tamarin, which indicates this system is suitable for serological and cell entry studies. Indeed, the humoral response in GBV-B infected animals is poorly understood. This is due in large part to the fact that there are no immortalised cell lines known to support GBV-B infection/replication *in vitro*. As seen in chapter 3, the development of the GBV-B PV overcame this hurdle and led to the development of a neutralization assay, which will allow the characterization of the role neutralizing antibodies play in the clearance of GBV-B. Deciphering the humoral response is a step forward in fully characterizing the GBV-B/new world monkey model for HCV infection and determining its usefulness going forward as a model system. This is particularly important as some of the more newly discovered hepaciviruses, such as the equine or rodent viruses, have potential as model systems [3,162,467,468]. However, one animal model may not fully recapitulate the HCV infection in humans, and different models may be employed based on which one best fits the research question. GBV-B infection in new world monkey is an established system for acute infection [168,172,195,469]. The characterization of the role of neutralizing antibodies in the clearance of the virus and comparison with the immune response to HCV infection in humans may give clues as to why GBV-B is generally cleared in the acute phase while the majority of HCV infections become chronic. Indeed, NAbs are indicated to play a role in the clearance of HCV but their role isn't fully understood [66–69,74].

The GBV-B PV was also used to investigate early steps in the viral entry. I've shown here that claudin-1 (CLDN-1) is a conserved entry factor between HCV

and GBV-B. At the time of writing this thesis, there were no published reports of any hepaciviral entry factors other than those for HCV, which would make this one the first described among the animal hepaciviruses. Exogenous expression of CLDN1 in HEK cells failed to confer susceptibility to $GBVB_{(MLV)}$ infection, indicating that there may be other entry factors needed for GBV-B cell entry. As HCV has multiple factors required for cell entry, it is not surprising that GBV-B may require additional entry factors. However, it is interesting that SRB1, CD81, and OCLN (known HCV receptors) do not appear to play a role in GBV-B entry. As shown in chapter 4, while both HCV and GBV-B use CLDN1 for cell entry, it is by different mechanisms. Further research into the interaction of GBV-B with CLDN1 is needed to determine the exact region of importance and if there is indeed direct interaction between E1E2 and CLDN1. It is not yet known if HCV directly interacts with CLDN1 or simply forms part of receptor complex needed for entry, deciphering the interaction between GBV-B and CLDN1 may provide clues to the mechanism by which HCV utilizes CLDN1. Furthermore, differences in usage of receptors and entry factors may explain the differences in infection outcome. Indeed, chimeric viruses with the structural proteins of HCV and the non-structural proteins of GBV-B have been shown to induce chronic infection [203].

More research into the entry factors of the other animal hepaciviruses may also give some indication whether HCV and GBV-B have divergent or convergent evolutionary paths. A convergent path where they evolved separately to utilize the same entry factor is presumably associated with both being hepatotropic viruses. Whereas if CLDN1 use originated with a common ancestor, divergent evolutionary paths would have resulted in different modes of interaction. Currently, little is known about origin of HCV. Understanding the evolutionary

history of hepaciviruses can inform how these viruses cross species barriers and disseminate into new hosts [470]. The equine hepacivirus is currently the most closely related virus to HCV [159]. Therefore, there is a possibility of a zoonotic transmission from horse to human especially given the close relationship between humans and horses throughout history [3]. There has also been the discovery of many hepaciviruses in bats and rodents which are well known reservoirs for cross-species transmission [471]. Ultimately the identification of more animal hepaciviruses along with characterization of their mode of infection and transmission will aid in deciphering the origins of HCV. Given the difficulties of propagating hepaciviruses in cell culture, producing more hepaciviral PVs may prove to be useful tools to conduct these studies, as seen with the GBVB PV in this thesis and the HCV PVs in the identification of CLDN1 and OCLN as entry factors [33,34].

In this thesis I have also been able to replicate data from recently published report of a dengue virus replicon used to pseudotype multiple flavivirus prME glycoproteins [339]. The $JEV_{(DENV)}$ was specifically neutralized by sera from JEV vaccinated individuals indicating that it is suitable as a surrogate to live virus in neutralization assays. To further confirm its utility in serological research, the $JEV_{(DENV)}$ pseudotyped virus neutralization assay should be compared with the traditional plaque reduction neutralization assay to determine if there is good correlation between the techniques. Additionally, I also show a novel method of pseudotyping JEV by using *in vitro* assembly. This method seems promising and overcomes the need to display the JEV envelope proteins at the cell surface or alter the proteins in any way. However, due to time constraints, the methods were not optimized enough to produce reliable results. Additionally, it needs to be determined if the PVs made by this method are neutralized by anti-JEV antibodies

to confirm their suitability for downstream research. PV production by this method has only been described in the literature for vesiculovirus G proteins, with both MLV and HIV vectors [443,444]. It would be interesting to determine if the JEV subviral particles could also pseudotype MLV and VSV cores via *in vitro* assembly. This is a feasible assumption, as they all bud from the plasma membrane, and therefore the lipid bilayer around the bald cores would be similar to that of HIV.

Another method that could be worth investigating to create flavivirus PVs with retroviral or VSV cores is attempting to increase the number of glycoproteins on the cell membrane. JEV as well as some other flaviviruses use KDEL receptor (KDLER) proteins to move from the ER to the Golgi, and knockdown of these proteins results in an accumulation of virions in the ER [14,15]. KDELRs are transmembrane proteins that cycle between the ER and Golgi to prevent leakage of ER resident proteins by bring them back to the ER [472]. Subviral particles with no core proteins are released from cells as part of the natural lifecycle of JEV as well as from cells transfected with only prME [251,252]. This suggests that there may not be an interaction between the prME and other viral factors required for the envelope proteins to move along the secretory pathway to be released. Therefore, overexpression of JEV prME in producer cells may not lead to 'leaking' from the ER but may instead result in more subviral particle formation. However, removing the cellular machinery co-opted to by the envelope proteins to move along the secretory pathway may indeed lead to an accumulation of prME in the ER and subsequent 'leaking' from which the proteins may localize at the plasma membrane (Figure 6.1). Therefore, investigating the effects of KDELR knockout on JEV prME cellular locations may warrant investigation. Indeed, this work was planned as part of my project and I have prepared the plasmids to create KDELR

KO cell lines via CRISPR/Cas9. However due to time restraints, these experiments could not be completed. A caveat to this approach could be that prME leaked from the ER to the plasma membrane, would bypass the maturation step in the Golgi in which immature prM to cleaved mature M protein, leading to the production of not functional PVs. However, this may be overcome by treatment of the PV with furin-like proteases after harvesting.

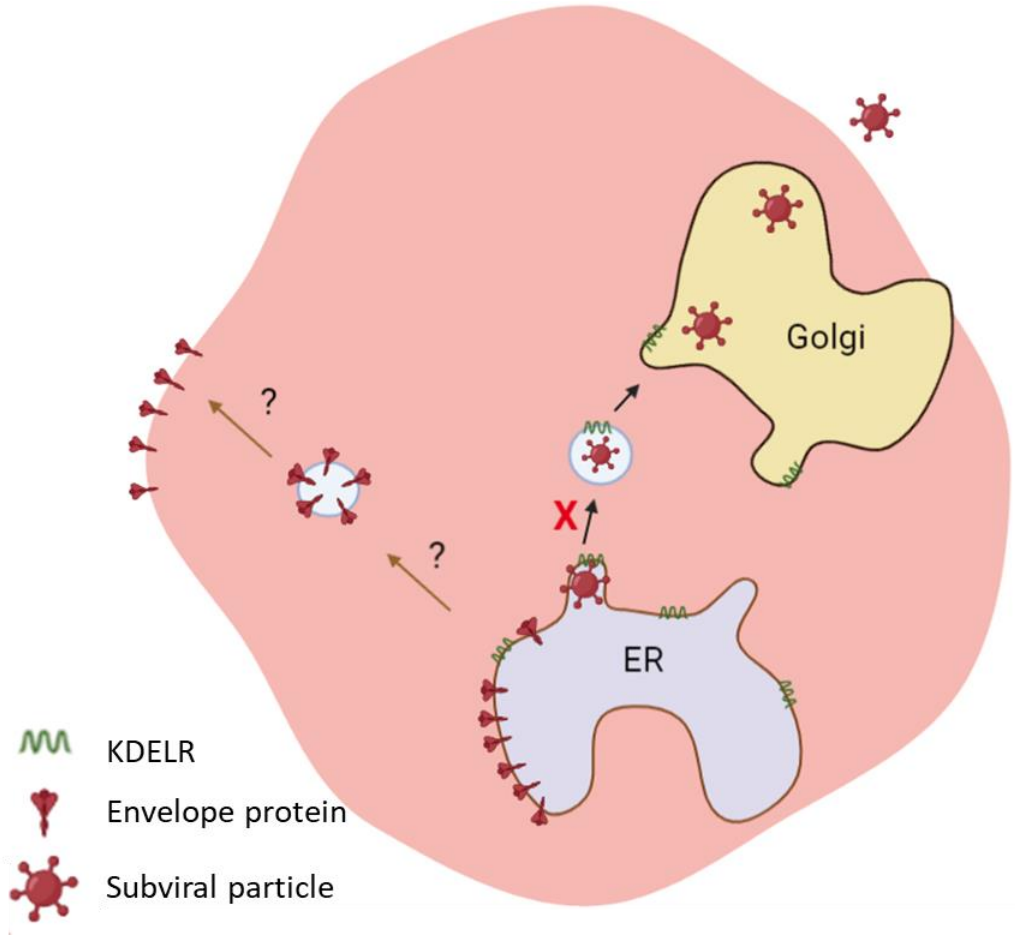


Figure 6.1. Effects of KDELR KO on Flavivirus envelope protein location

Some flaviviruses use KDELR proteins to move along the secretory pathway from the ER to the Golgi. If KDRLR proteins were knocked out in producer cells there is a possibility that this would lead to an accumulation of envelope proteins in the ER that may then leak out and localize at the plasma membrane. Image produced in BioRender.

While there are effective vaccines for JEV, these vaccines, all based on genotype 3, may not be as effective against new genotypes that may emerge. Indeed, the vaccines have been shown to be less effective against genotype 5 [473]. There is also some evidence that flaviviruses can recombine [474,475], which could potentially lead to emergence of genotypically distinct variants against which the vaccines are not effective. Development of JEV pseudotypes offers a safe tool to conduct serosurveillance studies, which aids in epidemic preparedness. Moreover, the flexibility of PVs allows for assessing the impact of genetic variability on vaccine efficacy. PVs can be mutated to include mapped or predicted mutation sites in the envelope proteins, therefore the immune responses elicited by the vaccine can be tested against the current different genotypes, and predicted future variants, with some indication of protection offered. Furthermore, JEV entry mechanisms are still unknown. The JEV PV described in this thesis can be used to study the early steps of JEV entry in a lower containment laboratory (BSL2 instead of BSL3). Elucidating the entry mechanisms can inform antiviral therapy development, which is of interest as there are no therapeutics available for JEV infection.

The use of pseudotyped viruses, while being versatile and generally safe, is not without drawbacks. The pseudotyped virus neutralization assay (PVNA) measures the presence of neutralizing antibodies in samples. They can also be used in assays to test for antibody-dependent enhancement of viral infection [476–478]. There are, however, other functions of antibodies that aid in viral clearance such as antibody-dependent cellular cytotoxicity (ADCC) [479], which is indicated in playing an important role in HCV clearance [480,481]. These types of *in vitro* assays do not fully measure the extent of the humoral response nor the

interplay between the humoral and cellular immune response. To address this, sera can be used in ADCC assays to be performed alongside the PVNA. Interestingly, the plaque reduction neutralisation assay, which is considered the gold standard for many viruses in determining neutralizing antibodies, particularly in immune reposes to vaccination, can also only detect neutralizing antibodies [482,483]. Indeed, ability of a vaccine to raise neutralizing antibodies to a virus as detected by *in vitro* assays is often used as the correlate of protection [484,485]. A PVNA also cannot detect antibodies that bind but do not neutralize or antibodies that bind to regions other than the envelope proteins, like the antibodies are raised to core proteins and non-structural proteins in HCV infection [486]. Antibodies that target non neutralizing epitopes may aid in opsonization. An ELISA can be used to detect and quantitate all binding antibodies in a sample. Interestingly, PVs can be used in ELISAs to capture antibodies to the envelope proteins.

Additionally, PVs contain the envelope proteins of the virus on interest, however, the arrangement and number of envelope proteins on the PV may not be reflective of the wildtype virus. For example, less tightly packed envelope protein on a PV may allow for antibodies to more easily access neutralizing epitopes resulting in higher neutralization titres than those measured against live virus. Conversely, envelope proteins packed too densely into the envelope may impair receptor binding, as seen with Ebola glycoproteins [487]. The glycoproteins on the surface of the PV may not have followed the same cellular route as the ones on the live virus so that may result in altered processing of the proteins, such as glycosylation patterns, which can also affect neutralization [488,489]. It has been shown that for HCV glycosylation pattern differ on envelope proteins in different cellular areas [321]. Further to that, as glycoproteins are expressed in the

absence of the other viral factors of the wildtype virus, the envelope displayed on PV may be missing interactions with these other factors. Such is the case with HCV PV, which do not have the characteristic association with lipoproteins [29]. Again, this may affect neutralization as the lipoproteins can shield neutralizing epitopes [99]. Therefore, whenever possible comparative studies should be undertaken to determine if the PVs are in good agreement with live virus assays. These types of studies have been done for a number of viruses and results vary depending on the glycoprotein used [463]. For example one study for influenza A showed that correlation between PVNA and the hemagglutinin inhibition assay with live virus ranged from poor (r=0.1171, A/Japan/305/57(H2N2)) to strong (r=0.8454, A/Brisbane/59/2007(H1N1)) depending on the envelope proteins used [490]. However, confirming correlation with live virus assays is not always feasible/possible as PVs are often employed when there have been no live virus assays developed or the facilities for those assays are not available.

Ultimately, I was successful in producing PVs for GBV-B and JEV, which can be used to further research into aspects of these viruses where assays were not available/easily accessible. However, creating an optimized protocol for pseudotyping all members of Flaviviridae was not achieved. One of the advantages of many PV systems is the ability to swap out one envelope plasmid for another to create a different PV. This type of ‘plug and play’ platform is advantageous for the quick development of new PV, especially for newly emerging viruses, which was recently demonstrated with the rapid development of SARS-CoV-2 PVs [348]. However, as demonstrated here, that approach is not feasible for some viral envelopes. Although hepaciviruses and flaviviruses have similar lifecycles and are within the same viral family, GBV-B and HCV pseudotyped retroviral cores while JEV did not, therefore there may be more

factors involved in creating a successful PV production other than compatible cellular location of components. More research into the parameters involved in successful PV generation could elucidate why some envelope proteins pseudotype and some do not. Even within genotypes of HCV, some isolates pseudotype and some do not [284,400]. Determining the differences in the envelope sequences that are responsible for the discrepancy, whether it be location, or interactions with cellular or core virus components, may give some indication as to what conditions need to be met for successful PV production with the established core systems. Currently, it is standard practice when viral envelope proteins fail to pseudotype one core, others are tested. Therefore, in addition to adapting/altering these envelope proteins or their cellular location to fit existing core systems, research could be undertaken to develop more core systems, particularly from viruses that do not bud from the plasma membrane, such as seen with the DENV system. This could put more tools in the virologist's toolbox for pseudotyping new viruses.

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