School of Biomedical Sciences

The Role of Genetic Diversity in the Replication, Pathogenicity and Virulence of Murray Valley Encephalitis Virus

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Doctor of Philosophy
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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:…….

Date:……

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ABSTRACT

Murray Valley encephalitis virus (MVEV) is the main causative agent of arboviral encephalitis in Australia. Of the four genotypes of MVEV (G1-G4), only G1 and G2 are found in Australia. G1 is dominant, while G2 is confined to Kununurra in the northeast Kimberley region of Western Australia (WA). Prior to this thesis, G2 MVEV had not been detected since 1995.

In order to accurately characterise the distribution of the different MVEV genotypes in WA, nucleotide sequencing and phylogenetic analyses were performed on the partial envelope gene of all (seventy one) MVEV isolates collected from mosquitoes in northern WA between 2005 and 2009. Four G2 isolates were identified (5.6% of all isolates sequenced) from Fitzroy Crossing and Broome in the west Kimberley region. This indicates that G2 continues to circulate in WA, and beyond its previously recognised geographic range of Kununurra. Of the sixty seven G1 isolates, forty five (67.2%) belonged to sublineage G1a, and twenty two (32.8%) belonged to sublineage G1b. To further characterise the genetic diversity within these isolates, sequencing and analysis of the full-length prM-E genes and the 3'-untranslated region (3'UTR) was performed on representative isolates. Analysis of the full-length prM-E genes indicated a similar phylogenetic relationship between all of the MVEV isolates compared to that obtained using a partial analysis of the envelope gene. Analysis of the 3'UTR sequences identified a unique 18-nucleotide deletion in all G2 isolates from 1991, 2006 and 2009, which may serve as a genetic marker of recent G2 strains.

In order to facilitate the detection and quantification of viral RNA from all four genotypes of MVEV in the laboratory, surveillance and clinical settings, a real-time quantitative RT-PCR (RT-qPCR) was developed and validated. This assay demonstrated a high level of efficiency, sensitivity and specificity to all four MVEV genotypes and detected MVEV in infected pools of mosquito homogenates, as well as infected brains and tissues of veterinary clinical specimens.

Next generation sequencing (NGS) was employed to characterise the depth of genetic diversity and mutation spectra in the G1 and G2 MVEV populations. The complexity of mutant spectra within each sample quasispecies was determined by calculating the percentage of sequence clones as well as nucleotides that differed from the consensus sequence. This approach has been used by many researchers to detect low-frequency mutations and variants in a viral population. NGS technology revealed that G1 samples are highly genetically diverse while G2 samples have a lower level of genetic complexity and sequence heterogeneity. The lower level of genetic diversity in G2 MVEV is associated with low abundance of this MVEV genotype in nature.

The phenotypic features of representative isolates from recent G1a, G1b and G2 were characterised and compared with those of the prototype viruses – the primary ancestral viruses – of G1 (MVE-1-51) and G2 (OR156) in a single-step growth curve assay and a mouse model of pathogenesis. There was no significant difference between the replication kinetics of G1 and G2 isolates in DF1 cells compared to their prototype viruses. In contrast, recent G1 isolates demonstrated significantly lower replication kinetics in C6/36 cells compared to G1 prototype virus; whereas, one recent G2 isolate displayed a significantly higher replicative fitness than the G2 prototype virus in these cells. In an *in vivo* mouse infection model, all G1 isolates demonstrated a dose-dependent mortality rate, characteristic of highly virulent strains. In contrast, G2 isolates demonstrated a significant reduction in neuroinvasiveness, and increases in average survival time and 50% humane dose end point (HD_{50}) values, indicative of attenuation.

In an experimental evolution study, the basis of MVEV restricted genetic diversity (the observation that MVEV has not been subjected to a high level of genetic variation) was explored. It was hypothesised that the genetic stability of MVEV results from alternate cycling between mosquito vectors and avian hosts. Genetically homogeneous MVEV G1 and G2 clonal populations were passaged sequentially in mosquito C6/36 or avian DF1 cells or alternately between the two cell lines. Passaging G1 and G2 MVEV in mosquito cells did not result in significant differences in genetic and phenotypic characteristics. In contrast, serial passage of

G1 and G2 MVEV in avian cells and alternately between mosquito and avian cells resulted in accumulation of mutations that were associated with significant differences in replicative ability in a single-step growth curve assay and an attenuation of virulence in mice. This suggests that the restriction of MVEV genetic diversity may be a result of alternating replication between mosquitoes, in which purifying selection is strong, and not as a result of replication in avian hosts, where purifying selection is relaxed.

Recent detection of G2 isolates from the Kimberley region of WA provided a unique opportunity to compare the genetic and phenotypic characteristics of this genotype to isolates belonging to the dominant Australian genotype (G1). Significantly, circulating G2 viruses were shown to have a low level of genetic diversity and an attenuated phenotype in a mouse model of MVE. In contrast, G1 viruses had relatively high levels of genetic diversity in circulating strains and a virulent phenotype in mice. It is therefore hypothesised that genetic diversity is an important contributing factor to the observed abundance and distribution of each of these genotypes. The body of work presented herein provides an insight into the genetic diversity, molecular epidemiology and geographical distribution of MVEV, the mechanisms that restrict MVEV evolution as well as the potential molecular determinants of attenuation in G2 MVEV. A new highly sensitive and specific diagnostic tool (RT-qPCR) capable of detecting all MVEV genotypes was developed.

LIST OF PRESENTATIONS

1. Aziz-ur-Rahman Niazi, Paul J. Costantino, Sinead M. Diviney, Cheryl A. Johansen and David T. Williams. 2010. Re-appearance of genotype 2 of Murray Valley encephalitis virus in Western Australia. *Australian Society for Medical Research*. Curtin University. P87.

2. Aziz-ur-Rahman Niazi, Paul J. Costantino, Sinead M. Diviney, Cheryl A. Johansen and David T. Williams. 2012. Detection of a rare genotype of Murray valley encephalitis virus in Western Australia. *Asia Pacific Congress of Medical Virologists*. Adelaide. P58.

3. Aziz-ur-Rahman Niazi, Beng Hooi Chua, Paul J. Costantino, Sinead M. Diviney, Cheryl A. Johansen and David T. Williams. 2012. Genotypic and phenotypic changes in Murray Valley encephalitis virus following serial passage in avian and mosquito cells. *Combined Biological Science Meeting: Australian Society of Microbiology*. The University of Western Australia. P4.

4. Aziz-ur-Rahman Niazi, Beng Hooi Chua, Paul J. Costantino, Sinead M. Diviney, Cheryl A. Johansen and David T. Williams. 2012. *In vivo* and *in vitro* phenotypic changes in Murray Valley encephalitis viruses following experimental evolution in avian and mosquito cells. *Mark Liveris Research Seminar*. Curtin University. P57.

5. Aziz-ur-Rahman Niazi, Beng Hooi Chua, Paul J. Costantino, Sinead M. Diviney, Cheryl A. Johansen and David T. Williams. 2010. Genetic and phenotypic characterisation of genotype 1 and 2 Murray Valley encephalitis. *Curtin Health Innovation Research Institute (CHIRI)*. Curtin University.

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Abbreviation	Full name
3D	Three dimensional
AAHL	Australian Animal Health Laboratory
ALFV	Alfuy virus
anchC	Anchor C
AST	Average survival time
BBB	Blood-brain barrier
C	Capsid protein
CAMBA	China-Australia Migratory Bird Agreement
CDV	Clone-derived virus
CF	Complement fixation
CFAV	Cell fusion agent virus
CHIKV	Chikungunya virus
cHP	Capsid hairpin
CNS	Central nervous system
CPE	Cytopathic effect
Cq	Quantification cycle
CS	Cyclization sequence
CSF	Cerebrospinal fluid
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT	Computed tomography
CV	Coefficient opf variation
DAR	Downstream of AUG region
DB	Dumbbell
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DI	Defective interfering
dpi	Days post infection
dsRNA	Double stranded RNA
DSS	Dengue shock syndrome
E	Envelope protein

Abbreviation	Full name
EHV	Edge Hill virus
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
emPCR	emulsion PCR
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
HD_{50}	50% humane end point dose
HI	Hemagglutination inhibition
HIV	Human immunodeficiency virus
hpi	Hours post infection
i.c.	Intracranial
i.p.	Intraperitoneal
ICTV	International Committee on Taxonomy of Viruses
IFA	Indirect fluorescent antibody
IFN	Interferon
IL	Interleukin
indels	Insertions/deletins
ISF	Insect-specific flaviviruses
JAMBA	Japan-Australia Migratory Bird Agreement
JC	Jukes-Cantor
JEV	Japanese encephalitis virus
K2+I	Kimura-2 model
kd	Kilodalton
KOKV	Kokobera virus
KOUV	Koutango virus
KUNV	Kunjin virus
LabVISE	The Laboratory Virology and Serology
LD_{50}	Lethal dose 50%
LOD	limit of detection
M	Membrane protein

Abbreviation	Full name
mAb	Monoclonal antibody
MID	Multiplex identifier
ML	Maximum likelihood
mNS1	Membrane-bound NS1 protein
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
MTase	Methyltransferase
MVEV	Murray Valley encephalitis virus
NASBA	Nucleic acid-sequence based amplification
NC	Nucleocapsid
NGS	Next generation sequencing
NJ	Neighbour joining
NNDSS	National Notifiable Disease Surveillance System
NS	Non-structural
NSW	New South Wales
NT	Neutralisation test
NT	The Northern Territory
NTC	No-template control
NTPase	Nucleoside triphosphatase
ORF	Open reading frame
PCR	Polymerase chain reaction
PCV	Palm Creek virus
PK	Pseudo-knot
PNG	Papua New Guinea
prM	Premembrane protein
PSEK	Porcine stable-equine kidney cells
QLD	Queensland
RC	Replication complex
RCS	Repeated conserved sequence
RdRp	RNA-dependent RNA polymerase

Abbreviation	Full name
RFU	Relative fluorescence unit
rmANOVA	Repeated measures analysis of variance
ROKAMBA	The Republic of Korea-Australia Migratory Bird Agreement
RTPase	RNA nucleoside 5’ triphosphatase
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Real-time quantitative reverse-transcription polymerase chain reaction
RVFV	Rift Valley fever virus
SA	South Australia
SD	Standard deviation
SEPV	Sepik virus
sfRNA	Subgenomic flavivirus RNA
sHP	Small hairpin
SL	Stem Loop
SLEV	Saint Louis encephalitis virus
SNP	Single nucleotide polymorphism
sNS1	Secreted NS1
STRV	Stratford virus
SVP	Subviral particles
TBEV	Tick borne encephalitis virus
$TCID_{50}$	50% Tissue culture infective dose
TC-SN	Tissue culture supernatants
TN93	Tamura-Nei model
UAR	Upstream of AUG region
USUV	Usutu virus
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VIC	Victoria
WA	Western Australia
WNV	West Nile virus
YFV	Yellow fever virus

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Acute viral encephalitis can be caused by numerous pathogens. In Australia, the main causative agent of arboviral encephalitis in humans is Murray Valley encephalitis virus (MVEV) that causes the disease Murray Valley encephalitis [MVE; (Mackenzie et al., 2002; Marshall, 1988)]. MVEV can also cause neurological disease in horses (Gard et al., 1977; Roche et al., 2013). MVEV is a member of the Japanese encephalitis (JE) serocomplex which comprises important viral pathogens including Japanese encephalitis virus (JEV), West Nile virus (WNV), Saint Louis encephalitis virus (SLEV) and Usutu virus [USUV; (Simmonds et al., 2012)]. In the last century, major MVEV outbreaks occurred in the eastern and south-eastern states of Australia (Broom et al., 2002a; Mackenzie et al., 1994; Mackenzie and Williams, 2009; Russell and Dwyer, 2000). However, MVEV is believed to currently exist in an enzootic cycle in the Kimberley region of Western Australia [WA; (Mackenzie and Broom, 1995; Spencer et al., 2001)], where most human cases of MVE now occur.

From early March 2011, significant rainfall and flooding in most parts of Australia resulted in an increase in the population of MVEV's main mosquito vector, *Culex annulirostris* (Knox et al., 2012). Subsequently, MVEV demonstrated higher levels of activity in endemic regions and a re-emergence in the southern and south-eastern states. During the 2011 outbreak of MVEV, there were sixteen confirmed human cases of encephalitis, including four deaths (Knox et al., 2012). Moreover, the 2011 MVEV outbreak saw substantial numbers of horse infections with significant mortalities and widespread seroconversion of sentinel chickens (Mann et al., 2013; Roche et al., 2013). Significantly, this was the first time since 1974 that MVEV infection was seen in humans in the south and south-eastern states (Knox et al., 2012).

The propensity of MVEV to spread and re-emerge is a major concern because of the serious public and veterinary health threat that MVEV outbreaks represent. The continued study of the distribution, spread and evolution of this virus will be important to understand the drivers of MVEV outbreaks.

1.2 Flaviviruses

The genus *Flavivirus* consists of a highly diverse group of mainly arthrobod-borne viruses (arboviruses) that are distributed worldwide (Gould et al., 2003). This genus comprises some of the highly virulent human pathogens such as MVEV, JEV, WNV and SLEV that cause encephalitis; as well as the dengue viruses (DENVs) and yellow fever virus (YFV) that cause haemorrhagic fever (Gubler, 2012; Gubler et al., 2007). These viruses cause significant morbidity and mortality rates among humans and animals (Ghosh and Basu, 2009; Knox et al., 2012; Murray et al., 2011; Roche et al., 2013). In addition, this genus contains many avirulent viruses such as Alfuy virus (ALFV) and Stratford virus (STRV) that do not appear to cause disease in humans or animals (May et al., 2006; Prow et al., 2011). A significant body of research has been conducted to investigate the genetic diversity, virulence, fitness and evolution of members of flaviviruses. These studies provided important insights into the epidemiology and pathogenicity of flaviviruses.

1.2.1 Taxonomy and Classification

Flavivirus is a genus of the family *Flaviviridae* (Simmonds et al., 2012). The prototype virus of this family is YFV, after which the genus and family were named. The Latin word *flavus* means yellow, signifying the jaundice caused by this disease. The taxonomy of the *Flavivirus* genus is somewhat confused since the International Committee on Taxonomy of Viruses (ICTV) frequently revises the classification. Prior to 2005, the ICTV classification listed over seventy viruses in the genus (Calisher and Gould, 2003; Calisher et al., 1989). This was mainly due to classifying subtypes and serotypes as separate species within the genus (Gubler, 2012). Calisher et al. (1989) classified flaviviruses into eight antigenic complexes and subcomplexes, based on their serological characteristics. One of the most important antigenic complexes of flaviviruses is the JE antigenic complex which comprises ten viruses including JEV, MVEV, WNV, SLEV, ALFV, STRV, USUV, Kunjin virus (KUNV), Koutango virus (KOUV), and Kokobera virus (KOKV), most of which are serious human pathogens (Calisher et al., 1989). However, many flaviviruses, including YFV, failed to fit into these categories. According to Kuno et al. (1998), one of the biggest challenges in the

classification of the *Flavivirus* genus arises from the diverse and widespread arthropod vectors and vertebrate hosts involved in the transmission of these viruses.

Advances in the studies of molecular phylogenetics, antigenic structures and geographic association of flaviviruses, coupled with knowledge of their vector and host associations, pathogenicity and ecological features, have enabled re-classification of these viruses into groups, species and subtypes/serotypes (Simmonds et al., 2012). The most recent ICTV classification (Simmonds et al., 2012) only lists fifty three species within the *Flavivirus* genus, forty of which are human pathogens (Table 1.1 and Appendix 1.1). In the recent classification, flaviviruses have been classified into three distinct groups, with associated subgroups. The three main groups are tick-borne viruses, mosquito-borne viruses, and viruses with no known vector. The tick-borne group contains twelve species, whereas the mosquito-borne group contains twenty seven. The remaining fourteen species belong to the group with no known vector (Table 1.1 and Appendix 1.1). Each main group contains viruses of medical and clinical significance that cause disease in humans and/or animals (Gubler, 2012). Some viruses in the family do not fit any of these groups and are yet to be classified.

1.2.2 Virion, Genome Organisation, Structure and Function

Flaviviruses have an enveloped spherical virion of approximately 50nm in diameter. The virion contains a positive-sense, single-stranded RNA genome that is surrounded by a host-derived lipid membrane which sediments between 170 and 210 S (Kuhn, 2012; Monath and Heinz, 1996; Schmaljohn and McClain, 1996). The viral genome spans approximately 11,000 nucleotides and consists of a single open reading frame (ORF) that encodes a polyprotein, flanked by 5′ and 3′ untranslated regions [UTRs; Fig. 1.1A; (Kuhn, 2012; Markoff, 2003)]. The genome is capped at its 5′ end, but unlike many cellular messenger RNA, it lacks a 3′ polyadenylated (poly A) tail.

Two forms of virus can be distinguished: an extracellular mature virion and an intracellular immature virion (Fig. 1.2A). Mature virions contain three structural proteins: the capsid (C) protein, the membrane (M) protein and the envelope (E)

Table 1.1. *Members of the genus Flavivirus.*

1. TICK-BORNE VIRUSES
Mammalian tick-borne virus group
Seabird tick-borne virus group (probably tick-borne)
Kadam virus group
2. MOSQUITO-BORNE VIRUSES
Aroa virus group
Dengue virus group
Japanese encephalitis virus group
Cacipacore virus
Japanese encephalitis virus
Koutango virus
Murray Valley encephalitis virus
Alfuy virus
St. Louis encephalitis virus
Usutu virus
West Nile virus
Kunjin virus
Yaounde virus
Kokobera virus group
Ntaya virus group
Yellow fever virus group
PROBABLY MOSQUITO-BORNE
Kedougou virus group
Edge Hill virus group
3. VIRUSES WITH NO KNOWN ARTHROPOD VECTOR (NKV)
Entebbe bat virus group
Modoc virus group
Rio Bravo virus group
4. TENTATIVE SPECIES IN THE GENUS (not approved)
Mammalian tick-borne viruses
Mosquito-borne viruses
Probably arthropod-borne viruses
Viruses with no known arthropod vector

The main groups of viruses are listed in bold UPPERCASE text, the subgroups are listed in bold lowercase text; and the names of virus species are listed in normal type script; subspecies names are listed as italics. Only members of the Japanese encephalitis virus groups are listed. For the complete list and classification of the *Flavivirus* genus, please refer to Appendix 1.1. Adapted from Simmonds et al. (2012).

protein (Rey et al., 1995). Intracellular immature virions contain the precursor to the M protein (prM) instead of M [Fig. 1.2A; (Heinz and Stiasny, 2012; Kuhn et al., 2002; Li et al., 2008)]. Flavivirus immature virions display a spiky surface (Fig. 1.2B), but unlike other mature RNA viruses that exhibit the spike-like projection on their surface, mature flaviviruses have a smooth surface [Fig. 1.2C; (Kuhn et al., 2002; Mukhopadhyay et al., 2005; Zhang et al., 2003)]. The surface of the flavivirus virion shows 30 copies of E protein dimers that are organised in an icosahedral symmetry [Fig. 1.2B, and Fig. 1.2C; (Mukhopadhyay et al., 2005; Zhang et al., 2003)].

1.2.2.1 Replication Overview

Flaviviruses replicate in the cytoplasm of host cells [Fig. 1.3; (Gubler et al., 2007; Lindenbach et al., 2007; Roby et al., 2012)]. Prior to cell entry, these viruses are capable of using several cellular molecules to mediate their attachment into the host cells (Jindadamrongwech et al., 2004 ; Krishnan et al., 2007; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). None of these molecules have yet been confirmed as functional flavivirus receptors (Roby et al., 2012). Upon attachment to the host cell surface, flaviviruses are internalised via clathrin-mediated endocytosis [Fig 1.3A; (Chu and Ng, 2004; Mosso et al., 2008; Suksanpaisan et al., 2009)]. Following internalisation, the low pH of the endosomes triggers the fusion of the viral envelope with the cell membrane, allowing the disassembly and release of the flavivirus RNA into the cytoplasm of the infected cells [Fig. 1.3B; (Bressanelli et al., 2004; Lindenbach et al., 2007; Roby et al., 2012; Stiasny et al., 2007)].

The translation of flavivirus RNA produces a large polyprotein of approximately 3400 amino acids (Fig. 1.3C) that is co- and post-translationally cleaved into ten proteins (C, prM-M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5; Fig. 1.1A) by virus-encoded protease and host cell signalases (Lindenbach et al., 2007; Mukhopadhyay et al., 2005; Roby et al., 2012)]. The structural proteins (C, prM/M and E) are encoded by the 5’ one-third of the ORF, while the non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded by the 3’ two-third (Fig. 1.1A). The NS proteins replicate the flavivirus genomic RNA [Fig. 1.3D; (Roby et al., 2012)]. The prM and E glycoproteins wrap the newly synthesised

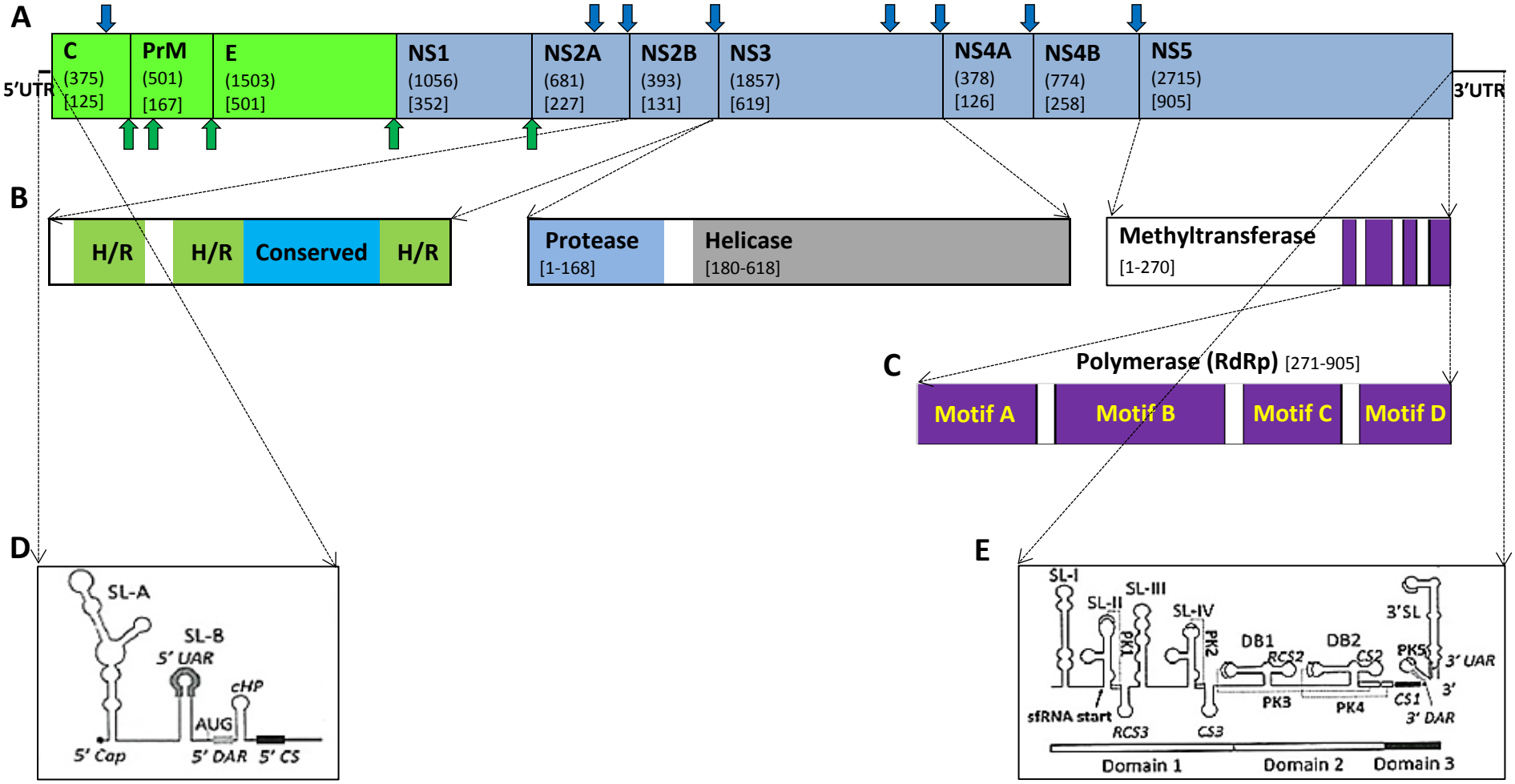
A
5'UTR
C (375) [125]
PrM (501) [167]
E (1503) [501]
NS1 (1056) [352]
NS2A (681) [227]
NS2B (393) [131]
NS3 (1857) [619]
NS4A (378) [126]
NS4B (774) [258]
NS5 (2715) [905]
3'UTR
B
H/R
H/R
Conserved
H/R
Protease [1-168]
Helicase [180-618]
Methyltransferase [1-270]
C
Polymerase (RdRp) [271-905]
Motif A
Motif B
Motif C
Motif D
D
SL-A
SL-B
5' UAR
cHP
AUG
5' Cap
5' DAR
5' CS
E
SL-I
SL-II
SL-III
SL-IV
PK1
PK2
DB1
RCS2
DB2
CS2
PK5
3'SL
3' UAR
3'
CS1
3' DAR
sfRNA start
RCS3
CS3
PK3
PK4
Domain 1
Domain 2
Domain 3

Fig. 1.1.

Fig. 1.1. *Schematic representation of the Flavivirus genome.*

A. There are three structural (C, prM, and E) proteins and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Numbers in parantheses **()** represent the length of each gene in nucleotide bases, whereas square brackets **[]** denote the lengths of each protein in terms of the number of amino acid. Structural proteins are green; non-structural proteins are blue. The cleavage sites of the *Flavivirus*-encoded NS2B-NS3 proteases are indicated by blue-filled down arrows. The cleavage sites of the host-produced proteases are indicated by green-filled up arrows.

B. The predicted composition of the NS2B protein is displayed. This contains a conserved hydrophilic region and three hydrophobic regions (H/R). Also shown is the protease and helicase domains of the NS3 protein. A schematic representation of the NS5 protein with its methyltransferase and polymerase (RdRp) domains is also shown. The relative positions of the conserved motifs of the polymerase domain is displayed below the NS5 protein.

C. The predicted secondary and tertiary structures of the 5'UTR and 3'UTR are displayed. The locations of the conserved secondary and tertiary elements in two untranslated regions at both ends of the genome are shown. **SL:** stem loop; **AUG:** start codon; **UAR:** upstream of AUG region; **DAR:** downstream of AUG region; **CS:** cyclisation sequences; **cHP:** the C protein hairpin; **sfRNA:** subgenomic flavivirus RNA; **RCS:** repeated conserved sequence; **DB:** dumbbell-like structure; **PK:** pseudoknot.

Fig. 1.1A and 1.1B are adapted from published data (Luo et al., 2012; Yu et al., 2007), Fig. 1.1C is reproduced from Roby et al. (2012).

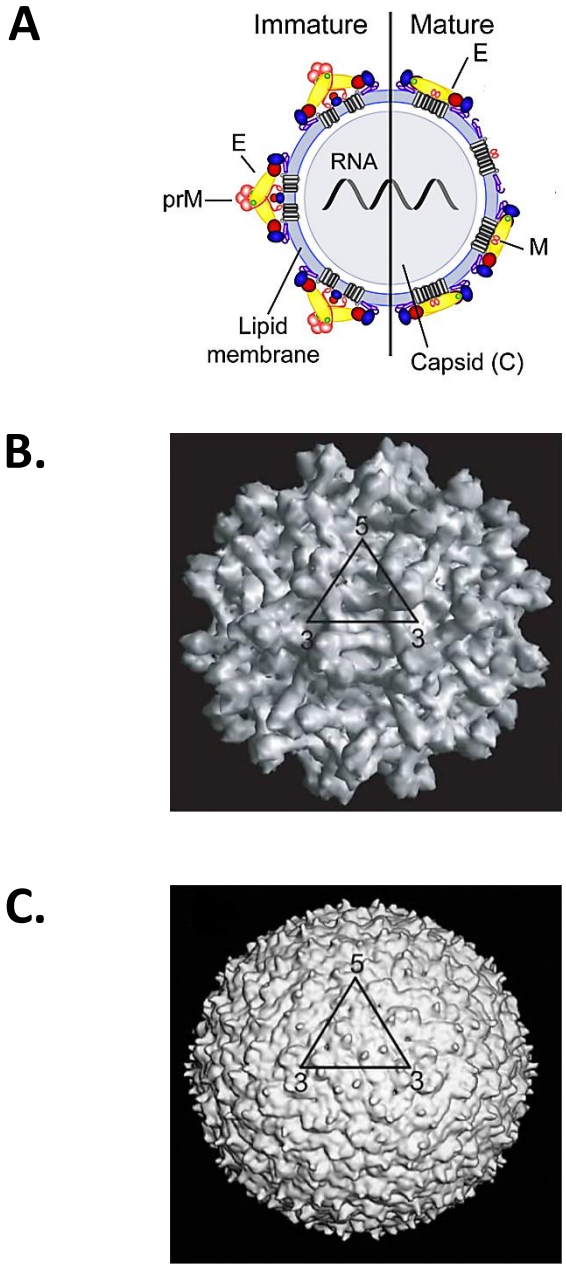

Fig. 1.2. *Schematic and three dimensional images of the Flavivirus virion.*

A. The spherical capsid contains the positive-sense single stranded genomic RNA and the capsid protein (C). The surface of immature protein (left) is covered by spike-like trimers of the pre-membrane (prM) and envelope (E) heterodimers. The mature particle (right) contains the mature membrane (M) instead of prM (Heinz and Stiasny, 2012). **B.** Surface shaded view of a cryo-electron microscopy reconstruction of DENV-2 immature virion at 12.5 Å resolution (Zhang et al., 2003). **C.** Cryo-electron microscopy reconstruction of the mature DENV-2 virion at 14Å resolution (Zhang et al., 2003). Triangles indicate one icosahedral unit.

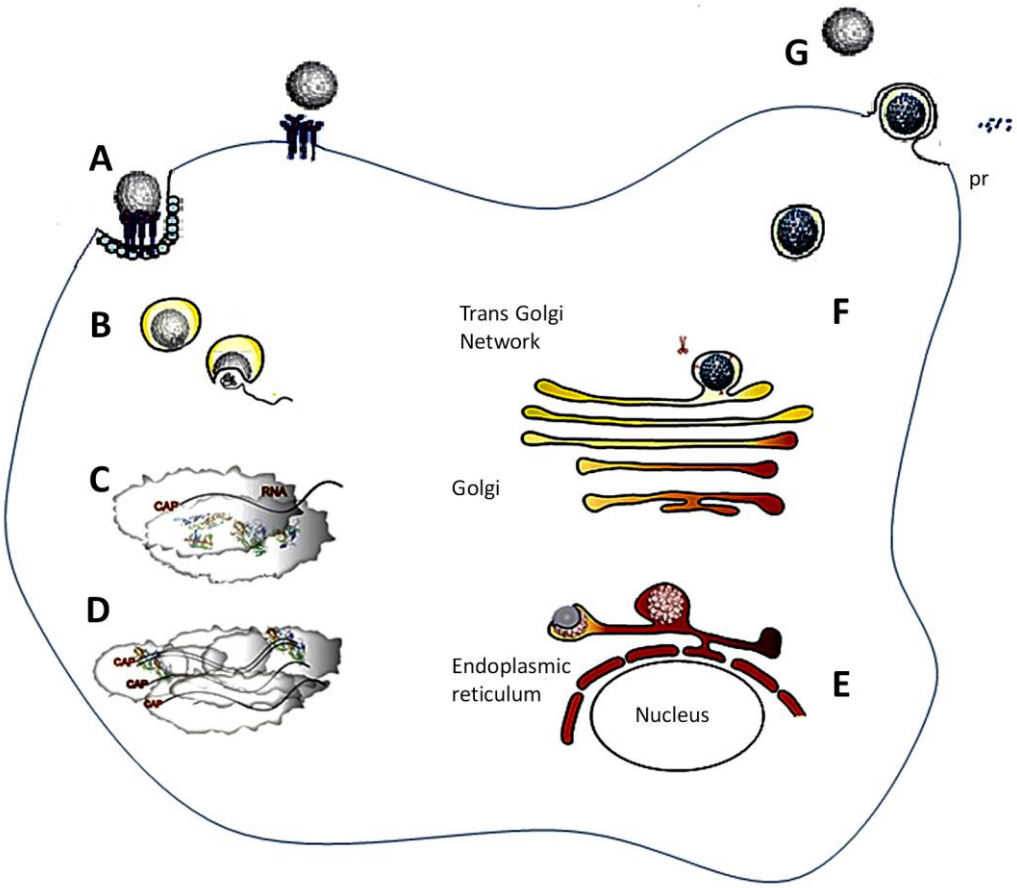

Fig. 1.3. *A schematic representation of the Flavivirus replication cycle.*

A. Virus attachment and entry
B. Disassembly and release of the viral RNA
C. Translation of flavivirus RNA
D. Replication of viral genomic RNA
E. Assembly of the immature virus particles and budding into the endoplasmic reticulum
F. Cleavage of pr segment from the M protein and maturation of the virus particle
G. Release of the mature virion from the cell.

Modified from Perera et al. (2008).

genomic RNA and the capsid protein to assemble the immature virus particles (Fig. 1.3E). These immature virions are budded into the endoplasmic reticulum (ER) before they are transported to the Golgi network via the secretory pathway (Mackenzie and Westaway, 2001; Welsch et al., 2009). The low pH of the trans-Golgi network causes the host enzyme furin to cleave the pr segment from the M protein and produce the mature virion [Fig. 1.3F; (Mukhopadhyay et al., 2005; Stadler et al., 1997; Welsch et al., 2009)]. The mature virion is immediately secreted at the plasma membrane [Fig. 1.3G; (Roby et al., 2012)].

Flaviviruses often produce defective genomes that lack most of the structural genes, and the 5′ half of the NS1 gene (Lancaster et al., 1998; Yoon et al., 2006). Defective genomes that encode stop codons or deletions have been found to form a part of the genetic diversity of many flavivirus populations (Aaskov et al., 2006; Lancaster et al., 1998; Yoon et al., 2006). The virus particles that carry these defective genomes are called defective interfering (DI) particles. Due to the defect in their genome, their replication and life cycle completely depends on the enzymes and helper functions of the parental virus (Aaskov et al., 2006; Lancaster et al., 1998; Yoon et al., 2006). Thus, defective genomes are unable to replicate in the absence of the complete genome of wild-type virus. However, they often compete with the parental viruses and disrupt the production of full-length infectious virions, resulting in an increased production of non-infectious particles (Lancaster et al., 1998; Poidinger et al., 1991). The DI particles are involved in significantly reducing the cytopathogenicity of the wild-type virus (Poidinger et al., 1991) and establishing a persistent infection (Poidinger et al., 1991; Yoon et al., 2006).

1.2.2.2 5′ and 3′ Untranslated Regions

The 5′ end of the flavivirus genome contains a type I $m^7GpppAmpN_2$ cap (Lindenbach et al., 2007). The 5′UTR demonstrates a high level of variation between viruses belonging to the different groups of flaviviruses (Brinton and Dispoto, 1988), however, the secondary structures in this region appear to be conserved and play an important role in RNA replication (Filomatori et al., 2006). Within the 5′UTR, there are two stem loops (SL) named SL-A and SL-B [Fig. 1.1D; (Lodeiro et al., 2009)].

SL-A, which is also known as 5'SL, is a necessary promoter element for RNA synthesis (Alvarez et al., 2005; Filomatori et al., 2006; Roby et al., 2012). SL-B contains a conserved sequence which is located upstream of the translation initiator (AUG) called the 5'UAR [upstream of AUG region; (Alvarez et al., 2005)]. The conserved sequences of the 5'UTR are complementary with their counterparts in the 3'UTR and are involved in the cyclisation of the flavivirus genome (Friebe and Harris, 2010; Khromykh et al., 2001a; Villordo and Gamarnik, 2009). The capped 5'UTR of flaviviruses can also direct the translational expression in cell culture (Chiu et al., 2005).

The 3'UTR of flaviviruses lack a poly A terminal and demonstrates considerable variation in size and sequence between flaviviruses (Poindinger et al., 1996). In mosquito-borne flaviviruses, the 3'UTR can be divided into three domains. Domain 1 is located immediately downstream of the stop codon and is highly variable in length and sequence (Mandl et al., 1998; Men et al., 1996; Poindinger et al., 1996). This domain contains four stem loops (SL-I, SL-II, SL-III and SL-IV) and two conserved hairpin structures: the repeated conserved sequence (RCS; RCS3) and the cyclisation sequence [CS; CS3; Fig. 1.1E; (Pijlman et al., 2008; Proutski et al., 1997)]. Since domain I demonstrates great variability, not all mosquito-borne flaviviruses contain all four SLs, RCS3 and CS3 of this domain. It has been reported that the highly structured RNA sequences in domain 1 of the 3'UTR of flaviviruses render resistance to cellular ribonucleases (Pijlman et al., 2008). The incomplete degradation of the flavivirus genome by cellular nucleases results in the production of subgenomic flavivirus RNA (sfRNA; Fig. 1.1E) that are important for the infectivity and the pathogenicity of these viruses (Pijlman et al., 2008).

Domain 2 is moderately conserved and contains several hairpin motifs such as RCS2 and CS2, that fold into dumbbell-like (DB) structures called DB1 and DB2 (Fig. 1.1E). Mosquito-borne flaviviruses possess at least one of these conserved structures (Roby et al., 2012).

Domain 3 is highly conserved and encompasses the 3'SL, CS1, 3' UAR and 3' DAR [downstream of AUG region; Fig. 1.1E; (Markoff, 2003)]. The 3'SL is the most

conspicuous structure at the 3' end of the flavivirus genome (Brinton et al., 1986). In addition to genome cyclisation, the conserved structures and RNA elements in the 3'UTR of flaviviruses are also involved in RNA synthesis (Alvarez et al., 2005; Tilgner et al., 2005), replication (Friebe and Harris, 2010) and translation (Holden and Harris, 2004; Li and Brinton, 2001). Apart from the structures in domain 3 of the 3'UTR shown in Fig. 1.1E, another secondary structure between 3' UAR and 3' DAR, called the small hairpin (sHP), has been recently described (Villordo et al., 2010; Villordo and Gamarnik, 2013). Significantly, the sequence of the loop of sHP is found to be essential in DENV replication only in mosquitoes, but not in mammalian cells (Villordo and Gamarnik, 2013). Mutations in the stem loop of sHP resulted in viruses that were unable to replicate in mosquito cells, whereas the same mutants replicated efficiently in mammalian cells (Villordo and Gamarnik, 2013). The presence and the role of sHP in other flaviviruses is not yet identified.

In addition to the above secondary structures within the 3'UTR of the flavivirus genome, up to five tertiary structures, named pseudoknots (PK), have also been predicted [Fig. 1.1E; (Olsthoorn and Bol, 2001; Pijlman et al., 2008; Shi et al., 1996)]. A PK is formed when single-stranded RNA within a hairpin loop base-pairs with an RNA sequence elsewhere in the genome (Roby et al., 2012).

1.2.2.3 Structural Proteins

Capsid (C): The C protein is an essential protein of about 11kd. It encapsidates the viral RNA to form the nucleocapsid (NC). Despite having a similar size in all flaviviruses, the sequence of the C protein shows little sequence conservation. According to Jones et al. (2003), the C protein is the least conserved protein in the genome of flaviviruses with only a maximum of 40% sequence identity between the genus members. The nascent form of C protein, also called anchor C (anchC), has a hydrophobic anchor on its C-terminal that functions as a signal sequence in the translocation of prM glycoprotein into the endoplasmic reticulum [ER; (Lindenbach et al., 2007). Analyses of the available sequence information of flaviviruses for predicting the secondary structure indicate that there are conserved RNA elements of small sizes that are complementary to their counterparts in domain 3 of the

3'UTR. 5' DAR is complementary to 3'SL (Friebe and Harris, 2010), whereas 5' CS is complementary to 3' CS1 [Fig. 1.1D and Fig. 1.1E; (Hahn et al., 1987). These two conserved structures (5' DAR and 5' CS) are separated by a 14-nucleotide sequence in the C protein that forms the capsid hairpin (cHP; Fig. 1.1D). The cHP directs the initiation of translation from the first AUG of the C protein (Clyde and Harris, 2006), and is essential for genome cyclisation and RNA synthesis (Clyde et al., 2008).

Pre-membrane/membrane (prM/M): The prM protein is the glycoprotein precursor to the M protein. After translocation of anchC/prM into the ER, the signal peptidase cleaves C to produce the mature C and M proteins. Cleavage is mediated by host proteases such as furin present in the Golgi-apparatus [Fig. 1.1A; (Stadler et al., 1997)]. However, this process is not undertaken until an adequately high level of viral serine protease is present (Amberg et al., 1994; Lobigs, 1993). Cleavage is also influenced by the level of expression of the E protein (Lorenz et al., 2002). The pr segment of the uncleaved prM protects the virus from a premature fusion to the cell membrane by covering the fusion loop of the E protein during egress from the cells (Li et al., 2008).

The cleavage of prM protein to the M protein and the N-terminal pr segment is associated with maturation and release of virus particles. The N-terminal of the pr segment contains six conserved cysteine residues. These cysteine residues form disulfide bridges (Nowak and Wengler, 1987) and participate in the formation of N-linked glycosylation sites (Kim et al., 2008). This site has been shown to be important in Golgi trafficking and secretion of the virion, and for the pathogenicity of flaviviruses in mice (Goto et al., 2005; Kim et al., 2008)

Envelope (E): The E protein is around 53kd (Lindenbach et al., 2007). It mediates receptor binding, membrane fusion, and induces immunity (Hall et al., 1996; Lobigs et al., 1990; Schneeweissa et al., 2011; Spohn et al., 2010; Sultana et al., 2009). The E protein contains 500 residues that are organised into three domains (Fig. 1.4), forming a head-to-tail homodimer on the surface of the virion (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004). Domain I is the central, eight-stranded, β-barrel domain, that contains approximately 120 residues arranged

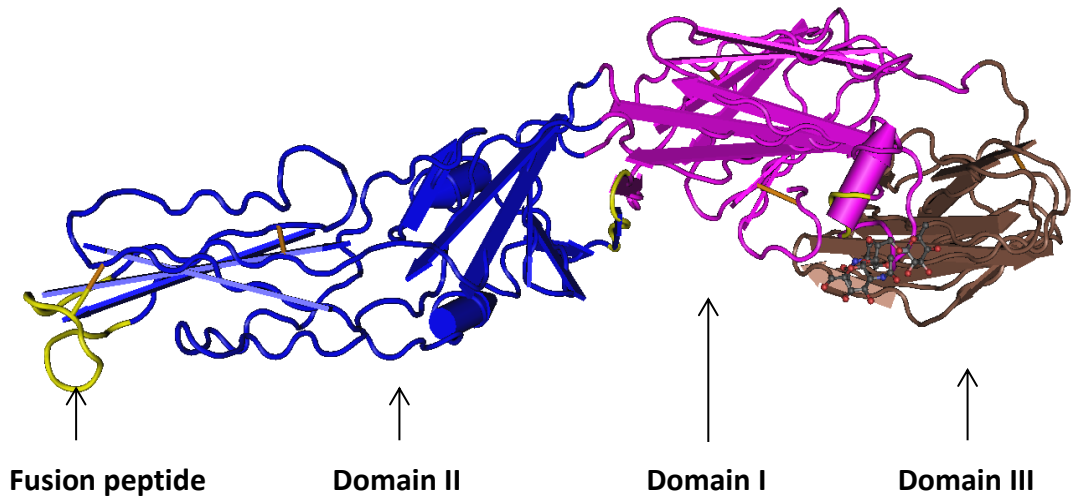

Fig. 1.4. *The three dimensional crystal structure of the E protein of WNV that shows the location of three distinctive domains.*

Adapted from Nybakken et al. (2006).

in three segments (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004). In WNV, the three segments span residues 1-51, 134-195 and 284-297 (Nybakken et al., 2006). There are four conserved cysteine residues in domain I, forming two disulfide bonds (Nybakken et al., 2006; Rey et al., 1995). The N-linked glycosylation site of the E protein (NYS; residues 154-156) is located within the second segment of domain I (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004). The glycosylation of E protein at N^{154} considerably enhances the assembly and secretion of flavivirus particles into the extracellular milieu and increases their infectivity (Hanna et al., 2005; May et al., 2006; Murata et al., 2010; Prow et al., 2011). Domain I acts as a hinge between domain II and domain III (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004).

Domain II contains two loops that connect the three segments of domain I [residues 52-133 and 196-283 (Nybakken et al., 2006)]. These loops fold together to produce a dimerisation domain. Domain II contains six cysteine residues that form three disulphide bonds (Nybakken et al., 2006; Rey et al., 1995). The tip of domain II contains the putative fusion peptide, a hydrophobic sequence that is rich in glycine [Fig. 1.4; (Nybakken et al., 2006; Rey et al., 1995; Zhang et al., 2004)]. The sequence of the fusion peptide is almost entirely conserved in all flaviviruses. The fusion peptide is 13 residues long (98-110) in mosquito-borne flaviviruses (Nybakken et al., 2006; Zhang et al., 2004). It is anticipated that the fusogenic activity of the virus is related to this sequence (Allison et al., 2001; Fritz et al., 2008; Kuhn et al., 2002; Seligman, 2008; Stiasny et al., 2007). In addition, the putative flexible hinge region (residues 273-277), which connects domain I and domain II, is also located on domain II. This region is also conserved in all flaviviruses (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995).

Domain III contains an immunoglobulin-like fold and is 103 residues long (residues 298-400) in mosquito-borne flaviviruses (Luca et al., 2012; Nybakken et al., 2006; Zhang et al., 2004). A 15-residue segment connects domain III to domain I. Domain III contains two cysteine residues and only one disulfide bridge. Domain III functions as a receptor binding domain (Chu et al., 2007; Rey et al., 1995) and is the key target of neutralising antibodies (Fritz et al., 2008; Lin and Wu, 2003; Martina et al.,

2008; Schneeweissa et al., 2011; Spohn et al., 2010). The RGD receptor binding motif (residues 388-390) is located in domain III of the E protein (Lobigs et al., 1990; Nybakken et al., 2006; Rey et al., 1995).

1.2.2.4 Non-Structural Proteins

NS1: The NS1 glycoprotein (46kd) demonstrates a high degree of homology among flaviviruses (Mackow et al., 1987; Mandl et al., 1989b). It contains twelve highly conserved cysteine residues (Blitvich et al., 2001; Wallis et al., 2004) and at least two or three N-linked glycosylation sites [N^{130}, N^{175} and N^{207}; (Muller and Young, 2012)]. The twelve conserved cysteine residues and their associated disulfide bridges are essential in the folding and function of the NS1 protein (Blitvich et al., 2001; Wallis et al., 2004). The N-linked glycosylation sites have an important role in RNA replication and viral pathogenesis (Muller and Young, 2012; Muylaert et al., 1996).

The NS1 glycoprotein can be found in three different cellular locations, including the vesicles within host cells (intracellular NS1), within the cell in the vesicular compartments associated with the cell membrane (mNS1), and as a secreted protein in the extracellular surface on the host cell [sNS1; (Muller and Young, 2012; Smith and Wright, 1985)]. Intracellular NS1 is important for viral replication and has been shown to co-localise with flavivirus double-stranded RNA (dsRNA) in replication complexes [RC; (Lindenbach and Rice, 1997; Mackenzie et al., 1996; Westaway et al., 1997)]. In addition, mNS1 and sNS1 are highly immunogenic (Falgout et al., 1990; Gould et al., 1986; Hall et al., 1996; Hall et al., 1995; Schlesinger et al., 1985). Several studies have also associated the NS1 protein and anti-NS1 antibodies in the disease pathogenesis and autoimmunity (Avirutnan et al., 2006; Cheng et al., 2009; Lin et al., 2006; Sun et al., 2007).

NS2A: The NS2A glycoprotein is a hydrophobic protein of comparatively small size (22kd). There is evidence that NS2A is involved in virus assembly, and a mutation in this protein can block virus production (Kummerer and Rice, 2002; Liu et al., 2003). However, this effect can be reversed by a second mutation in the NS3 helicase domain (Kummerer and Rice, 2002; Liu et al., 2003). The interaction of NS2A with

NS3, NS5, and the 3'UTR of flavivirus genomic RNA may co-ordinate the shift from RNA replication to RNA packaging (Khromykh et al., 2001b; Mackenzie et al., 1998b).

NS2B: NS2B is a hydrophobic protein of 14kd. The NS2B protein of flaviviruses contains a highly conserved, central and hydrophilic domain (40-residue long). This domain is flanked by three hydrophobic regions [Fig. 1.1B; (Falgout et al., 1993)]. The conserved domain is essential for flavivirus protease activity and NS3-mediated cleavage of the NS2B/NS3 cleavage site (Falgout et al., 1993; Jia et al., 2013). The protease activity of flaviviruses also involves the NS2B-NS3 serine protease which functions to release viral proteins that are crucial in viral replication and assembly (Chambers et al., 1991; Falgout et al., 1993; Rothan et al., 2012).

NS3: The NS3 protein of flaviviruses is a large, trypsin-like multifunctional protein of approximately 70kd. It is the second most conserved protein of the flavivirus polyprotein (Chambers et al., 1990; Luo et al., 2012). NS3 contains two structurally and functionally distinctive domains: the N-terminal (protease) domain and the C-terminal (helicase) domain (Fig. 1.1B).

The protease domain of the NS3 protein encodes serine protease that, in association with the conserved hydrophilic domain of the NS2B, is essential for the proteolytic cleavage of non-structural proteins of flaviviruses (except NS1; Fig. 1.1A). This process results in the release of mature NS proteins in the cytoplasm of flavivirus-infected cells. Additionally, the NS2B-NS3 complex cleaves within the C protein (Fig. 1.1A), releasing it from anchC (Chambers et al., 1990; Lobigs, 1993).

The helicase domain of the NS3 protein spans amino acids 180-618 (Fig. 1.1B) and has multiple functions including RNA helicase (Benarroch et al., 2004; Padmanabhan et al., 2006), RNA nucleoside 5' triphosphatase (RTPase), nucleoside triphosphatase (NTPase) activities (Benarroch et al., 2004; Wang et al., 2009; Warrener et al., 1993; Wengler and Wengler, 1991), increasing fatty acid synthesis (Heaton et al., 2010), and virus assembly (Liu et al., 2002; Patkar and Kuhn, 2008). In addition, NS3 has been shown to play a role in the induction of host cell

apoptosis (Duarte dos Santos et al., 2000) and the severity of DENV-associated disease in mice (Bordignon et al., 2007; Luo et al., 2012).

NS4A: NS4A is a hydrophobic transmembrane protein of 16kd that plays an important role in RNA replication (Mackenzie et al., 1998b; McLean et al., 2011). NS4A binds to NS3 as a cofactor and causes an increase in the catalytic activity of NS3 (McCoy et al., 2001). Moreover, together with some of the other NS proteins, NS4A is involved in blocking type I interferon signalling and the translocation of STAT2 into the nucleus of the host cell. This has the effect of modulating the innate immune response (Liu et al., 2005; Miller et al., 2006). Furthermore, it has been shown that NS4A co-localises with NS1 and dsRNA in the vesicle packets (Mackenzie et al., 1998b). This suggests a role for these proteins in RNA replication.

NS4B: NS4B is a highly hydrophobic protein of about 27kd that primarily resides in ER-derived cytoplasmic foci. NS4B also forms a complex with NS3 and dsRNA, driving the intermediate phase of viral RNA replication (Miller et al., 2006). Like NS4A, NS4B can block the interferon (IFN)-induced antiviral response by inhibiting type I IFN signalling (Liu et al., 2005; Munoz-Jordan et al., 2003).

NS5: NS5 is a large multifunctional protein of about 103kd, and is the most conserved protein of the flaviviruses (Davidson, 2009; Lindenbach et al., 2007). There are two distinct domains in this protein: the N-terminal domain [methyltransferase, (MTase)] and the C-terminal (polymerase) domain (Fig. 1.1). The MTase domain (residues 1-270) is involved in the modification of RNA 5' cap (Zhou et al., 2007). The polymerase domain (residues 271-905) contains the viral RNA-dependent RNA polymerase (RdRp), which is essential for viral RNA replication (Bartholomeusz and Wright, 1993; Davidson, 2009; Lescar et al., 2012; Yu et al., 2007). It is now known that the RdRp of flaviviruses is a primer-independent polymerase and initiates the *de novo* synthesis of negative-strand RNA without the help of other virus or host cofactors (Ackermann and Padmanabhan, 2001; Selisko et al., 2006). Sequence analysis of RdRp revealed four conserved motifs designated motif A, B, C, and D (Poch et al., 1989; Yu et al., 2007). It has been shown that

substitution in each of these motifs results in the complete inactivation of RdRp (Yu et al., 2007).

NS5 is also able to induce the transcription and subsequent secretion of interleukin 8 [IL-8; (Medin et al., 2005)]. The ability of NS5 to influence the expression of IL-8 prevents the antiviral effects of innate immunity enabling the spread of the virus in the host (Medin et al., 2005). Furthermore, NS5 can bind to IFN receptor complexes and inhibit IFN function (Best et al., 2005; Lescar et al., 2012). This highlights the importance of NS5 in the replication and pathogenesis of flaviviruses.

1.2.3 Pathogenesis

Most flaviviruses do not cause human or animal diseases. But emerging and re-emerging flaviviral infections around the world highlight their ability to become more pathogenic and cause large outbreaks (Bledsoe, 2004; Li et al., 2011; Mackenzie et al., 2004; Mackenzie and Williams, 2009). In this section, only the pathogenesis of encephalitic flaviviruses is addressed. Flaviviruses are usually transmitted by arthropod vectors. They typically enter via the skin through the bite of an infected arthropod. From the site of entry, the encephalitic flaviviruses progress to the regional draining lymph nodes (Kimura et al., 2010; McMinn et al., 1996). Within the first 48 hours post infection (hpi), flaviviruses amplify rapidly in the regional lymph nodes and peripheral organs, before migrating via the lymphatic system to the thoracic duct. Here, the viruses drain into the circulatory system and establish a primary viraemia (Kimura et al., 2010; Monath and Heinz, 1996). The virus is then transported to the extraneural tissues to replicate. Subsequently, the newly replicated viruses enter back into the bloodstream and cause a secondary viraemia within 60-72 hpi (Kimura et al., 2010). Approximately 4-5 days post infection (dpi) neurological infection can occur, whereby the virus enters the central nervous system by breaching the blood-brain barrier [BBB; (Hayes et al., 2005)], and/or invasion of the olfactory neurons (Andrews et al., 1999). Following replication in the CNS, clinical signs and symptoms appear after 7 dpi (Bennett, 1976; Kimura et al., 2010).

The disease caused by members of the JE serogroup ranges from mild fever and meningitis to acute destructive encephalitis (Hayes et al., 2005; Knox et al., 2012; Salcuni and Rizzo, 2011; Sejvar et al., 2003). The mortality rate of flavivirus infection depends on the species and genotypes involved, but can be as high as 25%. Most survivors experience permanent neurological sequelae (Ding et al., 2007; Ghosh and Basu, 2009; Knox et al., 2012; Murray et al., 2011).

1.2.4 Immunity

Following a flavivirus infection, both innate and adaptive immune systems are induced and interact with each other to eliminate the infection (Nash and Usherwood, 2000). The innate immune system initiates a rapid non-specific response against the pathogens. This may result either in completely abolishing or slowing the spread of infection (Nash and Usherwood, 2000). The adaptive immune system establishes the production of flavivirus-specific antibodies which is a slow but highly specific response. The E protein of flaviviruses is a major antigenic determinant and can provide a protective response (Hall et al., 1996; Schneeweissa et al., 2011; Spohn et al., 2010; Sultana et al., 2009). The antibody-eliciting epitopes on the E protein of flaviviruses induce the production of neutralising antibodies (Heinz, 1986; Oliphant et al., 2007; Roehrig et al., 1989). Non-neutralising, yet protective antibodies that recognise NS1 protein have also been reported (Calvert et al., 2006; Hall et al., 1996). Other flavivirus proteins such as C, prM, NS3, NS4B and NS5 are also immunogenic and have been shown to induce immunity (Brinton et al., 1998).

In areas where two or more flaviviruses co-circulate, secondary exposure to a related flavivirus results in an anamnestic response, in which a rapid and stronger immune response against the primary flavivirus infection is initiated (Calisher, 1994b; Mongkolsapaya et al., 2003; Williams et al., 2010). Serologically, it can be nearly impossible to distinguish the infections with different viruses (Halstead et al., 1983; Makino et al., 1994; Westaway et al., 1974; Williams et al., 2010). In many cases, the original antigenic sin phenomenon can be observed, in which the highest antibody response is provided by the virus responsible for the first infection

(Halstead et al., 1983; Midgley et al., 2011; Mongkolsapaya et al., 2003). Original antigenic sin is considered an important mechanism in the development of dengue haemorrhagic fever (DHF) and dengue shock syndrome [DSS; (Gibbons, 2010; Green and Rothman, 2006; Midgley et al., 2011)]. Original antigenic sin has also been reported in the experimental infection of guinea pigs with JEV and WNV (Inouye et al., 1984).

1.2.5 Diagnosis and Detection

Because flaviviruses can cause very severe diseases, their early diagnosis and detection is essential for improved outcome in the clinical setting. Although flavivirus diseases can be diagnosed based on clinical symptoms, geographical distribution and the patients' case history, many laboratory tests have been developed to assist with the early detection of flavivirus infection. Virus isolation, serology, and molecular tests are discussed below.

1.2.5.1 Virus Isolation

Flaviviruses can be isolated by using a variety of cell culture systems. Virus isolation from infected tissues of humans or animals can be achieved by inoculation of the chorioallantoic membranes of embryonated eggs, the intracerebral inoculation of suckling mice or serial culture in mosquito, mammalian or avian cell lines (French, 1952; French et al., 1957; Lehmann et al., 1976; Miles et al., 1951; Williams et al., 2010). Encephalitic flaviviruses such as JEV and MVEV can only be isolated from the cerebrospinal fluid (CSF) of <30% of human patients who manifest acute encephalitis (Monath and Heinz, 1996). The isolation of the virus from human and animal samples requires that the infection be at a very advanced stage. MVEV has been isolated post mortem from the brain tissue of fatal human cases (French, 1952; French et al., 1957; Lehmann et al., 1976; Miles et al., 1951).

For surveillance purposes, flaviviruses can be isolated from the homogenates of mosquito pools. Virus isolation from mosquito pools involves an initial cell culture in the *Aedes albopictus* C6/36 cell line, followed by culture in mammalian cells such as African green monkey cell line (Vero cells), and porcine stable-equine kidney [PSEK

cells; (Broom et al., 1989; Johansen et al., 2009)]. Subsequent identification can be made by several methods including enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), neutralisation with specific monoclonal or polyclonal antibodies and immunofluorescent staining, or by reverse-transcription polymerase chain reaction (RT-PCR).

Virus isolation remains an important method of detecting and characterising exotic, emerging and previously unidentified viruses. In addition, virus isolation is an important method for providing viral stocks for diagnostic and research purposes (Williams et al., 2010). Nonetheless, this is a time-consuming and labour-intensive technique.

1.2.5.2 Serological Tests

Classical serological techniques for flavivirus diagnosis include the serum neutralisation test [NT; (Gorman et al., 1975; Ksiazek and Liu, 1980)], haemagglutination inhibition [HI; (Clarke and Casals, 1958)], complement fixation [CF; (Casals and Palacios, 1941; Southam, 1956)], indirect fluorescent antibody [IFA; (Monath et al., 1981)] and ELISA (Burke et al., 1987; Martin et al., 2000; Solomon et al., 1998). The NT provides the most reliable results and is considered the best method for virus identification (Calisher, 1994a; Williams et al., 2010). Unfortunately, this technique is difficult to perform and the results can be difficult to interpret. The HI and CF tests are susceptible to considerable cross-reactivity between flaviviruses and, therefore, cannot be reliably used for the identification of flaviviruses to species level (Williams et al., 2010). Although the IFA test is easy to perform and the results can be obtained relatively quickly, it is not broadly commercially available (Williams et al., 2010). Therefore, ELISA and enzyme immunoassays (EIA) have been extensively used for the specific detection and identification of flaviviruses (Hogrefe et al., 2004; Pezzoti et al., 2011; Schmitz et al., 2011; Shi and Wong, 2003).

Problems are encountered when testing hyperimmune sera following multiple flavivirus infections. Serological identification of the infecting agent in a secondary flavivirus infection can be complicated because of the development of broadly

cross-reactive antibodies (Makino et al., 1994). This problem is more prominent in areas, such as northern Australia and the tropical Americas, where many flaviviruses co-circulate. It is important that positive serological test results in such areas are confirmed by multisampling or by other diagnostic techniques (Ledermann et al., 2011).

1.2.5.3 Molecular Tests

As discussed earlier, virus isolation and detection by immunoassay techniques are time-consuming, laborious, and sometimes lack sensitivity. Therefore, molecular diagnostic methods, such as RT-PCR, RT-nested PCR and real-time quantitative RT-PCR (RT-qPCR) techniques, are useful tools for the rapid and specific detection and identification of flavivirus infection (Huang et al., 2004; Jeong et al., 2011; Mendez et al., 2007; Patel et al., 2013).

RT-PCR provides additional information about the molecular epidemiology of flaviviruses in both routine surveillance (Jeong et al., 2011; Johansen et al., 2007) and outbreak settings (Huang et al., 2004; Nunes et al., 2011). Nested PCR, a modified version of the RT-PCR assay, involves an initial RT-PCR reaction using primers that cover a relatively wider region of the genome, followed by a nested PCR amplification that employs primers specific for a region within the initial RT-PCR amplified products. A nested PCR provides a definitive diagnosis of an etiological agent of a flavivirus infection when species-specific nested primers are employed (Jeong et al., 2011; Williams et al., 2010). The definitive identification of flaviviruses by conventional or nested PCR requires post-amplification manipulation of the PCR product such as analysis on agarose gels and DNA sequencing (Studdert et al., 2003; Williams et al., 2010). Therefore, they are time-consuming and can pose a risk of contamination. In addition, a conventional PCR principally produces the same amount of the end product at the completion of the amplification process making the quantification of the initial starting template extremely difficult (Bustin and Nolan, 2004; Kubista et al., 2006; Valasek, 2005). This would be useful for the determination of viral load in both clinical or surveillance situations.

Advances in fluorophore and probe design over the last two decades have prepared the way for designing more accurate, rapid, highly sensitive and specific RT-qPCR assays (Conceicão et al., 2010; Huang et al., 2004). RT-qPCR relies on the real-time detection of fluorescent signals produced during the amplification of the nucleic acid templates (Hugget et al., 2005; Kubista et al., 2006; Valasek, 2005). Two types of fluorophores are widely used: SYBR Green and target-specific probes labelled with various fluorescent dyes (Bustin and Nolan, 2004; Valasek, 2005).

The SYBR Green method does not require a fluorescence-labelled probe specific for a target sequence (Bustin and Nolan, 2004; Valasek, 2005). Therefore, an RT-qPCR using SYBR Green is easier to design and less expensive to run than the RT-qPCR, which requires target-specific probes. Moreover, the reaction performed with SYBR Green is reversible (Ririe et al., 1997; Valasek, 2005) allowing the generation of a melt curve after the completion of the PCR. Melt curve analysis is an essential tool to determine the melting temperature of the PCR product so as to ensure the specificity of primers, and to monitor for contamination and primer-dimerisation (Bustin et al., 2009; Bustin and Nolan, 2004; Ririe et al., 1997; Valasek, 2005). Moreover, melt curve analysis has been used for the serotyping of DENV (Yong et al., 2007). However, there are a few inherent shortcomings with the SYBR Green method. Since SYBR Green is not specific for a target sequence, it can indiscriminately bind to any double-stranded DNA during the amplification process (Bustin and Nolan, 2004; Valasek, 2005). Hence, it cannot be used for a multiplex PCR. In addition, if there are any primer-dimers present in the reaction, the SYBR Green will provide an incorrect fluorescence reading due to the binding to the dimers. This may result in fluorescence detection in the no-template control (NTC), giving a false-positive result (Bustin and Nolan, 2004; Valasek, 2005), if a melt curve analysis is not performed.

An alternative method is to use a probe-based RT-qPCR which employs probes that are highly specific for the target sequence. A significant advantage of probe-based RT-qPCR is that not only the primers but also the probe contributes to the specificity of the assay. Hence, an additional level of specificity is introduced (Bustin and Nolan, 2004; Valasek, 2005). In probe-based RT-qPCR assays, primer-dimers do

not emit a fluorescence signal; therefore the amount of signal emitted is proportionate to the amount of product amplified. Furthermore, hydrolysis probes can be labelled with various distinguishable dyes (Hugget et al., 2005). This allows the construction of a multiplex PCR for the amplification, detection, quantification and differentiation of different targets within a single PCR reaction (Chao et al., 2007; Hue et al., 2011; Lai et al., 2007; Pongsiri et al., 2012; Yong et al., 2007).

In addition to the various RT-PCR techniques, isothermal nucleic acid-sequence based amplification (NASBA) has been successfully used to detect flaviviruses (Lanciotti and Kerst, 2001; Wu et al., 2001). In this technique, the amplification of nucleic acid sequences are performed using three enzymes: a reverse transcriptase, T7 RNA polymerase, and RNase H. The amplified product is a single-stranded anti-sense RNA that can be detected by using a target-specific capture probe, a detector probe and an instrument which is capable of detecting and measuring electrochemiluminescence (Chan and Fox, 1999). This technique presents a more sensitive approach to detecting strains with low initial copy numbers (Jittmittraphap et al., 2006). Several studies have also used multi-analyte single-membrane biosensors to detect DENV. In this sensitive, rapid, and serotype-specific method, generic and serotype-specific DNA probes hybridise with the virus RNA that was amplified by NASBA (Baeumner et al., 2002; Zaytseva et al., 2004).

Finally, state-of-the-art next generation sequencing (NGS) has been used to detect flaviviruses (Bishop-Lilly et al., 2010; Mann et al., 2013; McMullan et al., 2012). Although NGS technology has not yet been reported extensively for the detection of flaviviruses in a diagnostic setting (McMullan et al., 2012), it has been more widely used for whole genome sequencing and for its ability to detect low frequency variants, providing valuable information about the depth of the genetic diversity within a virus population (reviewed below, Section 1.3.4). Therefore, it not only detects the virus but also provides valuable information about the depth of the genetic diversity within the virus population (Mardis, 2008; Radford et al., 2012; Su et al., 2011). This technique has some fundamental shortcomings. All steps involved in this process are subject to the introduction of errors, such as point mutations, insertions/deletions (indels), and recombinant chimeric sequences (Beerenwinkel et

al., 2012; Görzer et al., 2010b; Malet et al., 2003). Recent advances in NGS as well as statistical and computational analyses have allowed for the generation of longer reads, and adjustment of some of the confounding factors, including error correction (Beerenwinkel et al., 2012; Beerenwinkel and Zagordi, 2011; Yang et al., 2012). Hence, NGS technology not only can be used to detect flaviviruses, but also provide more accurate estimate of the genetic diversity within the virus population, as well as *de novo* sequencing of full-length genome.

1.3 Flavivirus Evolution and Genetic Diversity

Flaviviruses, like other arboviruses perpetuate themselves in nature by alternating replication (cycling) in arthropod vectors and vertebrate hosts (Ciota and Kramer, 2010; Marshall, 1988). The requirement of arboviruses, including flaviviruses, to replicate in distinct host environments may explain their need for a considerable genetic plasticity, which is the defining characteristic of RNA viruses (Ciota and Kramer, 2010; Weaver, 2006). This genetic plasticity is the result of a high mutation rate, a large population size and a high level of viral replication of RNA viruses (Domingo and Holland, 1994; Drake and Holland, 1999; Holland et al., 1990; Steinhauer et al., 1992)

It is well-established that, because the RdRp of RNA viruses lacks proofreading ability, these viruses acquire more mutations and endure higher genetic variation than DNA viruses (Steinhauer et al., 1992). The high error rate of RdRp is estimated to result in 10^{-3} to 10^{-5} substitutions per nucleotide in each round of replication (Domingo and Holland, 1994; Drake and Holland, 1999). However, even with this remarkable potential for sequence variation, arboviruses have experienced limited evolution over time (Davis et al., 2005; Ebel et al., 2004; Weaver, 2006).

1.3.1 The Mechanism of Restricted Molecular Evolution in Arboviruses

The generally accepted theory is that the evolutionary stasis of flaviviruses (and arboviruses) results from alternating replication in invertebrate and vertebrate hosts (Coffey et al., 2008; Coffey and Vignuzzi, 2010; Deardorff et al., 2011; Forrester et al., 2012; Scott et al., 1994; Weaver, 2006; Woolhouse et al., 2001).

This may be mainly due to the effect of various bottlenecks occurring during arbovirus transmission (Brackney et al., 2011; Forrester et al., 2012; Li and Roossinck, 2004; Sittisombut et al., 1997). Another theory associated with arbovirus evolutionary stasis is the fitness trade-off hypothesis. This hypothesis proposes that arboviruses maintain adequate replicative ability and fitness in both invertebrate and vertebrate hosts in preference to having a fitness increase in one host and a fitness decline in another (Ciota and Kramer, 2010; Deardorff et al., 2011). Therefore, only mutations that are either neutral or beneficial for both hosts are fixed, while those mutations that are deleterious for one or both hosts are eliminated by purifying selection (Domingo and Holland, 1997; Levins, 1968; Novella et al., 2012).

Many experimental studies have assessed the role of host cycling on arboviral adaptation and evolution both *in vitro* (Chen et al., 2003; Coffey and Vignuzzi, 2010; McCurdy et al., 2011; Vasilakis et al., 2009) and *in vivo* (Ciota et al., 2009; Ciota et al., 2008; Coffey et al., 2008; Deardorff et al., 2011; Fitzpatrick et al., 2010; Jerzak et al., 2007; Jerzak et al., 2008; Lin et al., 2004; McCurdy et al., 2011). However, these studies provide conflicting results. While some studies revealed that the genetic stability of arboviruses results from host cycling (Coffey et al., 2008; Coffey and Vignuzzi, 2010; Moutailler et al., 2011), others revealed that it is multiple passages through a single host type that contributes to the evolutionary conservation of arboviruses (Chen et al., 2003; Ciota et al., 2009; Lin et al., 2004; Vasilakis et al., 2009).

Research has revealed the rate of mutation and the genetic diversity is host-dependent. For example, in an *in vivo* study, Jerzak et al. (2007) revealed that more genetic changes developed in mosquito-passaged WNV than in chicken-passaged WNV. In contrast, subsequent research revealed that the rate of mutation in WNV was similar in viruses derived from sequential passaging either in chickens or mosquitoes compared with virus that underwent cycling between the two hosts (Jerzak et al., 2008).

In addition to host factors, a recent study revealed that the rate of mutation can be virus-dependent. McCurdy et al. (2011) demonstrated that passaging JEV in cell culture, eggs or mice introduced twenty two amino acid substitutions which resulted in its attenuation and improved survival rate in infected mice. However, in an identical experiment, Venezuelan equine encephalitis virus (VEEV) developed eleven amino acid changes which did not significantly affect the virulence of the virus in mice.

Numerous arbovirus experimental evolution studies carried out on WNV (Ciota et al., 2008; Ciota et al., 2007b; Deardorff et al., 2011), SLEV (Ciota et al., 2007b), DENV (Chen et al., 2003; Vasilakis et al., 2009), VEEV (Coffey et al., 2008), Chikungunya virus [CHIKV; (Coffey and Vignuzzi, 2010)] and Rift Valley fever virus [RVFV; (Moutailler et al., 2011)] suggested that releasing arboviruses from cycling, by sequential passaging in either vector or host cells, in a laboratory setting resulted in cell-specific adaptation. However, other studies do not support this phenomenon in single-host-specialised viruses (Ciota et al., 2009; Deardorff et al., 2011).

Therefore, the arbovirus evolution studies conducted so far, even with the same viruses, have produced varying results in an attempt to explain the reasons for the genetic stability of these viruses. According to Ciota and Kramer (2010), different outcomes of arbovirus evolution studies may be due to slight variations in factors such as the multiplicity of infection (MOI) applied in each study, the number and length of passages, the incubation temperature, and the source of parental virus used.

1.3.2 Selection Pressure and Flavivirus Evolution

Generally in RNA viruses, including flaviviruses, the unique features of viral biology, such as their mode of transmission, and their requirement to survive extreme conditions, may impose constraints on viral evolution (Scott et al., 1994; Weaver, 2006; Woelk and Holmes, 2002). Their evolution and genetic diversity is governed by the operation of different selection pressures (Hall, 2011; Lemey et al., 2009). Generally, the difference between the rates of synonymous (silent) mutations per synonymous sites (dS) to that of non-synonymous (replacement) mutations per

non-synonymous sites (dN) is used to estimate the nature of selection pressure in a molecular sequence. If dN > dS, positive selection is indicated, whereas a dN < dS reflects the operation of purifying selection. An equal dN and dS ratio (dN = dS) implies the action of neutral selection (Hall, 2011; Lemey et al., 2009). Both phylogenetic and genetic studies of flaviviruses strongly suggest the dominance of purifying selection over their evolutionary history (Holmes, 2003). The operation of strong purifying selection has been identified in JEV (Mohammad et al., 2011), WNV (Bertolotti et al., 2007; Jerzak et al., 2005; Jerzak et al., 2008; May et al., 2011), DENV (Holmes, 2003) and SLEV (Baillie et al., 2008). To date, only a low level of positive selection has been reported in WNV (Beasley et al., 2003; Bertolotti et al., 2007; Brault et al., 2007), SLEV (Baillie et al., 2008), JEV (Carney et al., 2012) and DENV (Bennett et al., 2006; Myat Thu et al., 2005; Twiddy et al., 2002a; Twiddy et al., 2002b).

1.3.3 Genetic diversity and Population Structure of Flaviviruses

Flaviviruses, like other RNA viruses, exist as a complex swarm of closely-related virus particles with non-identical genomes within a host. This genetically diverse population is generated as a result of the rapid replication kinetics of viruses with large population sizes via a low fidelity and error-prone RdRp (Domingo and Holland, 1994; Drake and Holland, 1999; Holland et al., 1990; Steinhauer et al., 1992). This ensemble of mutationally coupled variants is called quasispecies or mutant swarm (Biebricher and Eigen, 2006; Domingo et al., 2001; Lauring and Andino, 2010). The terms mutant spectrum (Ciota and Kramer, 2010; Ciota et al., 2007c; Perales et al., 2010) or mutant cloud (Perales et al., 2010; Roossinck and Schneider, 2006) have also been used to describe this dynamic swarm of mutants. Research has revealed that the viral quasispecies does not only describe an ensemble of individual variants, but a collection of interactive mutants that together define the phenotype of the virus (Biebricher and Eigen, 2005; Domingo et al., 2001; Lauring and Andino, 2010; Vignuzzi et al., 2006). The complexity of a mutant swarm (the number of various genomic sequences and the mean number of mutations in each genome) is governed by a balance between the mutation rate and selection forces (Ciota and Kramer, 2010; Perales et al., 2010), which are

controlled by the virus-host interaction (Schneider and Roossinck, 2001). The size, composition and complexity of the mutant swarm have a significant effect on various phenotypic characteristics of the virus such as its fitness (Domingo and Holland, 1997; Domingo et al., 2006; Perales et al., 2010), adaptability (Ciota et al., 2007a; Elena and Sanjuan, 2005; Lauring and Andino, 2010), virulence and pathogenesis (Jerzak et al., 2007; Lancaster and Pfeiffer, 2012; Vignuzzi et al., 2006) and response to antiviral drugs (Domingo et al., 2001; Domingo et al., 2006; Lauring and Andino, 2010; Perales et al., 2010).

Research on WNV has revealed that adaptation of the virus to a specific host cell is not solely due to the consensus changes, but it is mainly directly correlated with the breadth of mutant swarm in the virus population (Ciota et al., 2007b; Ciota et al., 2007c). Hence, a comprehensive understanding of the quasispecies dynamics is crucial in appropriate characterisation of arbovirus adaptation and evolution (Ciota et al., 2012; Ciota and Kramer, 2010).

Genetic diversity in flaviviruses can also manifest as genotypic diversity. Species that exist within different genotypes may be associated with unique phenotypes. For example, G1 and G3 of JEV are associated with JEV epidemics, while G2 and G4 are more commonly associated with endemic activity. During the last two decades, G1 has become the dominant genotype in Australasia (Pyke et al., 2001), Vietnam (Nga et al., 2004), China (Cao et al., 2011; Wang et al., 2010; Zhang et al., 2011), India (Fulmali et al., 2011), Japan (Ma et al., 2003), South Korea (Yun et al., 2010) and Thailand (Nitatpattana et al., 2008), suggesting a better fitness and/or virulence phonotype. Similarly, viruses belonging to different lineages and sublineages of WNV are also associated with distinct phenotypes. For example, lineage I encompasses strains distributed through much of the world and is subdivided into different clades. Clade Ia is distributed through much of the New World and contains the NY99 strain that caused severe encephalitis in humans and animals (Beasley et al., 2003; Davis et al., 2003a; Lanciotti et al., 1999; Murata et al., 2010; Nash et al., 2001), while clade Ib contains the WNV subtype KUNV that is restricted in circulation to the Australasian region and causes flu-like symptoms or mild

encephalitis in humans, and is a rare cause of neurological disease in horses (Broom et al., 2003; Gray et al., 2011; Mann et al., 2013).

1.3.4 Assessment of Genetic Diversity

The genotypic diversity of flavivirus populations has been characterised using the consensus sequences of either full-length genome or individual genes. In the majority of studies, the E gene and its deduced protein have been used for phylogenetic analyses of MVEV (Johansen et al., 2007; Lobigs et al., 1988), JEV (Ali and Igarashi, 1997; Paranjpe and Banerjee, 1996; Pyke et al., 2001; Uchil and Satchidanandam, 2001; Williams et al., 2000), WNV (Berthet et al., 1997; Jerzak et al., 2005; Scherret et al., 2001) and TBEV (Kovalev et al., 2010; Kovalev and Mukhacheva, 2012; Mandl et al., 1989a; Whitby et al., 1993). The E glycoprotein plays a significant role in the virulence, cellular tropism, protective host immune response, and replication cycle of flaviviruses (Beasley et al., 2005; Brault et al., 2004; Chu et al., 2007; McMinn, 1997; Prow et al., 2011; Sultana et al., 2009). As a result, this gene is subject to higher levels of natural selection than other genes, and shows higher levels of genetic variation (Twiddy et al., 2002a; Twiddy et al., 2002b), which makes it suitable for phylogenetic studies. In addition to the E protein, molecular determinants of pathogenesis in flaviviruses also reside in the prM protein (Hurrelbrink and McMinn, 2003; McMinn et al., 1995). As a result, both prM and E genes have been employed to better characterise genetic relationships of flaviviruses (Beasley et al., 2003; Blitvich et al., 2004; Chen et al., 1990; Davis et al., 2003b; Estrada-Franco et al., 2003; Williams et al., 2013; Williams et al., 2000). Other studies have used the NS1 gene (Jerzak et al., 2005), NS5-3'UTR sequences (Hobson-Peters et al., 2013; Poidinger et al., 2000; Poindinger et al., 1996) and complete genomes (Baillie et al., 2008; Jan et al., 1996; Mohammad et al., 2011) to characterise the genotypic or inter-population genetic diversity of flaviviruses.

The spectrum of heterogeneity within a viral population has been determined using standard molecular cloning approaches coupled with conventional (Sanger) sequencing of only a few biological clones isolated from a viral population (Ciota et al., 2012; Ciota et al., 2008; Ciota et al., 2007c; Jerzak et al., 2005; Jerzak et al.,

2007). However, because this procedure is both labour-intensive and time-consuming, very few studies have characterised the genetic diversity of flavivirus populations with adequate detail. The recent introduction of NGS technology has revolutionised this field of study (Beerenwinkel and Zagordi, 2011; Su et al., 2011; Wang et al., 2007). It has been successfully used in the characterisation of the genetic diversity and mutant spectra within discrete populations of HIV (Fleury et al., 2013; Tsibris et al., 2009; Wang et al., 2007; Yin et al., 2012), hepatitis B virus (Nishijima et al., 2012; Solmone et al., 2009), hepatitis C virus (Dietz et al., 2013; Escobar-Gutiérrez et al., 2012; Lauck et al., 2012) and influenza virus (Kuroda et al., 2010; Selleri et al., 2013; Watson et al., 2013).

Using this approach, low-frequency mutations and variants can be detected so as to provide a detailed picture of the genetic diversity of the viral population (Beerenwinkel and Zagordi, 2011; Chen-Harris et al., 2013; Eriksson et al., 2008). Due to the error-prone nature of the PCR and sequencing, library preparation and alignments of short reads, NGS data must be further processed and validated before it can be used for the estimation of the genetic diversity of a virus population (Mardis, 2008; Su et al., 2011). Recent advances in computational and statistical programs have overcome this challenge (Chen-Harris et al., 2013; Yang et al., 2012). Therefore, with appropriate post-sequencing data validation and processing, NGS offers the advantage of detecting minor, but clinically-relevant variants within virus populations that conventional sequencing might fail to detect in clinical and biological samples (Chen-Harris et al., 2013; Wang et al., 2007).

1.3.5 Molecular Determinants of Fitness and Pathogenesis

The engineering of infectious clones for various flaviviruses including MVEV (Hurrelbrink et al., 1999), JEV (Sumiyoshi et al., 1992), WNV (Yamshchikov et al., 2001), KUNV (Khromykh and Westaway, 1994), YFV (Rice et al., 1989), DENV (Lai et al., 1991; Mosimann et al., 2010; Pierro et al., 2006; Suzuki et al., 2007), and TBEV (Mandl et al., 1997) has facilitated the mapping of virulence determinants. These are distributed throughout the structural and non-structural proteins as well as the 5’ and 3’UTRs (Hurrelbrink and McMinn, 2003; McMinn, 1997). Some of the most

Table 1.2. *Molecular determinants of flavivirus virulence.*

Position	Mutation	Location	Flavivirus	Effects *	References
prM protein					
143	$E^{143}D$, $N^{144}R$	Glycosylation site	TBE/DENV-4	High NV in mice	(Pletnev et al., 1993)
162	$V^{162}A$	prM-E cleavage site	LGTV	Low NV	(Holbrook et al., 2001)
165	$Y^{165}H$		LGTV	Low NV	(Holbrook et al., 2001)
166	$T^{166}L$	prM-E signalase site	DENV-2	Loss of replication and virus recovery	(Pryor et al., 1998)
206	$S^{206}V$	Furin cleavage site	TBE/DENV-4	Low NV in mice	(Pletnev et al., 1993)
Envelope protein					
52	$Q^{52}R$ or $Q^{52}K$	Polar interface linking domain I and II, important for conformational changes	JEV	Low NI	(Hasegawa et al., 1992)
	$R^{52}G$		YFV	Low NI	(Schlesinger et al., 1996)
107	$L^{107}T$	Fusion peptide (98-113)	TBEV	Low fusogenic activity	(Allison et al., 2001)
	$L^{107}D$		TBEV	No fusogenic activity	(Allison et al., 2001)
126	$K^{126}E$	Between strands d and e on the surface, important for conformational changes and fusion activity	DENV-2	Loss of NV	(Gualano et al., 1998)
138	$E^{138}K$	External region of domain I, close to domain I and II junction, critical for interaction with cellular molecules	JEV	Low NI, and low NV	(Chen et al., 1996)
154	$N^{154}D$	Glycosylation site (154-156)	ALFV	Low NI	(Prow et al., 2011)
	$N^{154}S$		WNV	Low NI	(Beasley et al., 2005)
	$N^{154}L$		TBEV/DENV-4	Low NV	(Pletnev et al., 1993)
	$Y^{155}S$		WNV	Low NI	(Chambers et al., 2008)
159	$V^{159}A$	Adjacent to glycosylation site	WNV	Higher transmission efficiency by *Culex* mosquitoes	(Beasley et al., 2003; Moudy et al., 2007)

Table 1.2. *continued*

Position	Mutation	Location	Flavivirus	Effects *	References
270	$I^{270}S$	Adjacent to hinge region	JEV	Low NI	(Cecilia and Gould, 1991)
277	$S^{277}I$, $S^{277}N$, $S^{277}V$, $S^{277}P$	Flexible hinge region (273-277)	MVEV	Low NI, abnormal fusogenic activity, delayed growth in cell culture	(Hurrelbrink and McMinn, 2001; McMinn et al., 1995),
	$S^{270}*$		ALFV	Low NI	(McMinn et al., 1995; Prow et al., 2011)
305	$F^{305}V$	Lateral surface of domain III	TBEV	Higher NV	(Schlesinger et al., 1996)
308	$D^{308}N$		TBEV	Loss of NV	(Jiang et al., 1993)
310	$S^{310}P$		LIV	Loss of NV	(Jiang et al., 1993)
368	$G^{368}R$		TBEV	Loss of NI	(Holzmann et al., 1997)
380	$R^{380}T$		TBEV	Higher NV	(Schlesinger et al., 1996)
384	$Y^{384}H$		TBEV	Loss of NI	(Holzmann et al., 1997)
390	$D^{390}N$, $D^{390}Y$	RGD receptor-binding motif (388-390)	MVEV	Loss of NI	(Hurrelbrink and McMinn, 2001)
	$D^{390}G$, $D^{390}A$, $D^{390}H$		MVEV	Loss of NI	(Lee and Lobigs, 2000)
	$D^{390}G$, $D^{390}A$, $D^{390}H$		MVEV	Loss of NI, faster kinetics of blood clearance	(Lee and Lobigs, 2002)
	$D^{390}G$, $D^{390}A$, $D^{390}H$, $D^{390}N$		MVEV	Loss of NI	(Lobigs et al., 1990)
	$G^{389}A$, $D^{390}E$		YFV17D	Reduction of viral titre *in vitro*	(van der Most et al., 1999)
	$D^{390}H$, $D^{390}N$		DENV-2	Low NV in mice	(Sanchez and Ruiz, 1996)
NS1 protein					
30	$A^{30}P$		WNV	Low NV, Loss of NI	(Liu et al., 2006b)
903, 904	$N^{903}I$, $S^{904}R$,	N-linked glycosylation site	TBEV/DENV-4	Low NV	(Pletnev et al., 1993)
979, 980	$K^{979}R$, $N^{980}I$		TBEV/DENV-4	High NV	(Pletnev et al., 1993)
NS2B protein					
63	$E^{63}D$		JEV SA14	Loss of NV	(Ni et al., 1995)

Table 1.2. *continued*

Position	Mutation	Location	Flavivirus	Effects *	References
NS3 protein					
77	$D^{77}N$, $D^{77}A$,		YFV	Reduced NS3 protease activity	(Chambers et al., 1990)
138	$S^{138}A$		YFV	Reduced NS3 protease activity	(Chambers et al., 1990)
	$S^{138}C$		YFV	Abolished NS3 protease activity, No recovery of the infectious virus	(Chambers et al., 1990)
105	$A^{105}G$		JEV SA14	Loss of NV	(Ni et al., 1995)
249	$T^{249}P$		WNV	High NV in American Crows	(Brault et al., 2007)
NS4B protein					
101	$P^{101}L$		DENV-4	Low replication in mosquitoes, higher replication vertebrate cells	
102	$C^{102}S$		WNV	Low NV, Low NI	(Wicker et al., 2006)
NS5 protein					
536	$D^{536}A$	Motif A	JEV	Complete inactivation or severe loss of enzyme activity	(Yu et al., 2007)
605	$G^{605}A$	Motif B			
668	$D^{668}A$	Motif C			
669	$D^{669}A$				
669	$D^{669}N$				
691	$K^{691}A$	Motif D			
653	S653F			Inhibition of JAK-ATAT signalling. Enhanced replication of KUNV in the presence of IFN	(Laurent-Rolle et al., 2010)
The untranslated regions (UTRs)					
82-87	Deletion	**5'UTR**	DENV-4	Low translation efficiency	(Cahour et al., 1995)
172-143 3'd	Only retains 3' CS-2A, CS-1 and 3'SL	**3'UTR**	DENV-4	Smaller plaque size. No viraemia, moderate level of antibody response	(Men et al., 1996)

* NI: neuroinvasiveness; NV: neurovirulence. Comparisons have been made with either clone-derived or wild-type viruses. The NI and NV have been determined in the mouse model of pathogenesis.

important and well-studied flavivirus virulence determinants are described below and listed in Table 1.2.

In the prM protein, molecular determinants of virus replication and virulence reside in many important locations such as the glycosylation site, the prM-E signalase and cleavage sites and furin cleavage site. In the E protein, molecular determinants of viral, replication, fitness and virulence are found at various locations of all three domains. The most important and well-studied are the fusion peptide, the glycosylation site, the flexible hinge region and the receptor-binding RGD motif. Similarly, molecular determinants underlying enzymatic activities, virus replication, and virulence reside in the NS proteins, such as the glycosylation site of the NS1 protein, and the four motifs of the RdRp in NS5. The 5' and 3'UTRs of flavivirus genomes also harbour molecular determinants that are important in RNA translation, genome cyclisation, replication and immune response of flaviviruses.

1.3.6 Flavivirus Virulence Studies and Animal Model of Pathogenesis

The virulence of flaviviruses is typically determined by a mouse model of pathogenesis. Groups of three-week old mice (various breeds) are injected either via the intracranial (i.c.) or intraperitoneal (i.p.) route (Beasley et al., 2002; Hasegawa et al., 1992; Hurrelbrink et al., 1999; Lobigs et al., 1988; McMinn et al., 1995). The i.c. injection is carried out to determine the virus neurovirulence which is the ability of the virus to cause encephalitis following the initiation of a cytopathic infection in the central nervous system, (CNS). The i.p. injection is performed to characterise neuroinvasiveness, the ability of a virus to multiply in peripheral tissues, produce viraemia and invade the CNS.

According to Monath et al. (1980), flaviviruses can be classified into three different virulence categories, based on their ability to produce a lethal infection after i.c. and i.p. inoculation in an animal model. Highly virulent strains produce a high mortality rate after i.p. inoculation over all ranges of virus concentrations that are lethal via i.c. inoculation. Flavivirus virulence studies unequivocally reveal that, in highly virulent strains, the mortality rate is dose-dependent, with greater rates at higher doses (Beasley et al., 2002; Beasley et al., 2005; Chambers et al., 2008; Davis

et al., 2003a; Prow et al., 2011; Shirato et al., 2004). Intermediate virulent strains are those that produce scattered deaths in animals over a wide range of doses such that no 50% lethal dose (LD_{50}) via the i.p. route can be calculated for these viruses. Low virulent strains are those that produce no or minimal mortality over the entire range of virus concentrations tested, but produces death via the i.c. route. This classification is still used as the reference work on flavivirus virulence studies in animals.

Several studies have associated flaviviruses with a range of virulence characteristics, defined by different virus and host factors. For example, the dose and route of inoculation of flaviviruses impact on their virulence and the severity of the disease in mice. Flaviviruses demonstrate a high mortality rate when inoculated via i.c. route, whereas they exhibit a variable mortality pattern when administered via i.p. route (Beasley et al., 2005; Chambers et al., 2008; Coelen, 1988; Davis et al., 2003a; Hurrelbrink and McMinn, 2001; Lawson, 1988). It is hypothesised that inability of the virus to cross BBB after an i.p. injection is associated with a lower LD_{50} (Diamond and Klein, 2004).

Additional factors such as age, and the genetic background of the animal, can influence the virulence of flaviviruses. For example, it has been demonstrated that mice develop resistance to i.p. challenge with flaviviruses as they age (Fitzgeorge and Bradish, 1980; Lawson, 1988), but remain susceptible to i.c. challenge (Fitzgeorge and Bradish, 1980; Lawson, 1988; May et al., 2006). The development of resistance to infection with increasing age is believed to be due to the maturation of the BBB and differences in the function of T-cells and macrophages (Diamond and Klein, 2004; Fitzgeorge and Bradish, 1980).

Furthermore, significant differences in the virulence of flaviviruses have been observed between different strains of mice (Chambers et al., 2008; Lawson, 1988; Shueb, 2008). In a study on MVEV, Lawson (1988) demonstrated that inbred CBA/CaH mice exhibited a lower level of mortality compared to outbred CD1 mice when injected with high concentrations of the virus. In another study, Shueb (2008) revealed that DUB mice demonstrated a delayed time to death and a lower

mortality rate compared to HeJ mice when challenged with KUNV and MVEV. Similarly, Chambers et al. (2008) revealed that B-cell deficient mice displayed a lower mortality rate than the SCID mice when injected with WNV subcutaneously or intraperitoneally.

1.4 Flaviviruses of Australasian Region

Flaviviruses are the cause of a high number of viral infections in the Australasian region. Thirty different flaviviruses circulate in this region, ranging from highly pathogenic agents such as MVEV, JEV, WNV and DENV to less pathogenic viruses such as ALFV, STRV, KOKV (Mackenzie and Williams, 2009). The propensity of flaviviruses to emerge and establish in new geographic areas poses a significant public and veterinary health concern for the region. Given that some flaviviruses are pathogens of animals, disease outbreaks or emergence can also lead to economic losses and threaten trade status. Some of the most prevalent and important flaviviruses in the region are reviewed below.

1.4.1 Murray Valley Encephalitis Virus (MVEV)

Murray Valley encephalitis virus is a medically important flavivirus that is enzootic in the Kimberley region of WA (Mackenzie and Williams, 2009; Marshall, 1988). It is the most important causative agent of arboviral encephalitis in humans in Australasia (Mackenzie and Williams, 2009; Marshall, 1988) and also causes infection in horses leading to neurological diseases (Gard et al., 1977; Knox et al., 2012; Roche et al., 2013). Since MVEV is the focus of this thesis, a detailed review of this virus follows.

1.4.1.1 Clinical Picture

Infection with MVEV is mostly asymptomatic with only 1 in 200-1000 MVEV-infected individuals developing encephalitic manifestations (Burrow et al., 1998; Cordova et al., 2000; Knox et al., 2012; Mackenzie et al., 1993; Spencer et al., 2001). The incubation period is between 5 and 28 days (Bennett, 1976; Spencer et al., 2001). People of all ages can be affected but the severity of disease is more pronounced in young children and older adults (Burrow et al., 1998). Most likely,

MVEV enters the brain via the BBB and there is also evidence indicating that it can enter the brain via the olfactory neurons (Andrews et al., 1999). The pathogenic processes that mediate disease are yet to be fully elucidated.

MVEV causes both mild and severe infections. In mild cases, people may suffer from headache, fever (usually over 40°C), neck stiffness, irritability, photophobia, drowsiness, and some other non-specific febrile illness (Burrow et al., 1998; Cordova et al., 2000; Knox et al., 2012). In severe cases (encephalitis), patients may experience fits, coma, and respiratory failure. During the advanced phase of infection, patients may exhibit signs of cerebellar, brainstem and spinal cord involvement including flaccid paralysis, tremor and death. Seizures usually occur in children, but may also happen in adults (Burrow et al., 1998; Cordova et al., 2000; Knox et al., 2012; Mackenzie et al., 1993). Vomiting and macular erythematous rash are also common clinical features in children (Anderson, 1954; Burrow et al., 1998; Knox et al., 2012). The outcome of infection is worse in the elderly and young children (Burrow et al., 1998; Mackenzie et al., 1993). Physicians may not immediately associate these signs with MVE infection, especially in non-enzootic areas (Spencer et al., 2001). Computed tomography (CT) scan images are usually normal. However, dramatic abnormalities can be detected by magnetic resonance imaging (MRI). Because MRI is a more sensitive and specific system of imaging, it is the method of choice to detect early signs of disease (Cordova et al., 2000; Knox et al., 2012).

1.4.1.2 Morbidity and Mortality

There are no clear clinical features of MVEV that can be used to predict the outcome of the disease. Pioneer epidemiological studies on MVEV indicated a case mortality rate of 68% (Anderson, 1954). However, due to advances in modern medicine, the mortality rate has declined dramatically and it is currently 20-25% (Knox et al., 2012; Mackenzie and Broom, 1995). About 30%-50% of survivors will exhibit permanent brain damage and neurological sequelae (Bennett, 1976; Cordova et al., 2000; Spencer et al., 2001). Young male children from Aboriginal

communities in WA experience the worst outcome (Smith et al., 1997; Spencer et al., 2001).

1.4.1.3 Epidemiology

1.4.1.3.1 History and Geographic Distribution

Historical data suggest that the first cases of MVE were reported in 1916-1918 in New South Wales (NSW), Victoria (VIC), and Queensland (QLD) (Anderson, 1954; Mackenzie and Broom, 1995), however, an aetiological agent was not identified. Because of this, and the fact that the disease had not been described previously, it was called Australian X disease (Anderson, 1954; Mackenzie and Broom, 1995). The first large epidemics of MVE occurred in eastern Australia between 1917 and 1925 (Anderson, 1954), with 281 confirmed cases and a 68% fatality rate (Anderson, 1954; Mackenzie and Broom, 1995). No MVEV activity was recorded in Australia between 1925 and December 1950 (Anderson, 1954; Mackenzie and Broom, 1995).

The second major epidemic of MVE occurred in 1950-1951 in southern and south-eastern Australia. This was the first time the virus was detected in South Australia (SA) (Mackenzie and Broom, 1995; Miles et al., 1951). In this epidemic, forty five encephalitic cases were recorded with a mortality rate of 42% (Anderson, 1954; Mackenzie and Broom, 1995). During this time, MVEV was predominantly detected in the Murray Valley area, after which it was named. French (1952) first isolated the virus after inoculating brain material obtained from fatal cases onto the chorioallantoic membrane of embryonated chicken eggs. Miles et al. (1951) also isolated the virus from the brain of the only fatal case from SA. The isolation of MVEV from the brain of a deceased human MVE case from Papua New Guinea (PNG) in 1956 (French et al., 1957) marked its first detection in PNG, indicating its presence beyond Australia. After 1951, only sporadic cases of MVEV occurred in VIC (1956), WA (1969) and in QLD and NSW [1971; (Mackenzie and Broom, 1995)].

The next large epidemic of MVE took place in 1974 with 58 human cases including 12 deaths (20% mortality rate). The epidemic commenced in January and lasted for five months. The outbreak started around the Murray Valley and eventually spread

to all mainland states (Mackenzie and Broom, 1995; Spencer et al., 2001; Williams et al., 2010). After the 1974 outbreak, the disease was named Australian encephalitis (Mackenzie and Broom, 1995). However, since the term "Australian encephalitis" refers to infections caused by both MVEV and the closely-related KUNV, it was later recommended that the use of this term be discontinued. To avoid confusion, Murray Valley encephalitis and Kunjin encephalitis are now used accordingly (Spencer et al., 2001).

Prior to 1974, there was only one MVEV case reported in WA and none from the NT (Mackenzie and Broom, 1995). However, after 1974, there was a significant shift in the geographical distribution of MVE and the majority of cases since then have been reported in WA and the NT. There is substantial evidence suggesting that the Kimberley region of WA is the enzootic region for MVEV in Australia (Broom, 2003; Broom et al., 2002a; Broom et al., 1995; Broom and Whelan, 2005). The southern and eastern states (SA, NSW, VIC, and QLD) that had experienced epidemics prior to 1974 have since shown a low incidence of human clinical cases (Broom and Whelan, 2005; Knox et al., 2012; Spencer et al., 2001). Circumstantial evidence suggests that both environmental and ecological factors resulting from the damming of the Ord River in the Kimberley region of WA created favourable conditions for the virus to become enzootic in this region (Mackenzie and Broom, 1999; Spencer et al., 2001).

An increase in the activity of MVEV was observed in early 2000 as a consequence of heavy rainfall in WA. Significantly, in this year, a human case was reported in the town of Dongara, only 315km north of Perth, the southernmost point of WA, where an MVE case has been detected (Cordova et al., 2000). During the same year, antibodies to MVEV was detected in domestic chickens at Coorow, 240km north of Perth (Broom et al., 2002a).

The most recent outbreak of MVE occurred in 2011. Exceptional rainfall and heavy flooding increased the numbers of *Cx. annulirostris* mosquitoes, and led to the subsequent spread and re-emergence of MVEV in all mainland states. Severe human encephalitic cases were reported in WA (nine cases, one death), NT (four cases, one death), SA (two cases, one death), NSW (one case) and one suspected

fatal case from VIC (Knox et al., 2012). MVEV activity was also detected in horses in all mainland states resulting in a high incidence of mortality (Roche et al., 2013). MVEV antibodies were also detected in the serum of sentinel chickens at Dongara and Leonara (Knope et al., 2013). The 2011 epidemic highlighted the potential of MVEV to re-emerge and emphasises the need for vigilance and continued surveillance in order to control further outbreaks.

1.4.1.3.2 Factors That Facilitate the Spread of MVEV in Australia

It is hypothesised that the movement of infected waterbirds (Broom et al., 2002a; Guay et al., 2012; Mackenzie et al., 1994) and/or wind-blown infected mosquitoes (Broom et al., 1995; Johansen et al., 2000; Kay and Farrow, 2000; Ritchie and Rochester, 2001) facilitates the spread of MVEV and cause epizootic activities. Furthermore, similar to other flaviviruses, climatic factors may have a significant impact on the spread and the establishment of MVEV (Gould and Higgs, 2009; Martin et al., 2008; Russell et al., 2009; Weaver and Reisen, 2010). Environmental conditions, such as high temperature, heavy rainfall and flooding are likely to play a significant role in the spread, movement and maintenance of the virus (Broom et al., 2002a).

1.4.1.3.3 Molecular Epidemiology

Genetic variation among MVEV isolates representing different geographical regions, times of isolation and species of origin has been studied previously (Coelen and Mackenzie, 1988; Johansen et al., 2007; Lawson, 1988; Lobigs et al., 1986, 1988; Williams et al., 2013). As for other flaviviruses (Section 1.3.4), phylogenetic studies of MVEV have employed the prM, E, and NS5 genes as well as the 3'UTR to characterise the genetic diversity of MVEV (Johansen et al., 2007; Lobigs et al., 1988; Poindinger et al., 1996; Williams et al., 2013). These studies have identified four distinct genotypes (G1-G4). Of these, G1 is the dominant genotype. G1 strains have been isolated from all mainland Australian states and was also isolated from PNG in 1998 (Johansen et al., 2000), suggesting the movement of MVEV between PNG and mainland Australia. G2 has only been isolated from the Kununurra district of the Kimberley region of WA and appears to be restricted to this area. G2 strains

were last detected in 1995 (Johansen et al., 2007). This period of apparent dormancy suggests that this genotype may have altered biological characteristics compared to G1 viruses and that it exists in a distinct ecological niche (Johansen et al., 2007; Williams et al., 2010). Thus, G2 viruses may have been undersampled during mosquito trapping activities. Alternatively, G2 viruses may have not been detected since 1995 because they have become extinct (Williams et al., 2010). There is an 84.6% nucleotide identity translating to 94.4% amino acid similarity between isolates belonging to G1 and G2 (Johansen et al., 2007). A single isolate each of G3 and G4 were identified in 1966 (Lobigs et al., 1986) and 1956 (French et al., 1957), respectively, from PNG. Isolates of G3 and G4 are very closely related, to each other but distinct from G1 and G2 strains, demonstrating a 91.3% nucleotide identity (99.2% amino acid similarity) with each other and a maximum of 87.0% nucleotide identity (96% amino acid similarity) with G1 and G2 strains (Johansen et al., 2007). These two genotypes appear to be confined to this geographical region. However, due to the lack of an active and effective surveillance system in PNG, the accurate distribution of G3 and G4 is unknown.

1.4.1.3.4 Transmission Cycle

MVEV principally exists in a mosquito-waterbird amplification cycle, where mosquitoes are the vectors and waterbirds are the main hosts (Mackenzie and Broom, 1995; Marshall, 1988). Humans, horses and some other mammals are incidental or dead-end hosts (Gard et al., 1977; Holbrook and Gowen, 2008; Marshall, 1988; Roche et al., 2013). The main vector for MVEV is *Cx. annulirostris* which accounts for more than 90% of mosquito isolates (Mackenzie and Broom, 1995). MVEV was also isolated from other mosquito species, including *Cx. pullus*, *Cx. quinquefasciatus, Cx. sitiens, Aedes normanensis, Ae. pseudonormanensis,* and *Anopheles bancroftii* (Johansen et al., 2007; Kay et al., 1982). To date, vector competency (transmission efficiency) for MVEV has only been confirmed for *Cx. annulirostris* (Kay et al., 1984) and *Cx. quinquefasciatus* (Kay et al., 1982). The isolation of MVEV from *Ae. normanensis* (Broom et al., 1989) in arid regions of WA supports the hypothesis that, during episodes of drought, MVEV may be maintained

in desiccant-resistant eggs. When there is an improvement in the environmental conditions, viruses may then reactivate.

Little is known about the likely vertebrate hosts of MVEV. Seroepidemiological studies of a variety of animals and birds revealed that waterbirds were the likely host of MVEV (Anderson, 1954; Liehne et al., 1976). Marshall et al. (1982) indicated that waterbirds from the order *Ciconiiformes* are the main MVEV vertebrate host. Experimental studies have since confirmed that rufous night herons (Whitehead et al., 1968) and egrets (Boyle et al., 1983) play a role in the ecology and epidemiology of MVEV. Other vertebrates such as Grey kangaroos, rabbits, pigs, dogs, and chickens produce a high or moderate viraemic response to MVEV and may also have roles in the MVEV lifecycle (Kay et al., 1985). Further surveillance and investigation is needed to confirm the roles (if any) of these animals as either amplifying or maintenance hosts.

1.4.1.4 Control Measures

Since there is no specific cure available for MVE or vaccine to MVEV, it is essential to focus on surveillance and control measures aimed at preventing the spread of infection. To effectively control MVEV, a number of measures need to be undertaken concurrently. All these control measures are complementary and need to be carried out in parallel with each other to warrant the best outcome.

1.4.1.4.1 Control of Vectors

It is generally believed that barrier control (barrier fogging with both larvicides and adulticides) to create a buffer zone around communities that are at risk during an outbreak provides a good approach to controlling mosquitoes so as to protect human populations (Dale et al., 1998; Spencer et al., 2001; Whelan, 2011a). However, the success of this concept depends on knowledge of the habits and habitats of the mosquitoes, as well as the implementation of an efficient, intensive and effective mosquito control program. Large scale use of chemicals to eradicate MVEV in the endemic area is an impractical approach. Mosquito control programs consists of many strategies, including: physical, cultural, environmental, agricultural,

chemical and biological control of mosquitoes (Dale et al., 1998; Douglas, 2011; Lindsay and Harrington, 2011; Ng and Vythilingam, 2012; Spencer et al., 2001; Whelan, 2011a, b).

1.4.1.4.2 Control of Hosts

Although waterbirds have been implicated as MVEV hosts, there may be a number of other possible amplifying hosts that are yet to be identified [P. Neville (2012); personal communication]. The movement of waterbirds has been associated with the eruption of MVEV into new areas (Guay et al., 2012). Despite this knowledge, little has been done to control bird populations in endemic regions. This is a challenge given that the culling of birds, including migratory birds, is prohibited under the Japan-Australia Migratory Bird Agreement (JAMBA; 1974), China-Australia Migratory Bird Agreement (CAMBA; 1986), and the Republic of Korea-Australia Migratory Bird Agreement (ROKAMBA; 2006; (Australian Government, 2009; Neville, 2012)].

1.4.1.4.3 Surveillance Systems

All arbovirus infections, including MVEV, are reported to the Commonwealth Department of Health and Aged Care via the National Notifiable Disease Surveillance System (NNDSS). The Laboratory Virology and Serology (LabVISE) Reporting Scheme also contribute to the reporting and surveillance of MVE infection in Australia (Spencer et al., 2001). In addition, since the mid-1970s, a prospective surveillance program has been in place to detect evidence of MVEV infection in the serum of sentinel chickens in WA, the NT, NSW, and VIC (Fig. 1.5). Sera are collected and screened for the presence of antibodies against MVEV and other medically important arboviruses (Broom et al., 1998; Broom, 2003; Broom and Whelan, 2005; Broom et al., 2002b; Fitzsimmons et al., 2009; Johansen et al., 2009; Spencer et al., 2001). Furthermore, mosquito surveillance activities are carried out annually. Mosquitoes are trapped, speciated and processed for virus isolation to determine species that are likely to be involved in virus transmission (Johansen et al., 2009).

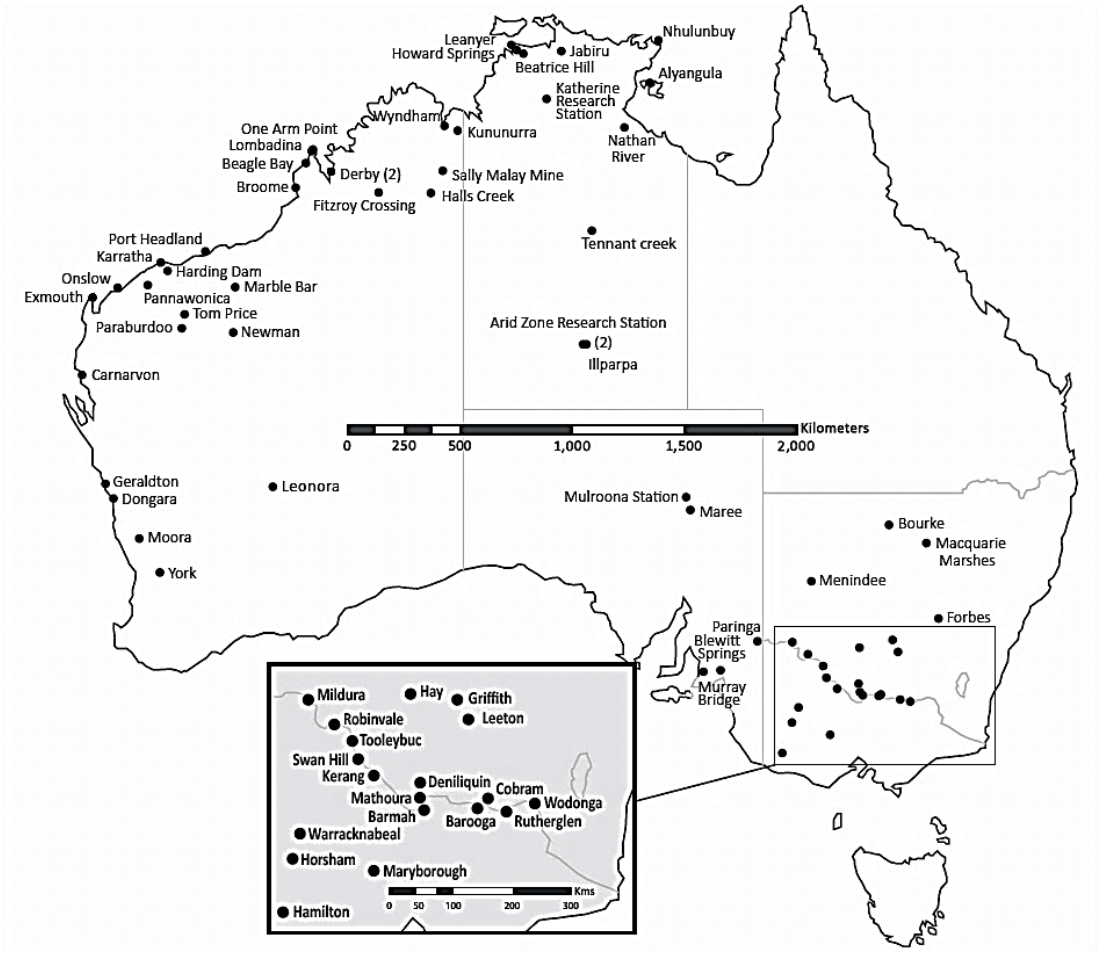

Fig. 1.5. *The location of sentinel chicken flocks in Australia in 2007-2008*

- Indicate the locations where sentinel chickens are located. Reproduced from Knope et al. (2013).

Some opportunistic surveillance has also been done for MVEV (Spencer et al., 2001). Sera collected during kangaroo and possum culling exercises were tested to help determine the extent of it geographical spread. In some parts of WA, opportunistic testing of domestic chickens has been used to determine the geographical limits of the spread of MVEV activity, where sentinel chickens were not available (Broom et al., 2002a; Spencer et al., 2001).

The surveillance of weather, including rainfall patterns, temperature, the Southern oscilation index (El Niño or La Niña events), and river flow levels are also useful tools for predicting MVEV activity and mosquito breeding capabilities (Spencer et al., 2001). Similar to RVFV (Martin et al., 2008)] and DENV (Russell et al., 2009; Weaver and Reisen, 2010), climatic factors may have a significant impact on the spread and the establishment of MVEV (Gould and Higgs, 2009). In fact, it is now evident that large outbreaks of MVEV, especially in WA, are related to extreme and abnormal weather (Broom et al., 2002a; Roche et al., 2013).

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1.4.1.4.4 Vaccines

Due to low incidence of MVE each year in Australia, it might be argued that the development of a vaccine is not warranted. However, this virus poses a high risk to the population of northern Australia and specifically to infants and children (Broom et al., 2000). Hence, the development of a vaccine against MVEV has been the subject of ongoing research for decades. Pioneer studies (Hall et al., 1996) revealed that when F1 hybrid mice were vaccinated with a recombinant vaccinia virus (expressing prM-E-NS1-NS2A), mice were 100% protected against a subsequent

challenge with a higher dose of MVEV. Mice that received a combination of (C-prM-E-NS1-NS2A), (NS1-NS2A without the structural genes) or pure NS1 were only partially protected. In another mouse study, an epitope of MVEV was expressed in an avirulent strain of *Salmonella* to stimulate the production of anti-MVEV antibodies (Whittle et al., 1997). The sera obtained six weeks post inoculation neutralised 60%-70% of a subsequent challenge with wild-type virus. No complete neutralisation was observed in this study (Whittle et al., 1997). Colombage et al. (1998) used a gene gun for the intradermal delivery of the prM and E proteins, as well as intramuscular injection of DNA. This provided resistance/protection against subsequent challenge with wild-type virus. When MVEV recombinant subviral particles (SVPs) were tested, they were found to provide 100% protection against a subsequent lethal dose of MVE-1-51 (Kroeger and McMinn, 2002).

In the absence of a commercially available vaccine, research has been undertaken to examine whether licenced vaccines for other members of the JEV serocomplex afford any protection against MVEV. Immunisation of mice with a low-dose of an inactivated JEV vaccine (JE-VAX) enhanced a subsequent infection with MVEV (Lobigs et al., 2009). In another study, the effect of immunisation against UV-inactivated MVEV on a subsequent JEV infection was examined. This study demonstrated that the antibodies produced after a primary challenge with MVEV may exacerbate the course of a secondary infection with JEV (Lobigs et al., 2003). Interestingly in two studies, a single high dose of a live chimeric JEV vaccine candidate-ChimeriVax-JE (Guirakhoo et al., 1999; Lobigs et al., 2009) was shown to elicit complete protection against a subsequent lethal challenge with MVEV infection. Therefore, the ChimeriVax-JE vaccine may be a good candidate to be used in the event of an MVEV outbreak, at times of vector abundance, or when considerable seroconversions are detected amongst sentinel chickens.

The effect of passive immunisation on MVEV infection has also been investigated. Broom et al. (2000) demonstrated that passive immunisation of mice with MVEV antisera provided complete protection against a subsequent MVEV infection. Moreover, at least two studies investigated the efficacy of passive immunisation of mice with the antiserum of KUNV (Broom et al., 2000) and JEV (Wallace et al., 2003)

on a later infection with MVEV. These studies revealed that prior inoculation of mice with subneutralising concentration of inactivated KUNV or JEV antiserum augments the course of MVEV infection resulting in a higher viraemia titre and a higher mortality rate. Therefore, passive immunisation cannot be regarded as an optimal choice for providing protection against MVEV infection. Moreover, it seems that prior infection with a closely-related flavivirus may render a person at risk of a more severe disease if a secondary infection with another flavivirus occurs.

1.4.2 Japanese Encephalitis Virus (JEV)

JEV is the most common cause of encephalitis in Asia, and it is estimated to cause nearly 45,000 cases of human disease in eastern and south-eastern Asia each year (Erlanger et al., 2009; Fischer et al., 2008; Lindenbach et al., 2007).

Most cases of human infection with JEV are asymptomatic. Less than 1% of JEV-infected people present with clinical disease (Gajanana et al., 1995; Vaughn and Hoke, 1992). The disease manifests with a wide range of clinical symptoms. In mild cases, patients may experience a febrile illness, whereas with encephalitic cases, people may suffer brain disorders, Parkinsonian syndrome, acute flaccid paralysis and death (Bhatt et al., 2012; Kalita and Misra, 2000; Kumar et al., 2006; Misra and Kalita, 1997; Rayamajhi et al., 2006; Solomon et al., 2002). The case fatality rate of JEV is approximately 20-30% (Bista and Shrestha, 2005; Ghosh and Basu, 2009; Kumar et al., 2006). Almost half of the survivors develop significant psychiatric and neurological sequelae (Ding et al., 2007; Ghosh and Basu, 2009; Mackenzie et al., 2006; Solomon et al., 2000). In pigs, JEV causes stillbirth and mummified foetus. Infected piglets that are born alive experience convulsions, tremors and die immediately after birth (Williams et al., 2012). Abortion is common in pregnant sows. The infection is asymptomatic in non-pregnant animals. The encephalitis symptoms are only occasionally seen until six months of age (Daniels et al., 2002; Williams et al., 2012).

In the early 1970's, more than 100,000 JEV cases were recorded each year, with China having the majority of these cases (Igarashi, 1992). However, over the last three decades, due to the use of a JEV vaccine together with urbanisation, host and

vector control, and systematic agricultural practices, the number of JEV cases has significantly declined. Currently, nearly 30,000 JEV cases are reported each year in China (Fischer et al., 2008). Though, because of poor surveillance and diagnostic systems in many countries, this number might represent an underestimate of the actual burden of the disease (Erlanger et al., 2009).

JEV is, presently, established in twenty four countries of Asia and the Pacific (Campbell et al., 2011). The reason for this huge geographical distribution has yet to be determined. It is thought that wind-blown mosquitoes, infected migratory birds, changes in agricultural practices and animal husbandry (especially in pig-rearing practices) and the establishment of irrigation for rice production could all contribute to this phenomenon (Erlanger et al., 2009; Hanna et al., 1996; Keiser et al., 2005).

In Australia, JEV was initially identified in the Torres Strait islands in 1995, and later spread to the Northern Peninsula Area and southwest Cape York in northern QLD (Hanna et al., 1999; Hanna et al., 1996; Mackenzie et al., 2004; Mackenzie et al., 2006). To investigate the origin of JEV infections in the Torres Straits, comprehensive seroepidemiological studies and mosquito surveys were conducted in PNG (Johansen et al., 1997; Johansen et al., 2000; Spicer et al., 1999). It was found that seroconversion against JEV was present in PNG as early as 1989 (Johansen et al., 1997), and in 1997-1998, JEV was isolated from *Cx. annulirostris* and *Cx. palpalis* in PNG (Johansen et al., 2000). Phylogenetic studies also revealed that PNG isolates were closely related to strains isolated from the Torres Strait Islands (Johansen et al., 2000). These results provided solid evidence that the 1995 outbreak of JEV in the Torres Strait Island of Australia originated from PNG (Johansen et al., 2000; Mackenzie et al., 2006). It is hypothesised that migratory birds and/or wind-blown mosquitoes may have carried the virus from PNG to the Torres Strait islands (Hanna et al., 1996; Johansen et al., 2000; Ritchie et al., 1997).

Phylogenetic analyses of the prM (Chen et al., 1992; Chen et al., 1990) and E genes (Li et al., 2011; Ni and Barrett, 1995; Paranjpe and Banerjee, 1996; Uchil and Satchidanandam, 2001; Williams et al., 2000) have classified JEV into five distinct

genotypes (G1-G5). There is a substantial genetic difference between the virus strains that circulate in endemic and epidemic areas. Strains of G1 and G3 are more commonly associated with JEV epidemics in temperate areas, whereas those of G2 and G4 have been detected in tropical areas where JEV is endemic. However, more G1 cases and isolates have been recorded in many Australasian countries in the past decades (Cao et al., 2011; Fulmali et al., 2011; Ma et al., 2003; Nga et al., 2004; Nitatpattana et al., 2008; Pan et al., 2011; Pyke et al., 2001; Wang et al., 2010; Zhang et al., 2011; Zhu et al., 2011). G5 contains isolates from Muar city of Malaysia, and very recently from China (Li et al., 2011; Mohammad et al., 2011; Takhampunya et al., 2011; Uchil and Satchidanandam, 2001). It is believed that ecological and epidemiological factors, together with differences in virulence, contribute to this distinct distribution of the JEV genotypes (Chen et al., 1992; Chen et al., 1990; Mackenzie et al., 2004).

JEV is transmitted between *Culex* mosquitoes and vertebrate hosts, mainly pigs and wading birds (Bista and Shrestha, 2005; Burke and Leake, 1988; Endy and Nisalak, 2002; Igarashi, 1992; Tang et al., 2010; Vaughn and Hoke, 1992). Other animals, such as cattle, goats, sheep, buffalo and horses, can also be infected with JEV but they usually do not develop high levels of viraemia, and hence are not considered amplifying hosts (Endy and Nisalak, 2002; Ilkal et al., 1988; Yang et al., 2007). Humans have no role in the maintenance and amplification of JEV and are only dead-end points in the JEV transmission cycle (Ting et al., 2004; Yang et al., 2007).

Control measures such as controlling mosquito populations and animal hosts can also reduce the number of human cases. Generally, these strategies are similar to those described for MVEV. One notable exception is the immunisation of pig populations with the JEV vaccine. Although the immunisation of pigs has been shown to have a profound effect on the incidence of JEV, it is not economically viable. Instead, the physical removal of pigs has been shown to have a significant impact in reducing JEV cases. It is recommended that pigs be kept at least five kilometres from human residents (Endy and Nisalak, 2002; Scherer et al., 1959).

At present, there are no specific treatments for JEV and therapy is limited to the management of complications and supportive care (Daniels et al., 2002; Guruprasad, 2011; Platt and Joo, 2006). Due to the lack of any specific antiviral therapy, the high incidences, the moderate fatality rate and the significant sequelae, prevention of JEV is considered very important. Human vaccination appears to be the most effective means of preventing JEV (Mackenzie et al., 2006; Tsai, 2000). In some countries, for example Japan and China, the implementation of JEV vaccination program has almost eradicated JE (Halstead and Thomas, 2010; Liu et al., 2006a; Tsai, 2000).

1.4.3 West Nile Virus (Kunjin Virus)

WNV is another medically important flavivirus of the JE serocomplex (Calisher et al., 1989) that presents both public and veterinary health concerns in Australia, in the form of the WNV subtype KUNV (Broom et al., 2003; Ebel and Kramer, 2009; Gray et al., 2011; May et al., 2011). WNV was originally isolated from the West Nile district of Uganda from the blood of a patient with fever (Smithburn et al., 1940). Its distribution now includes Australia, Europe and the Americas, Middle East and Asia (Bakonyi et al., 2006; Balenghien et al., 2006; Broom et al., 2003; Doherty et al., 1963; Ebel and Kramer, 2009; Gray et al., 2011; Kutasi et al., 2011; May et al., 2011; Monaco et al., 2011; Papa et al., 2012; Vazquez et al., 2010) .

KUNV can manifest as two types of clinical disease in humans. KUNV encephalitis is less common and the symptoms are milder than those of MVEV (Mackenzie et al., 1994; Mackenzie et al., 1993). In mild cases, fever and flu-like symptoms with polyarthralgia and polyarthritis are characteristic (Gray et al., 2011). In the last decade, 31 human cases of KUNV has been reported in Australia, mainly from the NT [only one case from NSW and three from VIC; (Gray et al., 2011)]. KUNV can also cause infection in horses (Frost et al., 2012; Gard et al., 1977; Mann et al., 2013; Roche et al., 2013). Horse infection by KUNV is subclinical in most cases. However, neurological symptoms such as ataxia, depression, hyperaesthesia, convulsion, paralysis and coma have been observed in some cases (Frost et al., 2012; Mann et al., 2013; Roche et al., 2013). In 2011, an outbreak of KUNV occurred in Australia

that caused devastating disease and death among horses, especially in southern and south-eastern states (Frost et al., 2012; Roche et al., 2013).

In Australia, KUNV is enzootic across the tropical north of Australia and can occasionally appear in other parts of mainland Australia (Broom et al., 2003; Ebel and Kramer, 2009; Gray et al., 2011; May et al., 2011). KUNV was first isolated in Australia from *Cx. annulirostris* mosquitoes in 1960 (Doherty et al., 1963; Krauss et al., 2003). The main mosquito vector of KUNV is *Cx. annulirostris* and ardeid birds are the main amplifying hosts (Boyle et al., 1983; Marshall, 1988). The ecology and epidemiology of KUNV is extensively monitored alongside MVEV in Australia. While KUNV is enzootic across northern Australia, it has been shown to have a focus in the Kimberley region of WA and the NT (Broom et al., 2003; Gray et al., 2011).

Within the WNV there have been up to seven lineages proposed that differ from each other by 5-25% (Bondre et al., 2007; Vazquez et al., 2010). KUNV belongs to lineage 1b (Pesko and Ebel, 2012; Scherret et al., 2001). According to Scherret et al. (2001), the Australian KUNV isolates are genetically homologous and are distinct from WNV strains found elsewhere.

The primary means of transmission of WNV/KUNV involves mosquitoes as the primary vectors and birds as the main amplifying hosts. In addition to birds, a large number of vertebrates such as humans, horses and reptiles may exhibit clinical symptoms. WNV can also be transmitted via blood transfusion (Papa et al., 2012; Pealer et al., 2003) and by organ transplant (Iwomoto et al., 2003). There is no specific treatment for WNV infection (Bhattacharya, 2003). Interferon-α (Kalil et al., 2005), Ribavirin (Loginova et al., 2009) and WNV-specific immunoglobulin (Hamdan et al., 2002) have been tried in the treatment of WNV infection; however, there is not enough data to support their efficacy.

No vaccine is available to prevent WNV infection in human. However, WNV vaccines have been licensed for use in horses (Garch et al., 2008; Ng et al., 2003; Seino et al., 2007). These vaccines have been designed against NY99 strain of WNV. To date, the efficacy of these vaccines for protection to KUNV has not been reported. Given that there is a close genetic and antigenic relatedness between KUNV and the NY99

strain of WNV, the use of these vaccines against KUNV may be an option. Other control measures include controlling mosquito populations, and are similar to those described for MVEV.

1.4.4 Alfuy Virus (ALFV)

ALFV is another member of the JE serocomplex of flaviviruses that circulates in Australia (Calisher et al., 1989; Mackenzie et al., 1994). ALFV shares a high degree of genetic and antigenic similarity with MVEV (Calisher et al., 1989; De Madrid and Porterfield, 1974; May et al., 2006; Poindinger et al., 1996). In fact, molecular studies have assigned ALFV as a subtype of MVEV (Simmonds et al., 2012; Thiel et al., 2005). However, sequencing of the complete genome of ALFV revealed a 73% nucleotide identity between ALFV and MVEV, corresponding to 83% amino acid similarity, indicating that ALFV is a distinct species in the JEV serocomplex(May et al., 2011).

Although ALFV is genetically and antigenically closely related to MVEV, it has not yet been associated with disease of human and animals (Mackenzie and Williams, 2009; May et al., 2006). *In vivo* studies with mice have revealed that ALFV is unable to invade the CNS. It has been suggested that the inability of ALFV to invade the CNS is due to mutations in the prM-E region of ALFV, compared to virulent MVEV (May et al., 2006; Prow et al., 2011). The glycosylation motif, which is associated with neuroinvasiveness in encephalitogenic MVEV, is absent in ALFV (May et al., 2006; Prow et al., 2011). Similarly, the conserved putative hinge region between domain I and domain II of the flavivirus E protein demonstrates a unique amino acid substitution in ALFV (May et al., 2006; Prow et al., 2011). Mutagenesis studies revealed that the absence of the glycosylation motif and changes to the hinge region in ALFV results in the loss of neuroinvasiveness in mice (May et al., 2011; Prow et al., 2011).

Since there is a high level of antibody cross-reactivity between infections caused by MVEV and ALFV, the chances of some MVEV cases having actually been caused by ALFV cannot be discounted. In fact, because there is an inadequate amount of information about the biological and clinical characteristics of this virus in humans

and animals, its potential as an etiologic agent may be unrecognised (May et al., 2006).

ALFV was first isolated in 1966 at Cape York in the north of Australia, from the blood of a swamp pheasant (Whitehead et al., 1968). The major vector for this virus is *Cx. annulirostris* and ardeid birds are believed to be the main vertebrate hosts (Mackenzie et al., 1998a; Mackenzie and Williams, 2009). The regular isolation of ALFV in *Culex* mosquitoes in northern Australia suggests that it has a similar ecology to other members of the JE serocomplex, especially MVEV (Johansen et al., 2003).

1.4.5 Other Flaviviruses

DENV is another member of the *Flavivirus* genus that causes some of the most important arboviral diseases around the globe. Over half of the earth's population live in areas at risk (Gubler, 2011). In Australia, DENV cases have been reported in QLD and NSW (Clenland and Bradley, 1918). There were 6,271 confirmed cases of DENV infection between 1993 and 2009, with the main outbreaks occurring in Townsville, Cairns and the Torres Strait Islands (Hanna et al., 2001; Hanna et al., 1998; Ritchie et al., 2013; Tropical Public Health Unit Network, 2004). DENV has four antigenically distinct serotypes (DENV-1 to DENV-4), all of which can produce dengue fever (DF) upon initial infection. A secondary DENV infection with a different serotype to that of the initial infection can cause more severe diseases, called dengue haemorrhagic fever (DHF) or dengue shock syndrome [DSS; (Gubler, 1998; Halstead et al., 1983)]. This is due to an antibody-dependent enhancement of the secondary DENV infection, a phenomenon called antigenic sin (Halstead et al., 1983). To date, no treatment or vaccine is available for DENV (Edelman, 2005).

DENV is not endemic in Australia, however, its main vector, *Aedes aegypti*, is native to northern QLD. Infected travellers introduce DENV to local mosquito populations and initiate outbreaks (Giele, 2011; Gubler, 2011). DENV's primary transmission cycle involves the *Aedes aegypti* mosquito as the main vector with humans as amplifying hosts (Gubler, 2011). Other *Aedes* species that act as vectors for DENV include *Ae. albopictus* and *Ae. scutellaris* (Hu et al., 2011; Russell et al., 2009). The principal risk factor associated with contracting DENV is exposure to the mosquito

vector (Gubler, 1998; Gubler and Clark, 1996; Gubler, 2011). However, DENV transmission has been reported in the nosocomial environment elsewhere in the world (Chen and Wilson, 2004; de Wazieres et al., 1988; Wagner et al., 2004), and recently in Australia (Clark et al., 2012).

The methods available for the diagnosis of DENVs are the same as those described for the other flaviviruses. The best way to control DENV is by raising public awareness and controlling mosquito populations (Gubler and Clark, 1996; Gubler, 2011).

KOKV is enzootic in Australia and PNG. It is another important member of the flaviviruses that can cause human infection (Mackenzie and Williams, 2009). Initially, it was classified as a member of the JE serocomplex. Later, based on both antigenic (Hall et al., 1991) and genomic (Poindinger et al., 1996) characteristics, KOKV was classified into its own serogroup together with STRV. KOKV was first isolated from *Cx. annulirostris* collected at Kowanyama in northern QLD in 1960 (Doherty et al., 1963). Since then, it has been detected in WA, the NT, QLD, NSW, and PNG (Mackenzie et al., 1994; Poidinger et al., 2000). Kangaroos and other macropods are believed to be KOKV's main reservoir hosts (Doherty et al., 1971). Human infections with KOKV (acute polyarticular disease) have been reported in NSW, QLD, VIC and PNG (Mackenzie et al., 1994; Mackenzie and Williams, 2009). The related STRV is enzootic in Australia and may also be found in PNG (Mackenzie and Williams, 2009). It was first isolated in Cairns, northern QLD from *Ae. vigilax* mosquitoes (Doherty et al., 1963). Later, it was also isolated from *Cx. annulirostris Ae. procax and Ae. notoscriptus.* Although human infection with STRV can occur (Hawkes et al., 1985), no human disease has so far been reported (Mackenzie and Williams, 2009).

Edge Hill virus (EHV) is another member of the *Flavivirus* genus that circulates throughout Australia. It was first isolated in Cairns, QLD, in 1961 (Doherty et al., 1963; Mackenzie and Williams, 2009), and has subsequently been found across Australia. EHV has been isolated from a range of mosquito species (Macdonald et al., 2001; Mackenzie et al., 1994; Russell and Dwyer, 2000). The main amplifying

hosts are marsupials (Doherty et al., 1964; Hawkes et al., 1985). Human infections have been reported in New South Wales (Hawkes et al., 1985) and disease can present as myalgia, arthralgia and muscle fatigue (Aaskov et al., 1993). Phylogenetic studies suggest that EHV is closely related to YFV (Macdonald et al., 2001; Macdonald et al., 2010).

Sepik virus (SEPV), which is phylogenetically the closest flavivirus to YFV (Kuno and Chang, 2006), was first isolated from the *Mansonia septempunctata* mosquito in the Sepik district of PNG (Karabatsos, 1985; Woodroofe and Marshall, 1971). Later, it was isolated from the *Cx. Sitiens* mosquito in the Western Province of PNG. Serological studies suggest that this virus may also be present in the Indonesian archipelago (Mackenzie and Williams, 2009). To date, SEPV has not been found in Australia.

Insect-specific flaviviruses (ISFs) have recently been isolated from the NT (Hobson-Peters et al., 2013). This is the first time an ISF has been isolated in Australia. The virus was isolated from *Coquillettidia xanthogaster* mosquitoes and was tentatively named Palm Creek virus (PCV), after its first place of isolation. Phylogenetic analyses of the NS5-3'UTR sequences of PCV and those of other known ISFs from other parts of the world revealed a close relationship between Australian PCV and the cell fusing agent virus (CFAV, Tentative *Flavivirus*; Appendix 1). Interestingly, a pre-infection of mosquito C6/36 cells with PCV suppressed the subsequent infection with MVEV and KUNV, via a superinfection exclusion phenomenon (Hobson-Peters et al., 2013). However, the detection of PCV-like viruses in *Coquillettidia* and *Culex* mosquitoes in the Kimberley region of WA [Nguyen, McLean, Hobson-Peters, Barnard, Johansen and Hall, unpublished data; cited in Hobson-Peters et al. (2013)] has invited speculation that PCV may be more broadly distributed than the NT. So far, PCV has not been associated with a human or animal disease in Australia.

1.5 Aims of This Study

Since MVEV is the most important encephalitogenic arbovirus in Australia, the aim of this thesis is to enhance the knowledge of this virus. This thesis will specifically focus on the following:

1. **Genetic characterisation of MVEV isolates from WA (2005-2009):** G2 of MVEV has not been detected since 1995. However, since only a small number of MVEV strains isolated each year have been sequenced, the first aim of this thesis is to sequence all MVEV strains isolated in WA during the five year period 2005-2009. This will address the continued circulation of G2 MVEV in the geographic region that is the enzootic focus of this virus and help to more accurately estimate genetic diversity at the genotype level.
2. **Development of an RT-qPCR capable of detecting and quantifying all four MVEV genotypes:** There is currently no rapid, sensitive and specific assay for the identification of all genotypes of MVEV. Such an assay is essential for comprehensive detection and identification of all MVEV strains in both routine surveillance and outbreak settings. The development of a rapid, sensitive and specific assay capable of detecting and quantifying all MVEV genotypes will be attempted. This assay will also be necessary to perform the RT-qPCR required for aim 3, 4 and 5.
3. **Characterisation of genetic diversity using NGS:** To date, the intra-population genetic diversity of flaviviruses has not been characterised using NGS technology. The third aim of this thesis is to characterise the genetic diversity and population structure within individual MVEV populations in order to gain an insight into the quasispecies phenomenon and mutant spectra of this virus.
4. **Phenotypic characterisation of recent isolates from G1 and G2:** Since 1988, MVEV research has focused on deciphering the phenotypic characteristics of the most prevalent Australian genotype (G1). Since the understanding of the actual differences between the phenotypic features of the co-circulating Australian genotypes (G1 and G2) is limited, the *in vitro* cytopathogenicity and *in vivo* virulence phenotypes of both G1 and G2, including recent isolates, will be characterised and compared.
5. **Experimental evolution of MVEV:** Previous research has provided conflicting findings on the reasons for the evolutionary stasis and restricted genetic diversity within flavivirus populations. In this thesis, an *in vitro* experimental

evolution study will be carried out to investigate these factors for G1 and G2 of MVEV.

It is anticipated that the results obtained in this thesis will lead to a better understanding of the prevalence and distribution of currently circulating MVEV genotypes, as well as the depth of genetic diversity within individual virus populations. This study also aims to address the biological factors underlying the restricted evolution of MVEV through the characterisation of the phenotypic features of the two co-circulating MVEV genotypes (G1 and G2). Taken together, this study aims to further enhance the knowledge of both the genetic and phenotypic characteristics of MVEV and to provide insights into the spread and pathogenesis of this virus.

CHAPTER 2

GENETIC AND GENOTYPIC CHARACTERISATION OF RECENT MVEV STRAINS FROM WESTERN AUSTRALIA: DETECTION OF GENOTYPE 2

2.1 Introduction

MVEV is endemic in the Kimberley region of WA and can occasionally cause epizootic activity in other regions of WA (Broom, 2003; Broom et al., 2002a; Marshall, 1988), and sporadic outbreaks in other states of Australia (Broom and Whelan, 2005; Knope et al., 2013; Knox et al., 2012; Roche et al., 2013). The major vector of MVEV is *Cx. annulirostris,* accounting for over 90% of isolates (Mackenzie and Broom, 1995; Russell and Dwyer, 2000). However, other mosquito species such as *Cx. pullus* (2.6%)*, Ae. normanensis* (1.6%) *and Cx. quinquefasciatus* (1.2%) have yielded isolates and may also transmit the virus [C. Johansen and A. Broom, unpublished data, cited in Williams et al. (2010)].

Of the four distinct genotypes of MVEV, only G1 and G2 strains have been isolated in Australia. G1 is the dominant genotype and comprises isolates from all mainland states. Only five strains belonging to G2 have been detected, between 1973 and 1995 from Kununurra in the Kimberley region of WA, and appear to be restricted to this area (Johansen et al., 2007; Liehne et al., 1976). No G2 isolates have been detected since 1995. However, it is important to note that since 1995, only a small proportion of all MVEV isolates collected from field mosquitoes have been sequenced [C. Johansen (2010), personal communication], such that an accurate estimation of the genetic diversity of MVEV has not been properly characterised. It has been suggested that G2 strains may exhibit altered biological features that confine them to the north-eastern Kimberley region (Johansen et al., 2007). Furthermore, it has been hypothesised that G2 viruses may be extinct or occupy a rarely-sampled ecological niche with a low level of activity (Johansen et al., 2007; Williams et al., 2010).

In the present study, the latter hypothesis was tested by nucleotide sequencing and phylogenetic analyses of all MVEV isolates collected from WA between 2005 and 2009. This chapter reports the first detection of G2 isolates since 1995 and their first appearance outside the northeast Kimberley region of WA. Results obtained from this study help describe the distribution and pattern of activity of different

genotypes of MVEV in WA. They also provide an insight into inter- and intra-genotypic variation between MVEV isolates.

2.2 Materials and Methods

2.2.1 Cells and Viruses

C6/36 cells were grown at 28°C in medium 199 (M199; Life Technologies, Australia) supplemented with 1% L-glutamine (Life Technologies, Australia) and 10% foetal bovine serum (FBS; Life Technologies, Australia). Vero and PSEK cells were grown at 37°C in M199 medium (Life Technologies, Australia) supplemented with 1% L-glutamine (Life Technologies, Australia) and 5% or 10% FBS (Life Technologies, Australia), respectively. All incubations were carried out in the presence of 5% CO_2.

MVEV isolates included in this study were kindly provided by the Arbovirus Surveillance and Research Laboratory, University of Western Australia, and consisted of eighty three isolates from mosquitoes collected from various locations in WA between 2005 and 2009, as part of an annual survey of mosquito fauna and arbovirus activities in WA (Fig. 1.5). Virus isolates were derived from the infected homogenates of mosquito pools, as described previously (Johansen et al., 2009; Quan et al., 2011). Briefly, homogenates were used to inoculate monolayers of C6/36 cells in 96-well plate (Greiner Bio-one, Australia) followed by passages in Vero and PSEK cells (Fig. 2.1). Monolayers were incubated for four days and examined microscopically for CPE. The isolation of MVEV strains were confirmed by enzyme immunoassay using an MVEV-specific monoclonal antibody (Broom et al., 1998; Johansen et al., 2009; Quan et al., 2011). Isolated viruses were further grown in PSEK cells in 25cm^2 tissue culture flasks (Greiner Bio-one, Australia) and incubated for four days. The tissue culture supernatants were clarified by centrifugation at 1000*xg* for 5 min, and aliquots were stored at -80°C.

2.2.2 RNA Purification, and RT-PCR

Viral RNA was extracted from 140µl of infected tissue culture supernatants using a QIAamp® Viral RNA mini kit (QIAGEN, Australia), according to the manufacturer's

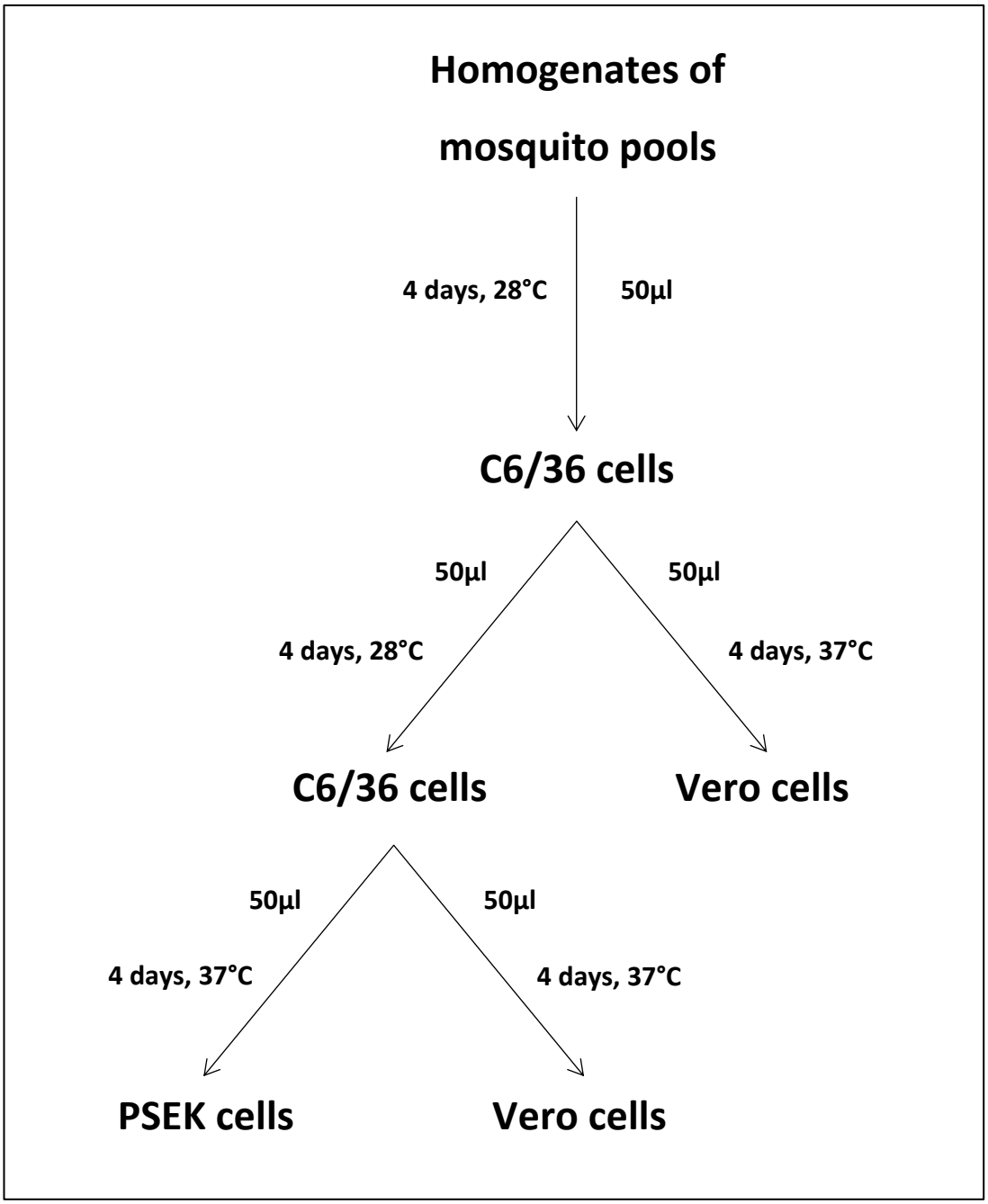


Fig. 2.1. *The isolation process of MVEV strains from the homogenates of mosquito pools.*

Adapted from (Johansen et al., 2009)

protocol. Purified RNA was reverse-transcribed using the flavivirus universal primer VD8 (Pierre et al., 1994) and SuperScript® III Reverse Transcriptase kit (Life Technologies, Australia), according to the manufacturer's protocol. Briefly, 10µl of purified RNA was combined with 2µM of VD8 and 10mM dNTP mix in a 0.2ml thin-walled PCR tubes and incubated at 65°C for 5 min; followed by cooling on ice for 5 min. Subsequently, 4µl of 5X RT buffer, 1µl of DTT, 40U of RNase OUT™ and 200U of SuperScript® III RT were added to the reaction, and incubated at 55°C for 60 min, followed by 70°C for 15 min. The cDNA was aliquoted and stored at -30°C for subsequent use. Reverse transcription was done in an Eppendorf AG thermal cycler.

Initially, a 675bp region within the envelope (E) gene of all virus isolates was amplified. This genomic region included nucleotides 1293-1967 (amino acids 431-655) of the full-length MVEV (GenBank accession No. NC000943), corresponding to nucleotide 319-993 (amino acid 107-331) of the E gene. The amplification was carried out using the forward primer MVE-E1269F and the reverse primer MVE-E1990R (Table 2.1).

For the amplification of the full-length pre-membrane (prM) and E genes (prM-E), three overlapping sets of primers were used. The first set contained the forward primer prM-E-368F and the reverse primer prM-E-1086R; the second set consisted of the forward primer prM-E-991F and the reverse primer prM-1795R; and the third set comprised of the forward primer prM-E-1691F and the reverse primer prM-E-2514R (Table 2.1).

A 678bp region corresponding to 279bp of the 3' end of the NS5 gene and the first 399bp of the 3'UTR of the full-length MVEV (GenBank accession number: NC000943) was amplified using flavivirus universal primers EMF1 and VD8 (Pierre et al., 1994). Table 2.1 presents the details of all oligonucleotide primers used in this chapter.

All PCR amplifications were carried out using 1µl of the cDNA, 8µM of the primers and 22µl of the PCR SuperMix (Life Technologies, Australia), as per manufacturer's

Table 2.1. *Details of oligonucleotide primers used in this study.*

Primers	Region *	Size	Sequence (5'→3')	References
prM-E-368F	C	24	GATTGATGTGGTGAACAAAAGGGG	(Williams et al., 2013)
prM-E-1086R	E	21	GTTTRTCAGCRGCCATGATGG	(Williams et al., 2013)
prM-E-991F	E	22	GYAGCCGTGAYTTYATTGAAGG	(Williams et al., 2013)
prM-1795R	E	25	GHRAACTCGACTGGTAYGGCTCCAG	(Williams et al., 2013)
prM-E-1691F	E	21	GGAGTTTGAAGAGCCACATG	(Williams et al., 2013)
prM-E-2514R	NS1	20	TRAGCTCYCTYCTGGTGATG	(Williams et al., 2013)
MVE-E1269F	E	19	GCTGGGGYAAYGGATGTGG	This study
MVE-E1990R	E	24	GATATTGGRATTTTGCATGGTCCA	This study
EMF1	NS5	21	TGGATGACSACKGARGAYATG	(Pierre et al., 1994)
VD8	3'UTR	20	GGGTCTCCTCTAACCTCTAG	(Pierre et al., 1994)

*** C:** Capsid gene; **E:** Envelope gene; **NS1:** NS1 gene; **NS5:** NS5 gene; **3'UTR:** 3' untranslated region.

protocol. The reactions were performed using the following thermocycling conditions: a denaturation step at 94°C for 2 min; followed by 35 cycles of 94°C for 30 sec; annealing at 56°C for 30 sec; and elongation at 72°C for 1 min, plus a final extension at 72°C for 10 min. The amplification was carried out in thin-walled 0.2ml PCR tubes using an Eppendorf AG thermal cycler. PCR products were visualised on a 2% w/v agarose gel stained with ethidium bromide. The RT-PCR products were then purified using the QIAquick® PCR purification kit (QIAGEN, Australia), according to the manufacturer's protocol.

2.2.3 Sequencing

The purified PCR products were sequenced using Big Dye version 3.1 chemistry and a 3730xl DNA Analyser with a 96-capillary array (Applied Biosystems, Australia). The sequencing was carried out at the Australian Genome Research Facility (AGRF) in Perth.

2.2.4 Sequence Analysis and Multiple Alignment

Sequences were edited using BioEdit 7.2.0 Sequence Alignment Editor software (Hall, 1999). The sequences of all isolates in this study were aligned with the reference sequences of MVEV strains previously isolated and sequenced from Australia and PNG [Table 2.2; (Johansen et al., 2007; Williams et al., 2013)]. Multiple alignments of the nucleotide and deduced amino acid sequences were performed using ClustalW in MEGA 5.2.1 (Tamura et al., 2011). The NS5-3'UTR sequences obtained in this study were aligned with the reference sequences of MVEV and other closely-related flaviviruses such as ALFV, JEV and WNV (Table 2.3). The nucleotide sequences obtained in this study were deposited in the GenBank database.

2.2.5 Phylogenetic Analyses

The phylogenetic analyses in this study were performed using MEGA 5.2.1 software. Initially, two different methods were employed to construct phylogenetic trees for these sequences: maximum likelihood (ML) and neighbour joining (NJ). To construct the ML tree, all sequences were subject to a model test in MEGA 5.2.1 to find the

Table 2.2. *Details of Murray Valley encephalitis sequences used as reference strains in the analyses of E and prM genes in this study.*

Isolate	Year of isolation	Location of isolation *	Species of origin †	GenBank accession No.
MVE-1-51	1951	Mooroopna, VIC	Human brain	NC000943
NG156	1956	Brown River, Central Province, PNG	Human brain	JN119801
MK6684	1966	Maprik, East Sepik Province, PNG	Mixed Culicines	JN119800
T69	1969	Northern Australia	Human brain	JN119813
OR2	1972	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119805
OR155	1973	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119803
OR156	1973	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119804
TC123130	1974	Culgoa, VIC	Human brain	JN119814
OR1109	1977	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119802
PH491	1981	Newman, E Pilbara, WA	*Cx. annulirostris*	JN119810
K109	1986	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119770
K5686	1989	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119784
K6454	1991	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119788
K6521	1991	Kununurra, NE Kimberley, WA	*An. bancroftii*	JN119789
K16963	1994	Billiluna, SE Kimberley, WA	*Cx. annulirostris*	JN119773
K21413	1995	Kununurra, NE Kimberley, WA	*Cx. pullus*	JN119774
18403C	1996	Mitchell River, Cape York Peninsula, QLD	*Cx. annulirostris*	JN119762
PNG6910	1998	Balimo, Western Province, PNG	*Cx. sitiens* group	JN119812
PNG6523	1998	Balimo, Western Province, PNG	*Cx. sitiens* group	JN119811
CY1189	1999	Pormpuraaw, Cape York Peninsula, QLD	*Cx. sitiens* group	JN119769
K36687	1999	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119776
K41994	2000	Billiluna, SE Kimberley, WA	*Cx. annulirostris*	JN119777
GU0019	2000	Normanton, QLD	*Cx. annulirostris*	JN119765
K47457	2001	Derby, NW Kimberley, WA	*Cx. annulirostris*	JN119778
K50609	2003	Billiluna, SE Kimberley, WA	*Ae. normanensis*	JN119782
K66339	2008	Kununurra, NE Kimberley, WA	*Cx. annulirostris*	JN119790
K67517	2008	Willare, NW Kimberley, WA	*Cx. annulirostris*	JN119791
K67812	2008	Broome, W Kimberley, WA	*Cx. annulirostris*	JN119792
611W/WA/08	2008	Kununurra, NE Kimberley, WA	Human brain	JN119767
08-154300	2008	Monto, QLD	Horse brain	JN119766
145694	2008	Leeton, NSW	*Cx. annulirostris*	JN119755
145507	2008	Griffith, NSW	*Cx. annulirostris*	JN119759
145649	2008	Griffith, NSW	*Cx. annulirostris*	JN119758
145705	2008	Leeton, NSW	*Cx. annulirostris*	JN119760
V11-10	2011	Callawadda, VIC	Horse brain	JX123032

*** NSW:** New South Wales; **VIC:** Victoria; **QLD:** Queensland; **WA:** Western Australia; **PNG:** Papua New Guinea; **E:** east; **W:** west; **NE:** North-east; **NW:** northwest; **SE:** southeast

† Cx: *Culex*; **An:** *Anopheles*; **Ae:** *Aedes*

Table 2.3. *Details of flavivirus sequences used in the analysis of a highly variable region of the 3'UTR in this study.*

Isolate	Year of isolation	Location of isolation	Species of origin	GenBank accession No.
MVEV MVE-1-51	1951	Australia	Human	NC000943
MVEV OR156	1973	Australia	Mosquito	L48976
MVEV MK6684	1966	PNG	Mosquito	L48974
MVEV NG156	1956	PNG	Human	L48975
ALFV K7827	1991	Australia	Mosquito	L48965
ALFV MRM3929	1966	Australia	Pheasant	L48966
JEV K94P05	1994	Korea	Mosquito	AF045551
JEV FU	1995	Australia	Human	L48968
JEV Nakayama	1935	Japan	Human	EF571853
JEV JKT6468	1981	Indonesia	Mosquito	AY184212
JEV Muar	1952	Malaysia	Human	HM596272
WNV NY99	1999	USA	Human	AF196835
KUNV MRM61C	1960	Australia	Mosquito	L48978
WNV Sarafend	unknown	unknown	unknown	L48977
WNV Rabensburg	1997	Czech Republic	Mosquito	AY765264
WNV LEIV-Krnd88	1998	Russia	Tick	AY277251
KUNV Sarawak	1966	Borneo	Mosquito	L49311

best-fit substitution model. To construct an ML tree for the 106 partial E gene sequences included in this study, the Kimura-2 model of evolution with the proportion of invariable sites (K2+I) was found to be the best model for the data, and was therefore used. For the NJ tree, the suitability of the data was first determined in MEGA 5.2.1 by calculating the average pairwise Jukes-Cantor (JC) distance of all sequences included in the study. According to Nei and Kumar (2000), if the average pairwise JC distance is <1.0, the data is suitable for the NJ tree. For the sequences of the partial E gene, the average JC distance between the sequences was 0.059, which indicated that the data was suitable for constructing the NJ tree. To construct the NJ tree, the Maximum Composite Likelihood model was applied, as recommended by Hall (2011).

For the full-length prM-E sequences, only an ML tree was constructed. Fifty three sequences first underwent a model test as described above. Consequently, the Tamura-Nei model with gamma distribution with invariant sites (TN93+G+I) was used. The robustness of all trees constructed was assessed by applying a bootstrapped support analysis of 1000 replicates. In this study, two criteria were used to determine whether a group of sequences formed a distinct lineage: the visual inspection of the tree and a percentage cut-off >4% difference.

A nucleotide difference of <92% was used as a cut-off value to determine different genotypes. Within each genotype, sequences with a ≥95.5% nucleotide identity were considered the same lineage. The cut-off value was determined based on the visual inspection of the tree and the nucleotide identity between different strains belonging to different clades/sub-clades.

2.2.6 Assessment of Selection Pressures

The operation of selection pressure was tested on the complete 53 prM-E sequences of MVEV isolates representing all four genotypes. To detect adaptive evolution in these sequences, the hypotheses of positive selection, purifying selection and neutral selection were tested using a codon-based Z-test of selection in MEGA 5.2.1. Z-test analyses were performed averaging over all as well as between sequence pairs. Z values (dN – dS) were estimated using the Nei-Gojobori

(proportion) model, applying 1000 bootstrap replicates. Probability values of $p<0.05$ were considered significant. In addition, to find codons encoded by the prM-E genes that had been under selection pressure, at each codon level, a HyPhy analysis (Pond et al., 2005) was performed in MEGA 5.2.1. The test statistic dN - dS was calculated using an ML method and the Tamura-Nei model.

2.2.7 Three Dimensional Modelling

The full-length E amino acid sequences of representative G1 and G2 isolates were aligned with the E amino acid sequences of closely-related flaviviruses, including JEV (Luca et al., 2012), WNV (Nybakken et al., 2006), DENV (Zhang et al., 2004) and TBEV (Rey et al., 1995), for which crystal structures have previously been solved. Furthermore, the non-conservative amino acid substitutions of the G2 consensus sequence were mapped on the three dimensional model of WNV [PDB accession number: 2HG0; (Nybakken et al., 2006)] using the Cn3D 4.3 software (National Center for Biotechnology Information, 2011).

2.3 Results

2.3.1 RT-PCR and Sequencing

RT-PCR and sequencing was performed to compare the genetic diversity between MVEV isolates that were obtained from mosquitoes collected from WA between 2005 and 2009. Of the eighty three isolates included in this study, twenty were not amplified by the initial RT-PCR and underwent a re-isolation process in cell culture (Fig. 2.1). Eight of these isolates were re-isolated and were subject to the RT-PCR and sequencing. Therefore, seventy one MVEV isolates that were obtained between 2005 and 2009 were used for sequence analyses (Table 2.4). Of these, fifty nine (83%) were isolated from *Cx. annulirostris*, eight (11.3%) from *Cx. pullus* and four (5.6%) from other mosquito species, such as *Ae.* (Macleaya) species, *An. amictus* and *Ae. normanensis*.

2.3.2 Phylogenetic Analyses

2.3.2.1 Partial E Gene

Sequencing of nucleotide 378 to 1050 of the MVEV E gene has been used previously for phylogenetic analyses of MVEV strains (Johansen et al., 2007), and was also employed in this study (Table 2.4). Both ML and NJ methods produced trees of similar topology, with minor differences observed in the order of the terminal nodes and in bootstrap support. Four genotypes were clearly identified. The recent isolates from WA formed two distinct lineages (G1 and G2). Furthermore, two separate lineages for G1 (designated G1a and G1b) were evident.

Four new G2 isolates were identified (Fig. 2.2C, Appendix 2.1C). Three of these were from mosquitoes collected at Fitzroy Crossing (2006 and 2009) and one from Broome (2006). This is the first time that a G2 strain has been detected outside Kununurra in the northeast Kimberley region of WA. Three recent G2 isolates were isolated from *Cx. annulirostris* and one from *Cx. pullus*. These strains formed a subclade within the G2 lineage. G2 comprises this subclade, as well as a cluster of five distinct isolates from 1973 to 1995. The partial E sequences of G2 isolates shared at least 95.5% nucleotide identity with each other and 99.1% amino acid similarity. G2 isolates were more closely related to G3 and G4 viruses than those of G1 (Table 2.5). There was a maximum of 91.8% nucleotide identity and 99.1% amino acid similarity between isolates of G2, G3 and G4. Whereas, there was a maximum of 86.8% nucleotide identity and 95.1% amino acid similarity between the isolates of G1 and G2 (Table 2.5).

G1 was further classified into two separate lineages: G1a and G1b (Fig. 2.2 and Appendix 2.1). G1a included strains from 2008 and 2009, as well as the two recent PNG strains from 1998 [PNG6910 and PNG6523; (Johansen et al., 2007)]. The Australian strains within G1a are exclusively from the Kimberley and Pilbara regions of WA. Within the 675bp region of the E gene (partial), G1a isolates exhibited a high level of genetic variation with a minimum of 96.0% nucleotide identity, translating to at least 97.7% amino acid similarity (Table 2.5). In contrast, G1b comprised

Table 2.4. *Details of Murray Valley encephalitis virus isolates collected between 2005 and 2009 from different regions of Western Australia and used in this study.*

Isolate	Year of isolation	Location of isolation	Species of origin	GenBank accession No.		
				Partial E	prM-E	NS5-3'UTR
K56445	2005	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867202	JN119783	ND
K59532	2006	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	JX867132	KC206092	JX867211
K59536	2006	Fitzroy Crossing (W Kimberley)	*Cx. pullus*	JX867133	KC206093	JX867212
K62017	2006	Broome (W Kimberley)	*Cx. annulirostris*	JX867134	KC206094	JX867213
K61375	2006	Derby (W Kimberley)	*Cx. annulirostris*	JX867191	ND	ND
K60555	2006	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867192	ND	ND
K59582	2006	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	JX867193	ND	ND
K61299	2006	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867194	ND	ND
K60748	2006	Wyndham (NE Kimberley)	*Cx. annulirostris*	JX867195	ND	ND
K61396	2006	Derby (W Kimberley)	*Cx. annulirostris*	JX867196	ND	ND
K60119	2006	Halls Creek (SE Kimberley)	*Ae. (Macleaya)* sp.	JX867197	JN119785	ND
K60365	2006	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867198	JN119786	ND
P8891	2006	Karratha (W Pilbara)	*Ae. normanensis*	JX867199	ND	JX867210
P8400	2006	Newman (E Pilbara)	*An. amictus*	JX867200	ND	ND
P8372	2006	Newman (E Pilbara)	*Cx. annulirostris*	JX867201	JN119807	ND
K62899	2007	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867190	JN119787	ND
K67259	2008	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867166	ND	JX867209
K67317	2008	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867167	ND	ND
K67238	2008	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867168	ND	ND
K66298	2008	Kununurra (NE Kimberley)	*Cx. pullus*	JX867187	ND	ND
K67234	2008	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867188	ND	ND
K67235	2008	Parry's Lagoon (NE Kimberley)	*Cx. annulirostris*	JX867189	ND	ND
K68196	2009	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	JX867135	KC206095	JX867214
P9771	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867136	ND	ND
P9781	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867137	ND	ND
P9929	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867138	ND	ND
P9904	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867139	ND	ND
P9783	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867140	ND	ND
P9937	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867141	ND	ND
P9831	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867142	ND	ND
P9833	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867143	ND	ND
P9883	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867144	ND	ND
P9943	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867145	ND	ND
P9765	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867146	ND	ND
P9978	2009	Newman (E Pilbara)	*Cx.* sp. (damaged)	JX867147	ND	ND
P9808	2009	Newman (E Pilbara)	*Cx.* sp. (damaged)	JX867148	ND	ND
P9950	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867149	ND	ND

Table 2.4. ***continued***

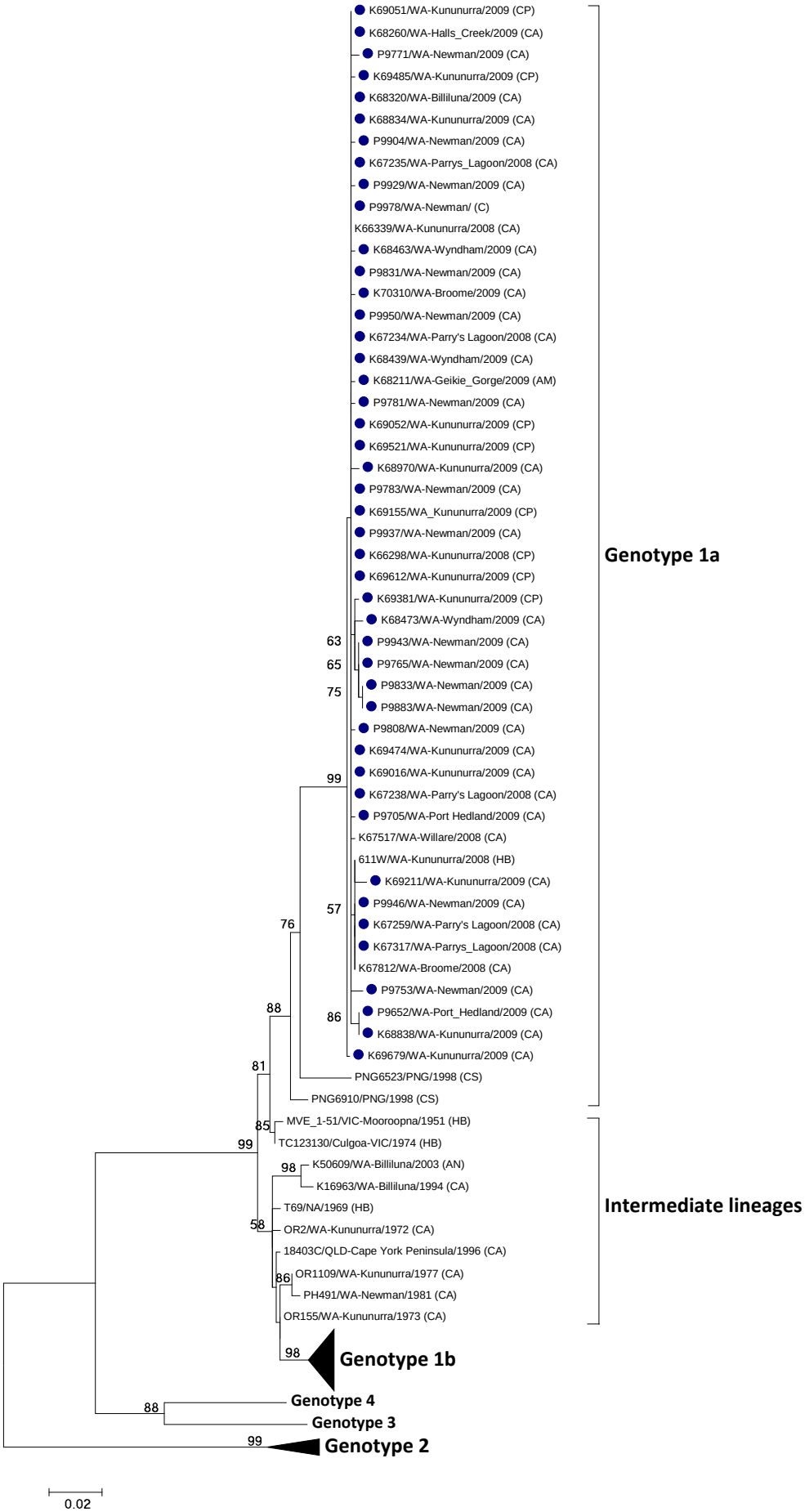
Isolate	Year of Isolation	Location of isolation *	Species of origin †	GenBank accession No. ‡		
				Partial E	prM-E	NS5-3'UTR
P9705	2009	Port Hedland (W Kimberley)	*Cx. annulirostris*	JX867150	ND	ND
P9946	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867151	ND	ND
P9753	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867152	ND	ND
P9652	2009	Port Hedland (W Kimberley)	*Cx. annulirostris*	JX867153	ND	ND
P9990	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867154	JN119809	ND
P9777	2009	Newman (E Pilbara)	*Cx.* sp. (damaged)	JX867155	ND	JX867203
P9986	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867156	ND	JX867204
P9754	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867157	ND	ND
P9901	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867158	ND	ND
P9749	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867159	ND	ND
P9862	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867160	JN119808	ND
P9992	2009	Newman (E Pilbara)	*Cx. annulirostris*	JX867161	ND	ND
K68834	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867162	ND	ND
K69679	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867163	ND	ND
K69211	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867164	ND	ND
K68150	2009	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	JX867165	JN119793	JX867205
K70310	2009	Broome (W Kimberley)	*Cx. annulirostris*	JX867169	JN119799	JX867206
K68439	2009	Wyndham (NE Kimberley)	*Cx. annulirostris*	JX867170	ND	ND
K68320	2009	Billiluna (SE Kimberley)	*Cx. annulirostris*	JX867171	JN119795	JX867208
K69016	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867172	ND	ND
K68260	2009	Halls Creek (E Kimberley)	*Cx. annulirostris*	JX867173	ND	ND
K68838	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867174	JN119797	ND
K68463	2009	Wyndham (NE Kimberley)	*Cx. annulirostris*	JX867175	ND	ND
K68473	2009	Wyndham (NE Kimberley)	*Cx. annulirostris*	JX867176	JN119796	ND
K68474	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867177	ND	ND
K68970	2009	Kununurra (NE Kimberley)	*Cx. annulirostris*	JX867178	ND	ND
K69485	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867179	JN119798	ND
K69155	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867180	ND	ND
K69051	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867181	ND	ND
K69052	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867182	ND	ND
K69521	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867183	ND	ND
K69381	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867184	ND	ND
K69612	2009	Kununurra (NE Kimberley)	*Cx. pullus*	JX867185	ND	JX867207
K68211	2009	Geikie Gorge (W Kimberley)	*Ae. (Macleaya)* sp.	JX867186	JN119794	ND

*** E:** east; **NE:** North-east; **SE:** Southeast; **W:** west;

† Cx: *Culex*; **An:** *Anopheles*; **Ae:** *Aedes;* **sp***.* species

‡ Partial E: partial envelope gene; **prM-E:** full-length pre-membrane and envelope genes; **NS5-3'UTR:** the 3' end of the NS5 gene with the 5' end of 3'UTR; **ND:** Not determined.

A.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18403C/QLD-Cape York Peninsula/1996 (CA)
OR1109/WA-Kununurra/1977 (CA)
PH491/WA-Newman/1981 (CA)
OR155/WA-Kununurra/1973 (CA)
Genotype 1a
Intermediate lineages
Genotype 1b
Genotype 4
Genotype 3
Genotype 2
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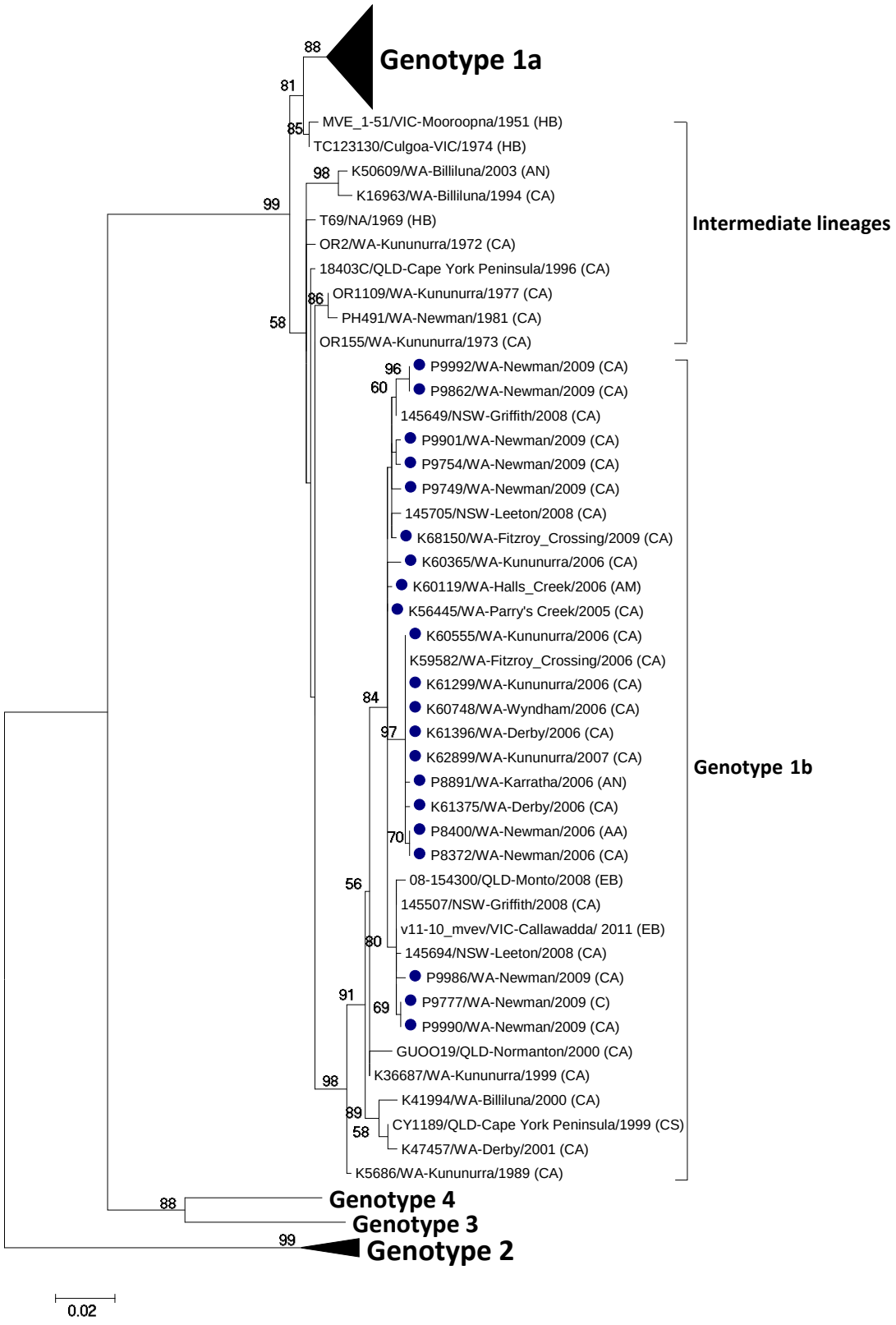
B.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TC123130/Culgoa-VIC/1974 (HB)
K50609/WA-Billiluna/2003 (AN)
K16963/WA-Billiluna/1994 (CA)
T69/NA/1969 (HB)
OR2/WA-Kununurra/1972 (CA)
18403C/QLD-Cape York Peninsula/1996 (CA)
OR1109/WA-Kununurra/1977 (CA)
PH491/WA-Newman/1981 (CA)
OR155/WA-Kununurra/1973 (CA)
Intermediate lineages
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P9862/WA-Newman/2009 (CA)
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P9754/WA-Newman/2009 (CA)
P9749/WA-Newman/2009 (CA)
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Genotype 1b
Genotype 4
Genotype 3
Genotype 2
0.02

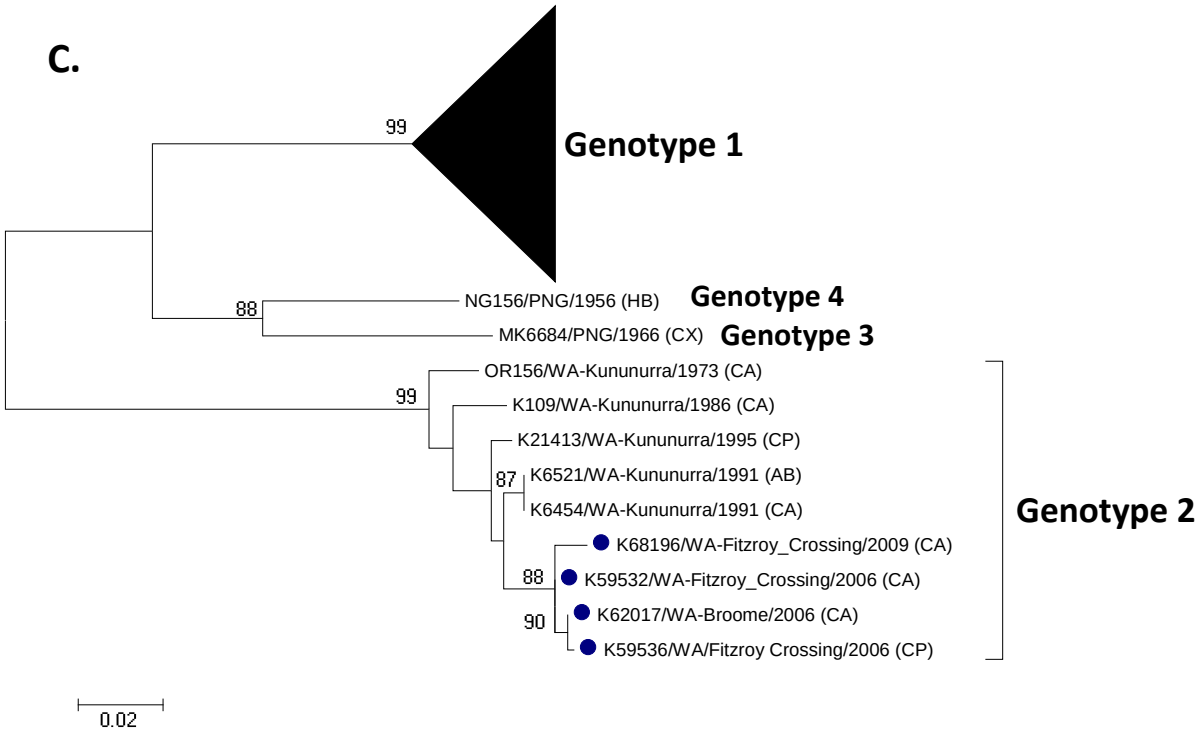


Fig. 2.2. *Maximum likelihood phylogenetic tree constructed using partial E gene sequences.*

Trees are constructed using nucleotide 319-993 of the E gene sequences. Four genotypes are clearly identified (Genotype 1 to Genotype 4). Genotype 1 is further classified as genotype 1a and 1b. Genotypes 1b, 2, 3 and 4 are collapsed in **(A)**. Genotypes 1a, 2, 3 and 4 are collapsed in **(B)**. Genotype 1 is collapsed in **(C)**. Numbers above the internal nodes are the bootstrap support values from 1000 replicates. The scale represents 0.02 substitutions per nucleotide site. **WA:** Western Australia; **NSW:** New South Wales; **VIC:** Victoria; **QLD:** Queensland; **PNG:** Papua New Guinea; **NA:** Northern Australia; **CA:** *Cx. annulirostris*; **CP:** *Cx. pullus*; **CS:** *Cx. sitiens* group; **CX:** Mixed culicine; **C:** Culex species (damaged); **AM:** *Aedes* (Macleaya) species; **AN:** *Aedes normanensis*; **AB:** *Anopheles bancroftii*; **AA:** *Anopheles amictus*; **HB:** Human Brain; **EB:** Equine brain;

● indicates the isolates that were sequenced in this study.

Table 2.5. *Nucleotide and deduced amino acid identities within and between partial E gene sequences of genotypes of Murray Valley encephalitis virus.*

Genotypes	**Nucleotide identity %**	**Amino acid identity %**
Within G1a	96.0-100	97.7-100
Within G1b	97.4-100	98.2-100
Within G1	92.6-95.4	96.0-98.6
Within G2	95.5-99.8	99.1-100
G1 vs G2	84.3-86.8	92.4-95.1
G1 vs G3 and G4	87.5-90.1	94.6-97.3
G2 vs G3 and G4	86.2-91.8	94.6-99.1
Within all MVEV strains	84.1-100	92.4-100

isolates from 1989-2011 from WA, NSW, VIC and QLD. When the partial E sequences were compared, G1b isolates showed a slightly lower level of variation compared to G1a, with at least 97.4% nucleotide identity translating to a minimum of 98.2% amino acid similarity (Table 2.5).

2.3.2.2 Full-Length prM-E Genes

Previous studies have used the full-length prM-E genes to better characterise the phylogenetic relationships in flaviviruses, such as MVEV (Williams et al., 2013), JEV (Williams et al., 2000), and WNV (Blitvich et al., 2004). To confirm and expand the partial E gene phylogeny, full-length prM-E genes of representative strains of G1a, G1b and G2 from different regions, dates and sources of isolation, were sequenced and aligned with reference strains of MVEV (Table 2.2) to build an ML tree.

Similar tree topology and groupings to the partial E gene ML tree (Figure 2.2) were observed using the full-length prM-E sequences (Appendix 2.2). However, contrary to the partial E gene analyses, when the level of variation between different genotypes was examined for the full-length prM-E sequences, G1a demonstrated slightly higher variation than G1b (Appendix 2.3). G1a isolates displayed a minimum of 97.5% nucleotide identity, translating to at least 99.7% amino acid similarity. G1b demonstrated at least 98.0% nucleotide identity translating to a minimum of 99.1% amino acid similarity (Appendix 2.3). Moreover, similar to partial E gene analysis, G2 strains were more closely related to the G3 and G4 strains when the full-length prM-E sequences were compared (Appendix 2.3). There was a maximum of 88.0% nucleotide identity translating to 96.8% amino acid similarity between the isolates of G2, G3, and G4. Whilst a maximum of 87.5% nucleotide identity and 96.7% amino acid similarity was observed between the isolates of G1 and G2. The isolates of G1, G3 and G4 shared a maximum of 90.6% nucleotide and 98.9% amino acid similarity, indicating that G1 isolates are also more closely related to G3 and G4 PNG strains instead of the co-circulating strains of G2 (Appendix 2.3).

Nonetheless, the differences observed in the level of variation within and between different genotypes of MVEV were not so significant as to alter the tree topography.

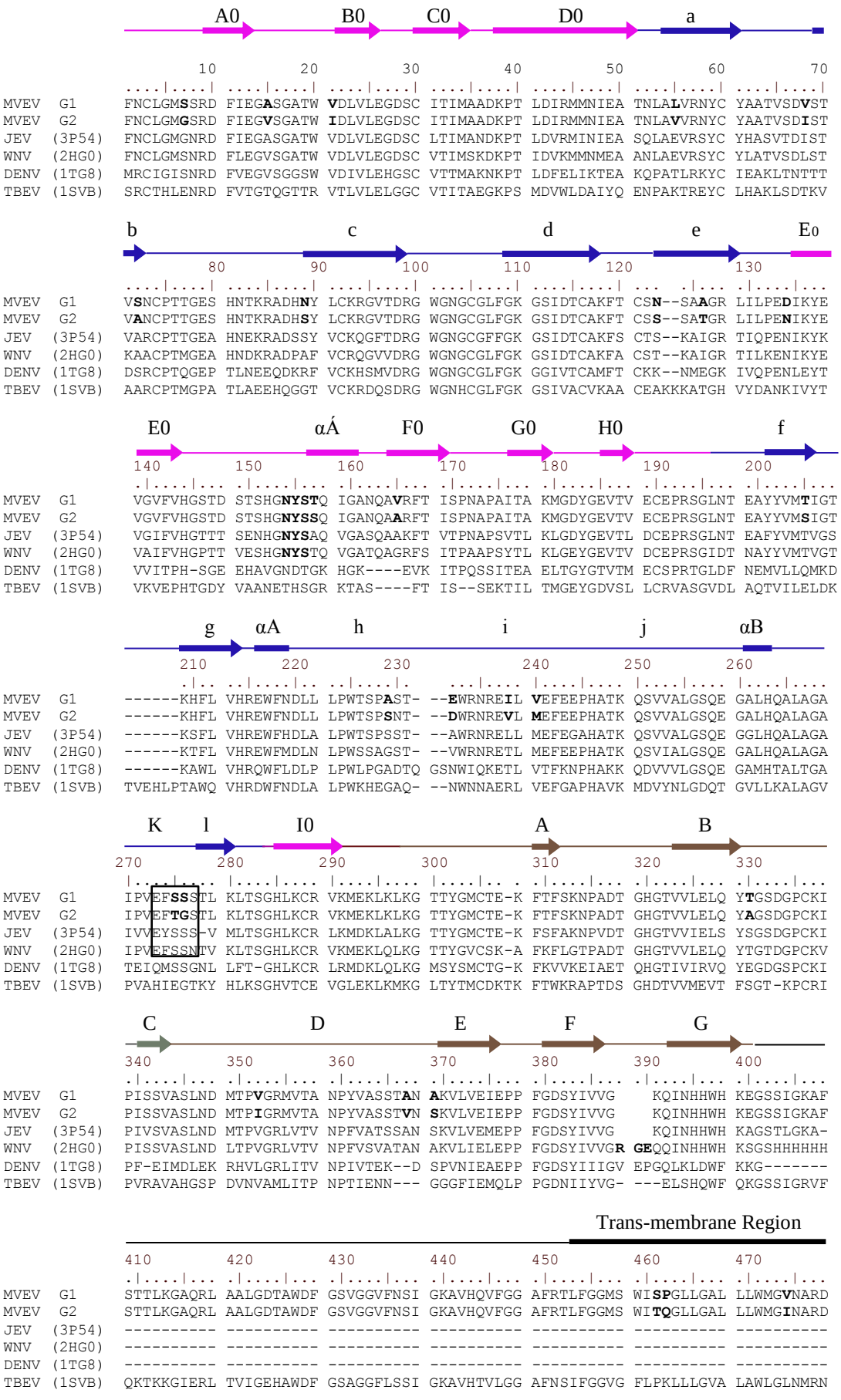
Hence, for the purpose of phylogenetic analyses, the partial E gene sequences provided adequate information to build the trees.

2.3.3 Three Dimensional Modelling

Multiple alignments of the prM-E amino acid sequences of G1 and G2 strains were performed to identify biologically significant substitutions or differences. Since no G3 and G4 strains were detected in this study, these genotypes were not included in the three dimensional analysis. Alignment of the prM amino acid sequences revealed a high level of conservation in this protein. Only a single amino acid substitution was observed for strain K21413 ($T^{98}I$) that resulted from the $C^{293}T$ mutation in the nucleotide sequence (data not shown). However, many silent nucleotide mutations were observed in the prM gene of the G2 that did not affect the deduced amino acid sequence. The multiple alignment of the E protein revealed that there were twenty six unique amino acid substitutions between G1 and G2 isolates, of which fifteen were non-conservative (Table 2.6). All cysteine residues, the putative fusion peptide, the glycosylation site, and the receptor binding RGD motif were completely conserved in MVEV isolates (Fig. 2.3).

No three dimensional (3D) structure of MVEV has yet been solved. However, the 3D crystal structures of the E glycoprotein of JEV (Luca et al., 2012), WNV (Nybakken et al., 2006), DENV (Zhang et al., 2004) and TBEV (Rey et al., 1995) have been previously described. In order to locate the observed mutations in the G2 strains of MVEV on the 3D structure of the E protein of flaviviruses, a multiple alignment of the consensus sequences of the E proteins of G1 and G2 of MVEV was carried out with reference sequences of JEV, WNV, DENV and TBEV. Of the twenty six unique substitutions in the E protein between G1 and G2, thirteen are located in domain II, six in domain I and four in domain III. The remaining three mutations are located in the trans-membrane region, which is not part of the soluble structure of the E glycoprotein (Fig. 2.3).

Since MVEV is genetically closely-related to WNV, the fourteen non-conservative amino acid substitutions were mapped on the 3D structure of the E glycoprotein of WNV [Fig. 2.4; (Nybakken et al., 2006)]. Four of these were located in domain II,




```
                    10        20        30        40        50        60        70
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
MVEV  G1      FNCLGMSSRD FIEGASGATW VDLVLEGDSC ITIMAADKPT LDIRMMNIEA TNLALVRNYC YAATVSDVST
MVEV  G2      FNCLGMGSRD FIEGVSGATW IDLVLEGDSC ITIMAADKPT LDIRMMNIEA TNLAVVRNYC YAATVSDIST
JEV   (3P54)  FNCLGMGNRD FIEGASGATW VDLVLEGDSC LTIMANDKPT LDVRMINIEA SQLAEVRSYC YHASVTDIST
WNV   (2HG0)  FNCLGMSNRD FLEGVSGATW VDLVLEGDSC VTIMSKDKPT IDVKMMNMEA ANLAEVRSYC YLATVSDLST
DENV  (1TG8)  MRCIGISNRD FVEGVSGGSW VDIVLEHGSC VTTMAKNKPT LDFELIKTEA KQPATLRKYC IEAKLTNTTT
TBEV  (1SVB)  SRCTHLENRD FVTGTQGTTR VTLVLELGGC VTITAEGKPS MDVWLDAIYQ ENPAKTREYC LHAKLSDTKV

                    80        90        100       110       120       130
                    ....|....| ....|....| ....|....| ....|....| ....|....| ...  .|... .|....|...
MVEV  G1      VSNCPTTGES HNTKRADHNY LCKRGVTDRG WGNGCGLFGK GSIDTCAKFT CSN--SAAGR LILPEDIKYE
MVEV  G2      VANCPTTGES HNTKRADHSY LCKRGVTDRG WGNGCGLFGK GSIDTCAKFT CSS--SATGR LILPENIKYE
JEV   (3P54)  VARCPTTGEA HNEKRADSSY VCKQGFTDRG WGNGCGFFGK GSIDTCAKFS CTS--KAIGR TIQPENIKYK
WNV   (2HG0)  KAACPTMGEA HNDKRADPAF VCRQGVVDRG WGNGCGLFGK GSIDTCAKFA CST--KAIGR TILKENIKYE
DENV  (1TG8)  DSRCPTQGEP TLNEEQDKRF VCKHSMVDRG WGNGCGLFGK GGIVTCAMFT CKK--NMEGK IVQPENLEYT
TBEV  (1SVB)  AARCPTMGPA TLAEEHQGGT VCKRDQSDRG WGNHCGLFGK GSIVACVKAA CEAKKKATGH VYDANKIVYT

                    140       150       160       170       180       190       200
                    .|....|... .|....|... .|....|... .|....|... .|....|... .|....|... .|....|...
MVEV  G1      VGVFVHGSTD STSHGNYSTQ IGANQAVRFT ISPNAPAITA KMGDYGEVTV ECEPRSGLNT EAYYVMTIGT
MVEV  G2      VGVFVHGSTD STSHGNYSSQ IGANQAARFT ISPNAPAITA KMGDYGEVTV ECEPRSGLNT EAYYVMSIGT
JEV   (3P54)  VGIFVHGTTT SENHGNYSAQ VGASQAAKFT VTPNAPSVTL KLGDYGEVTL DCEPRSGLNT EAFYVMTVGS
WNV   (2HG0)  VAIFVHGPTT VESHGNYSTQ VGATQAGRFS ITPAAPSYTL KLGEYGEVTV DCEPRSGIDT NAYYVMTVGT
DENV  (1TG8)  VVITPH-SGE EHAVGNDTGK HGK----EVK ITPQSSITEA ELTGYGTVTM ECSPRTGLDF NEMVLLQMKD
TBEV  (1SVB)  VKVEPHTGDY VAANETHSGR KTAS----FT IS--SEKTIL TMGEYGDVSL LCRVASGVDL AQTVILELDK

                    210       220       230       240       250       260
                    .|.. ..|....|.. ..|....|.    ...|.... |....|.... |....|.... |....|....
MVEV  G1      ------KHFL VHREWFNDLL LPWTSPAST- --EWRNREIL VEFEEPHATK QSVVALGSQE GALHQALAGA
MVEV  G2      ------KHFL VHREWFNDLL LPWTSPSNT- --DWRNREVL MEFEEPHATK QSVVALGSQE GALHQALAGA
JEV   (3P54)  ------KSFL VHREWFHDLA LPWTSPSST- --AWRNRELL MEFEGAHATK QSVVALGSQE GGLHQALAGA
WNV   (2HG0)  ------KTFL VHREWFMDLN LPWSSAGST- --VWRNRETL MEFEEPHATK QSVIALGSQE GALHQALAGA
DENV  (1TG8)  ------KAWL VHRQWFLDLP LPWLPGADTQ GSNWIQKETL VTFKNPHAKK QDVVVLGSQE GAMHTALTGA
TBEV  (1SVB)  TVEHLPTAWQ VHRDWFNDLA LPWKHEGAQ- --NWNNAERL VEFGAPHAVK MDVYNLGDQT GVLLKALAGV

                    270       280       290       300       310       320       330
                    |....|.... |....|.... |....|.... |....|.. .|....|... .|....|... .|....|...
MVEV  G1      IPVEFSSSTL KLTSGHLKCR VKMEKLKLKG TTYGMCTE-K FTFSKNPADT GHGTVVLELQ YTGSDGPCKI
MVEV  G2      IPVEFTGSTL KLTSGHLKCR VKMEKLKLKG TTYGMCTE-K FTFSKNPADT GHGTVVLELQ YAGSDGPCKI
JEV   (3P54)  IVVEYSSS-V MLTSGHLKCR LKMDKLALKG TTYGMCTE-K FSFAKNPVDT GHGTVVIELS YSGSDGPCKI
WNV   (2HG0)  IPVEFSSNTV KLTSGHLKCR VKMEKLQLKG TTYGVCSK-A FKFLGTPADT GHGTVVLELQ YTGTDGPCKV
DENV  (1TG8)  TEIQMSSGNL LFT-GHLKCR LRMDKLQLKG MSYSMCTG-K FKVVKEIAET QHGTIVIRVQ YEGDGSPCKI
TBEV  (1SVB)  PVAHIEGTKY HLKSGHVTCE VGLEKLKMKG LTYTMCDKTK FTWKRAPTDS GHDTVVMEVT FSGT-KPCRI

                    340       350       360       370       380       390       400
                    .|....|... .|....|... .|....|... .|....|... .|....|... .|....|... .|....|...
MVEV  G1      PISSVASLND MTPVGRMVTA NPYVASSTAN AKVLVEIEPP FGDSYIVVG    KQINHHWH KEGSSIGKAF
MVEV  G2      PISSVASLND MTPIGRMVTA NPYVASSTVN SKVLVEIEPP FGDSYIVVG    KQINHHWH KEGSSIGKAF
JEV   (3P54)  PIVSVASLND MTPVGRLVTV NPFVATSSAN SKVLVEMEPP FGDSYIVVG    KQINHHWH KAGSTLGKA-
WNV   (2HG0)  PISSVASLND LTPVGRLVTV NPFVSVATAN AKVLIELEPP FGDSYIVVGR GEQQINHHWH KSGSHHHHHH
DENV  (1TG8)  PF-EIMDLEK RHVLGRLITV NPIVTEK--D SPVNIEAEPP FGDSYIIIGV EPGQLKLDWF KKG-------
TBEV  (1SVB)  PVRAVAHGSP DVNVAMLITP NPTIENN--- GGGFIEMQLP PGDNIIYVG- ---ELSHQWF QKGSSIGRVF

                    410       420       430       440       450       460       470
                    .|....|... .|....|... .|....|... .|....|... .|....|... .|....|... .|....|...
MVEV  G1      STTLKGAQRL AALGDTAWDF GSVGGVFNSI GKAVHQVFGG AFRTLFGGMS WISPGLLGAL LLWMGVNARD
MVEV  G2      STTLKGAQRL AALGDTAWDF GSVGGVFNSI GKAVHQVFGG AFRTLFGGMS WITQGLLGAL LLWMGINARD
JEV   (3P54)  ---------- ---------- ---------- ---------- ---------- ---------- ----------
WNV   (2HG0)  ---------- ---------- ---------- ---------- ---------- ---------- ----------
DENV  (1TG8)  ---------- ---------- ---------- ---------- ---------- ---------- ----------
TBEV  (1SVB)  QKTKKGIERL TVIGEHAWDF GSAGGFLSSI GKAVHTVLGG AFNSIFGGVG FLPKLLLGVA LAWLGLNMRN
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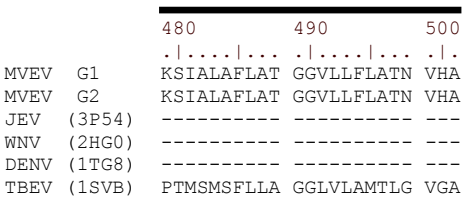


Fig. 2.3. *Multiple alignment of the E protein amino acid consensus sequences of G1 and G2 Murray Valley encephalitis virus (MVEV) with closely related flaviviruses and tick-borne encephalitis virus (TBEV).*

Domains are colour coded as: domain I, dark purple; domain II, dark blue; domain III, grey. The C-terminal region which is not part of the soluble envelope fragment is in black. The trans-membrane region is shown in thick black line. Important amino acids are highlighted as follows: turquoise: conservative substitutions; red: non-conservative substitutions; yellow: glycosylation site; grey: putative fusion peptide; bright green: conserved cysteine residues; gray: conserved histidine residues; blue with white font: conserved RGD motif; Black square indicate putative flexible hinge region between domain I and domain II. Substitution types are categorised as conservative or non-conservative as described previously (Chelikani et al., 2007; Vrati et al., 1999). Assignments of domains are based on the 3D structure of West Nile virus (PDB accession number: 2HG0). Numbers on the top of the alignment indicate the position of amino acid residues based on the MVE-1-51 complete genome (NC000943). Arrows indicate the β-sheets; lines between arrows represent connecting loops. Nomenclatures are indicated above the sheets and loops.

Table 2.6. *Unique amino acid substitutions in the envelope glycoprotein of the G2 compared to the G1 of Murray Valley encephalitis virus. Substitutions are colour coded according to their domain as per figure 2.6.*

Substitutions*	Substitution type†	Location‡
$S^{7}G$	Non-conservative	D I
$A^{15}V$	Conservative	D I, loop Ao-Bo
$V^{21}I$	Non-conservative	D I, loop Ao-Bo
$L^{55}V$	Conservative	D II, sheet a
$V^{68}I$	Non-conservative	D II, loop a-b
$S^{72}A$	Non-conservative	D II, sheet b
$N^{89}S$	Conservative	D II, loop b-c
$N^{123}S$	Conservative	D II, sheet e
$A^{126}T$	Non-conservative	D II, sheet e
$D^{134}N$	Non-conservative	DI1, loop e Eo
$T^{157}S$	Conservative	D I, loop αÁ
$V^{165}A$	Conservative	D I, sheet Fo
$T^{205}S$	Conservative	D II, sheet f
$A^{229}S$	Non-conservative	D II, loop h
$E^{232}D$	Conservative	D II, loop i
$I^{238}V$	Non-conservative	D II, loop i
$V^{240}M$	Non-conservative	D II, loop i
$S^{275}T$	Conservative	D II, loop k
$S^{276}G$	Non-conservative	D II, loop k
$T^{330}A$	Non-conservative	D III, B-C loop
$V^{352}I$	Non-conservative	D III, loop D
$A^{367}V$	Conservative	D III, loop D
$A^{369}S$	Non-conservative	D III, loop D
$S^{461}T$	Conservative	Trans-membrane region
$P^{462}Q$	Non-conservative	Trans-membrane region
$V^{474}I$	Non-conservative	Trans-membrane region

*Amino acid locations are based on MVE-1-51 complete genome (NC000943).

† Substitution types are categorised as conservative or non-conservative as described in Fig. 2.3.

‡ Domain locations are based on West Nile virus envelope crystal structure (PDB accession no. 2HG0).

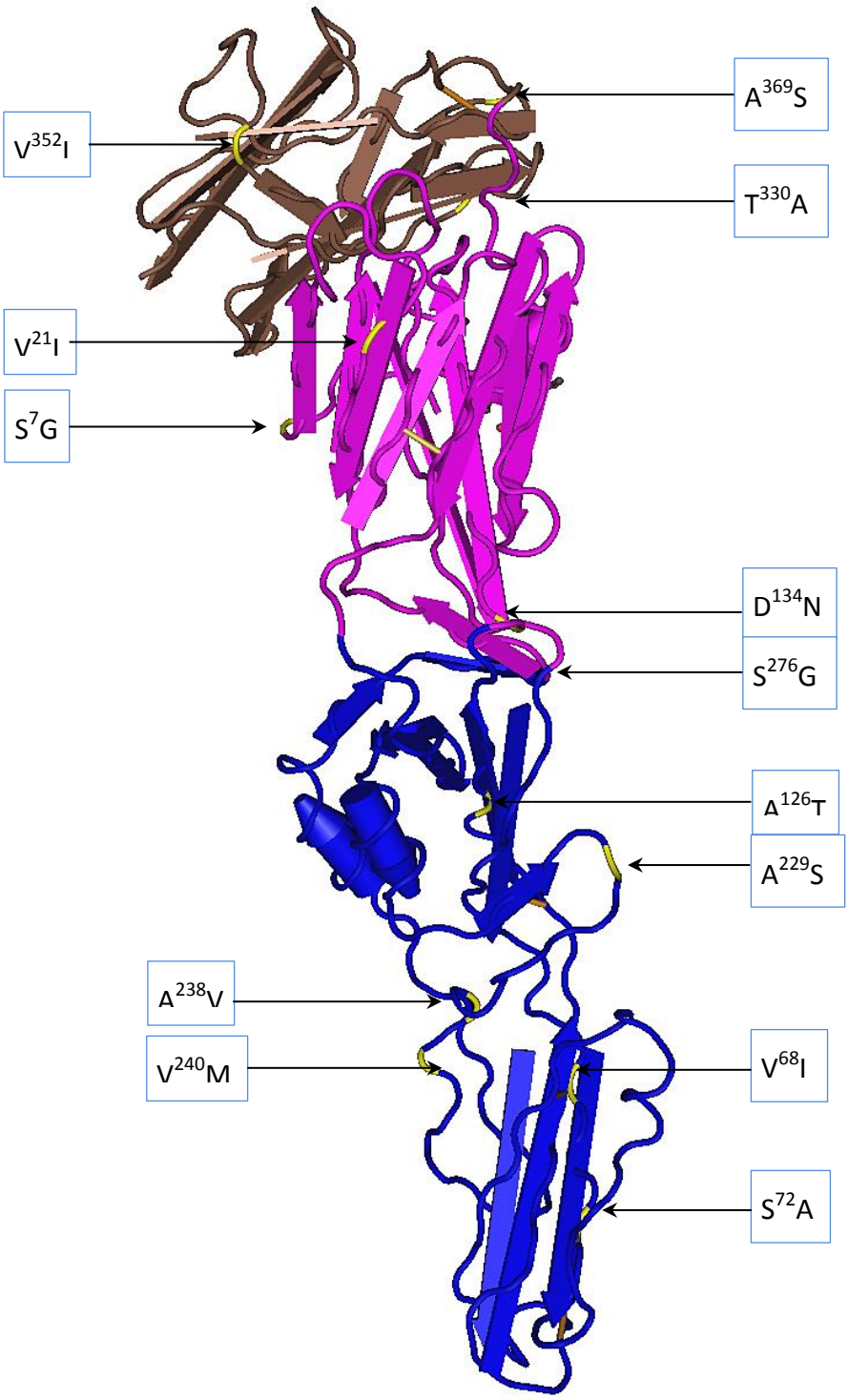


Fig. 2.4. *Diagram showing the predicted 3D folded structure of West Nile virus E protein with homologous unique non-conservative substitutions of the G2 of Murray Valley encephalitis virus highlighted in yellow.*

The diagram is adapted from the closely-related West Nile virus (PDB accession number: 2HG0). Domains are color-coded as per Fig. 2.3. The ball-and-stick structures indicate the carbohydrate on N^{154} which is part of NYS glycosylation site. Ribbons with arrowheads represent the β-sheets. Connecting loops are shown with thin tubes.

suggesting this domain is more variable for MVEV than the other two domains. Significantly, the flexible hinge region (residues 273-277) which connects domain I and domain II (Rey et al., 1995; Zhang et al., 2004) exhibited two mutations: a conservative $S^{275}T$ substitution and a non-conservative $S^{276}G$ substitution (Table 2.6 and Fig. 2.3, Fig. 2.4). Although the glycosylation site (NYS; 154-156) was fully conserved, a conservative substitution ($T^{157}S$) adjacent to this site was detected in the G2 isolates.

2.3.4 Operation of Selection Pressures between Various Genotypes

The operation of positive selection was assessed using either the Z-test or HyPhy. No evidence of positive selection was found using either method. However, there was evidence of neutral or purifying selection in the prM-E genes of MVEV isolates included in this study (Table 2.7).

2.3.5 NS5-3'UTR Sequence Analysis

In order to compare the highly variable polymorphic region at the beginning of the 3'UTR of G1 and G2 strains, a multiple alignment of the first 67 nucleotides of the 3'UTR was carried out along with the same region of ALFV, JEV and WNV (Fig. 2.5). The multiple alignment revealed a unique 18-nucleotide deletion in the 3'UTR of the recent G2 isolates just downstream of the stop codon (Fig. 2.5), which may serve as a genetic marker for the recent G2 strains. A 64-nucleotide deletion in OR156, which is regarded as the prototype strain of G2 of MVEV, has been previously identified (Poindinger et al., 1996). The OR156 deletion pattern closely resembled the deletion in ALFV strains K7827 and MEM3929 that exhibited a 56-nucleotide deletion at a very similar genomic region. Interestingly, one of the G2 isolates (K6521) displays a one-nucleotide insertion at position 10414, which aligns with the same nucleotide in MK6684 (G3 of MVEV), JKT6468 (G4 of JEV) and Rabsenburg (lineage III of WNV).

One of the recent PNG isolates (PNG6910) that belongs to G1a isolates shares a two-nucleotide deletion at positions 10458 and 10459 with another PNG isolate, NG156 (G4 of MVEV). Two of the recent G1b isolates (K68150 and P8891) exhibit a

Table 2.7. *Analysis of the operation of selection pressures averaged over all full-length prM and E gene sequences included in this study.*

Type of Selection	**dN – dS ***	***p*-value †**
Positive	-24.108	1.000
Purifying	24.094	0.000
Neutral	-23.867	0.000

* dN – dS (Z test of selection).

† A *p*-value of >0.05 was considered not to be significant.

Fig. 2.5. *Multiple nucleotide alignment of the 3'UTR of representative strains of G1a, G1b, G2, G3 and G4 of Murray Valley encephalitis virus with the representative strains of closely related flaviviruses such as Alfuy virus, Japanese encephalitis virus and West Nile virus.*

Black square indicates the position of the unique 18-nucleotide deletion in the recent G2 MVEV isolates. Dotted square indicate the 64-nucleotide deletion of OR156. Dashes represent gaps; numbers on the top indicate nucleotide position according to the MVE-1-51 complete genome (NC000943).

three-nucleotide deletion at the same position (10458-10460). Interestingly, deletions at this position are also observed in MK6684 (G3 of MVEV) and the representative strains of all WNV lineages, except Sarafend strain (lineage II). None of the JEV strains exhibit a deletion at this position (Fig. 2.5). No specific deletions were observed to separate G1a strains from G1b. These results highlighted the sequence variability in this region of the 3'UTR of flaviviruses.

2.4 Discussion

The major finding of this study was the identification of four more G2 isolates; three from mosquitoes collected from Fitzroy Crossing and one from Broome. This supports the hypothesis that G2 strains of MVEV continue to circulate in the Kimberley region of WA and may exist in a rarely sampled ecological niche. It is the first report of G2 isolates in the west Kimberley and extends the known geographic range of this genotype by approximately 600km west (Fig. 1.5). The isolation of three G2 strains in one year (2006) from mosquitoes in Fitzroy Crossing and Broome, approximately 400km apart, provides evidence that this genotype circulates over a broad geographic region. No G2 isolate was detected in mosquitoes collected from the Pilbara region of WA, suggesting that this genotype may not yet have become established or has not been sampled in that region. Given the intrinsic role of mosquitoes in the lifecycle and ecology of MVEV, isolates from mosquitoes may be viewed as representative of the ecosystem from which they were sampled. However, since these are only collected from specific sites over a selected period of time in WA, they are only a sample of the population. Although isolates retrieved from mosquito collections may not definitively represent the MVEV population in nature, they can be regarded as a random sampling. It should also be noted that some strains circulating in nature may not be cultureable; therefore, subpopulations of MVEV may have been left undetected in this study. This is difficult to assess and further studies are required to evaluate PCR-positive, isolation-negative mosquito pools

The detection of G2 strains beyond the Kununurra region invites speculation as to whether these viruses have spread from this region or have circulated more broadly

than initially thought, but simply not been detected during surveillance activities. The fact that G2 has not been detected from Kununurra since 1995 may indicate that the ecological and environmental conditions in that area are no longer favourable for the G2. However, because the complete prM-E sequence comparison of all G2 isolates revealed a maximum of 1.1% amino acid divergence in these strains, it is unlikely that biological characteristics of these structural proteins are involved in this phenomenon.

Similar to the previous G2 isolates that were collected from different mosquito species (*Cx. annulirostris, Cx. pullus, and An. bancroftii*), recent G2 isolates were also isolated from *Cx. annulirostris* and *Cx. pullus.* The observed amino acid substitutions in the E gene of G2 strains, as compared to G1 strains, could affect the transmission efficiency of this genotype by other mosquito vectors that transmit G1. Studies on the closely-related WNV revealed that a single substitution in the E gene ($A^{159}V$) resulted in a shorter extrinsic incubation period in the mosquito vector leading to an explosive westward expansion of the geographic range of WNV in North America (Davis et al., 2005; Ebel et al., 2004; Kilpatrick et al., 2008; Moudy et al., 2007). Moreover, Moudy et al. (2009) revealed that a mutation at position 154 ($N^{154}I$), of the WNV glycosylation site (Luca et al., 2012; Nybakken et al., 2006; Prow et al., 2011; Rey et al., 1995), severely affected the vector competency of *Cx. pipiens* and *Cx. tarsalis* and restricted virus transmission. Although G2 of MVEV did not show mutation at either of these positions, it demonstrated a mutation at position 157 ($T^{157}S$) which is in close proximity of residues 159 and 154 [adjacent to the glycosylation site; (Luca et al., 2012; Nybakken et al., 2006; Prow et al., 2011; Rey et al., 1995)]. This mutation may affect one or more stages of virus replication in the vector. Further investigation is required to assess whether $T^{157}S$ mutation affects the competence of mosquito vectors for G2 virus transmission.

Moreover, the inherent genetic diversity of *Cx. annulirostris* and *Cx. pullus* may also influence the vector competence of these species for G1 and G2 MVEV strains. In a study on the closely-related JEV, Hemmerter et al. (2007) provided circumstantial evidence that certain lineages of *Cx. annulirostris* may not efficiently transmit JEV, thereby preventing the establishment of JEV in mainland Australia. The authors

revealed that biodiversity of *Cx. annulirostris* coincided with the southern limits of JEV activity in Australasia. Further studies may reveal whether differences in the biodiversity of *Cx. annulirostris* and *Cx. pullus* mosquitoes that circulate in different regions of WA and various states of Australia impacts on the activity and establishment of different MVEV genotypes or subgenotypes. Moreover, Hemmerter et al. (2007) demonstrated that the south-Australian lineage of *Cx. annulirostris* can only be found in some parts of SA, NSW, and south-eastern QLD. Coincidentally, no G1a and G2 strains have been detected in these states, yet. Therefore, it can be speculated that the south-Australian lineage of *Cx. annulirostris* may be poorly or non-permissive to G1a and G2 strains, thus preventing their transmission and spread to these areas. Vector competency studies will be required to address this possibility.

Recent studies on JEV revealed that the viral host preference is affected by molecular determinants that are unique for each specific genotype (Schuh et al., 2010; Solomon et al., 2003). According to Solomon et al. (2003), JEV strains that belong to G4, such as JKT6468 and JKT7003, demonstrated unique molecular determinants in the C and E proteins. These genotype-defining viral molecular determinants may presumably be responsible for restricting G4 of JEV to Indonesian region. The G2 strains of MVEV may possess such molecular determinants that limit them to a particular host species or smaller range of hosts. As a result, G2 strains could be restricted to particular vertebrate hosts in which they cannot amplify efficiently.

Previous studies on the genetic relationships of MVEV have been limited in that small numbers of viruses were considered or isolates from a restricted endemic region were studied (Coelen and Mackenzie, 1988; Johansen et al., 2007; Lawson, 1988; Lobigs et al., 1986, 1988). Nonetheless, all phylogenetic studies divided MVEV isolates into four distinct genotypes. Furthermore, recently Williams et al. (2013) further classified G1 isolates into G1a and G1b sublineages. G1a comprises strains collected in 2008 and 2009 from WA and two recent PNG strains collected in 1998. G1b contains MVEV strains collected between 1989 and 2011 from all mainland states of Australia. Some G1 isolates occupied intermediate lineages, which

clustered interchangeably with either G1a or G1b subgroups, depending on the region of the genome or the algorithm used for phylogenetic analyses (Williams et al., 2013). These isolates, therefore, could not reliably be classified into either G1 subgroups. Phylogenetic studies of the MVEV isolates reported in this chapter found a similar pattern and supported the recent proposed classification.

The majority of MVEV strains collected from WA between 2005 and 2009 were isolated from *Cx. annulirostris* (Table 2.4), confirming that this species is the main mosquito vector of MVEV. The results also revealed that in recent years, the isolation of MVEV from this species has slightly decreased. While 83% of recent MVEV strains were isolated from *Cx. annulirostris*, 11.3% were isolated from *Cx. pullus*, and 5.6% from the other minority mosquitoes. This indicates that more MVEV strains have been isolated from *Cx. pullus* in recent years, compared to 2.6% isolation from this mosquito in previous years (Williams et al., 2010). It may simply be due to under-trapping of *Cx. pullus* in previous years, or it may indicate the relative recent abundance of this mosquito compared to previous years. No MVEV was isolated from *Cx. quinquefasciatus* in this study, which has previously been shown to account for 1.2% of transmission of MVEV (Williams et al., 2010).

The E glycoprotein of flaviviruses consists of three domains: domain III, a receptor-binding domain that acts as major target of neutralizing antibodies, domain II, the dimerisation domain that contains the putative fusion peptide and the flexible hinge region, and domain I which acts as a hinge between domain II and III and houses the glycosylation site (Luca et al., 2012; Nybakken et al., 2006; Rey et al., 1995). G2 strains of MVEV encoded twenty six unique amino acid substitutions in the E glycoprotein, fourteen of which are non-conservative. Half of these mutations are located in domain II (Table 2.6; Fig. 2.3, and Fig. 2.4), suggesting that this domain is subject to a higher level of selection pressures than the other two domains. However, evidence of selection pressure operating on the E protein was not found in this study. The biological importance of many of these substitutions, if any, is yet to be confirmed.

Significantly, the glycosylation site that is crucial for the neuroinvasive phenotype of flaviviruses (Beasley et al., 2005; Kim et al., 2008; Prow et al., 2011; Shirato et al., 2004), the putative fusion peptide that is essential for the fusogenic activity of the virus (Allison et al., 2001; Seligman, 2008), and the RGD motif which is the receptor binding region within the E glycoprotein (Hurrelbrink and McMinn, 2003; Lee and Lobigs, 2000; Lobigs et al., 1990) are fully conserved in all MVEV isolates. Similarly, all twelve cysteine residues that form intramolecular disulphide bridges in the 3D folded structure of flaviviruses (Nowak and Wengler, 1987) are fully conserved in all MVEV isolates (Fig. 2.3). Thus, even when some other mutations act on the surface of the molecule affecting its hydrophobicity, charge, shape and intermolecular interaction, the intramolecular structure of the virus remains greatly conserved (Nowak and Wengler, 1987; Tennessen, 2008). In contrast, the putative flexible hinge region that links domain I and domain II of the E protein (Rey et al., 1995; Zhang et al., 2004) is not conserved in the G2 isolates of MVEV. This region exhibited $S^{275}T$ (conservative) and $S^{276}G$ (non-conservative) substitutions (Table 2.6; Fig. 2.3). Mutations at this region were shown to result in significant decreases in the flexibility of the hinge, fusion efficiency, and neuroinvasiveness in mice (McMinn, 1997; McMinn et al., 1995).

Analysis of the 3'UTR sequences of MVEV isolates identified a unique 18-nucleotide deletion downstream of the stop codon in the G2 strains isolated after 1991. This may serve as a genetic marker of the recent strains. A similar pattern of deletions has been found in the closely-related flaviviruses such as ALFV, JEV and WNV (Poindinger et al., 1996). However, ALFV demonstrated the most similar deletion (56-nucleotide deletion) to OR156, which has a 64-nucleotide deletion. One explanation of the differing sizes of this deletion in G2 strains is that the ancestral G2 strain may have contained the smaller 18-nucleotide deletion, but through time, a larger deletion was introduced in the OR156 lineage. Alternatively, it can be speculated that the ancestral G2 strain had the larger deletion, which was subsequently replaced with sequence encoded by the recent G2 via a recombination event. Due to the relatively high degree of nucleotide variation in this region between the recent strains of G2 and other genotypes, this is unlikely to

have occurred with latter. The biological significance of this deletion remains to be characterised. However, recently it has been shown that the highly structured RNA sequences at the beginning of the 3'UTR of flaviviruses renders resistance to nucleases (Pijlman et al., 2008). This results in the production of subgenomic flavivirus RNA that are important for infectivity and pathogenicity of these viruses (Pijlman et al., 2008). The observed variability and polymorphism at the beginning of the 3'UTR does not affect the known 3'SL secondary structure of flaviviruses which is essential for the genome cyclisation and RNA synthesis (Alvarez et al., 2005; Khromykh et al., 2001a; Tilgner et al., 2005; Villordo and Gamarnik, 2009).

Finally, within the prM and E genes of MVEV, no codon position was subject to positive selection. This indicates that similar to other flaviviruses (Baillie et al., 2008; Holmes, 2003; Jerzak et al., 2005; Jerzak et al., 2008; May et al., 2011; Mohammad et al., 2011; Woelk and Holmes, 2002), the genome of MVEV has been subject to purifying selection over time.

2.5 Conclusion and Future Direction

In conclusion, the analysis of all MVEV isolates resulted in the detection of four new G2 isolates comprising 5.6% of all recent MVEV isolates sequenced in this study. The results reported in this chapter support the hypothesis that G2 strains of MVEV have a smaller and restricted circulating population or have less efficient transmission cycles than G1 strains (Johansen et al., 2007; Williams et al., 2010) and G2 may exist in a unique ecological cycle (Johansen et al., 2007). This suggests that in order to understand the pattern of MVEV activity and geographic variation, it is essential to sequence all MVEV strains that are isolated each year. However, this may not be feasible or practical in arboviral surveillance activities. Further sequence analyses, especially using the highly sophisticated NGS technique can provide valuable information on the depth of genetic diversity and population structure in MVEV isolates, which is addressed in Chapter 4. The observed amino acid substitutions in the G2 of MVEV are likely to affect the phenotypic characteristics of this genotype, such as differences in replication kinetics in the cell culture,

pathogenicity and virulence in animal models. This will also be explored as part of this thesis in Chapter 5.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A FLUOROGENIC ONE-STEP REAL-TIME QUANTITATIVE PCR FOR THE DETECTION AND QUANTIFICATION OF ALL GENOTYPES OF MURRAY VALLEY ENCEPHALITIS VIRUS

3.1 Introduction

The laboratory diagnosis of MVEV infections, as for other flaviviruses, is typically performed using serological assays to detect antibody responses (Hall et al., 1996; Kuno, 2003), virus isolation techniques (French, 1952; French et al., 1957; Lehmann et al., 1976; van den Hurk et al., 2002) and/or RT-PCR to detect viral nucleic acid (Harnett and Cattell, 2010; McMinn et al., 2000; Patel et al., 2013; Pyke et al., 2004; Studdert et al., 2003; Tanaka, 1993; Williams et al., 2010). Serological assays, especially neutralisation tests are regarded as the gold standard method of detection because they provide the most accurate and conclusive results. However, these assays are technically difficult to perform and the results can be difficult to interpret unless the operator is highly experienced (Calisher, 1994b; Williams et al., 2010). Serological tests are also unable to distinguish antibody responses to secondary flavivirus infections due to a complex anamnestic response to common epitopes of the primary infecting virus (Halstead et al., 1983; Makino et al., 1994). Virus isolation can take a long period of time and may require multiple passages to culture an isolate. In addition, virus isolation techniques commonly fail to provide results if viable virus is absent or of low titres in an infected sample (Johansen et al., 2009). Disadvantages also exist for molecular methods of MVEV detection such as conventional RT-PCR and nested PCR. Conventional RT-PCR requires post-amplification manipulation to visualise PCR products using electrophoresis and also requires sequencing of amplicons for definitive identification (Studdert et al., 2003; Tanaka, 1993; Williams et al., 2010). Nested PCR is also subject to the risk of contamination, because of the requirement of two rounds of PCR.

Real-time quantitative PCR (qPCR) assays offer the advantage of lower contamination risk, accurate quantitative measurement and easy standardisation (Bustin and Nolan, 2004; Kubista et al., 2006). Since its development in 1992 (Higuchi et al., 1992), qPCR has been used for the detection (Cavrini et al., 2011; Harnett and Cattell, 2010; Patel et al., 2013; Pyke et al., 2004; Zaayman et al., 2009), quantification (Gurukumar et al., 2009; Mantel et al., 2008; Santhosh et al., 2007), genotyping and serotyping (Chien et al., 2006; Johnson et al., 2005; Leparc-Goffart

et al., 2009; Shu et al., 2003) of many flaviviruses. While flavivirus universal RT-qPCR assays (Moureau et al., 2008; Patel et al., 2013) can detect MVEV, final identification is only achieved by sequencing. Two specific RT-qPCR assays for the detection of MVEV have been reported (Harnett and Cattell, 2010; Pyke et al., 2004). Both assays utilise primers and probes that contain several mismatches in the genomic sequences of G2, G3 and G4. The recent detection of G2 isolates from the Kimberley region of WA, reported in Chapter 2, highlighted the need to detect all genotypes of MVEV for comprehensive molecular diagnostic capability. This chapter outlines the development and validation of a fluorogenic one-step RT-qPCR assay for the detection and quantification of all genotypes of MVEV.

3.2 Materials and Methods

3.2.1 Viruses, Clinical and Environmental Samples

Flavivirus isolates that represent each of the recognised genotypes or phylogenetic lineages of available members of the JEV group were used in the validation of the assay. These viruses included strains of MVEV, ALFV, JEV, WNV, KUNV, and KOUV. In addition, KOKV, STRV, EHV, YFV 17D, and isolates of each of four DENV serotypes were tested.

The environmental samples used in this study consisted of homogenates of mosquito pools that previously tested positive for MVEV, ALFV, KUNV, and EHV. Mosquito homogenates were kindly provided by the Arbovirus Surveillance and Research Laboratory, the University of Western Australia. The clinical samples tested in this study included homogenates of horse and duck tissues that tested positive for MVEV and KUNV at CSIRO, Australian Animal Health Laboratory (AAHL). The clinical samples were kindly provided by CSIRO, AAHL.

3.2.2 Titration

All virus stocks were titrated in PSEK cells using 96-well micro-titre plates (Greiner Bio-one, Australia). The titre was determined by the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938).

3.2.3 Assay Design

3.2.3.1 Primers and Hydrolysis Probe

To design oligonucleotide primers specific for MVEV, available sequences of all four genotypes of MVEV were aligned using ClustalW in MEGA 5.2.1 software (Tamura et al., 2011), along with the sequences of representative genotypes and lineages of ALFV, JEV, WNV, KUNV, KOUV, STRV, YFV, and DENV (Table 3.1). Primers and the hydrolysis probe were selected to target the 3'UTR that was conserved within the four MVEV genotypes but demonstrated variation amongst other flaviviruses. The forward primer MVEV-10593F, reverse primer MVEV-10703R and the hydrolysis probe MVEV-10656 were synthesised by Fisher Biotech (Table 3.2). The probe was labelled with a 5' 6-carboxy-fluorescein (FAM) reporter and a 3' MGBNFQ minor groove binding non-fluorescent quencher. A nucleotide BLAST search (BLASTn) was carried out to ensure that primers and probe were specific for MVEV.

3.2.3.2 RNA Extraction and Melt Curve Analysis

Viral RNA was extracted from 140µl of samples using the QIAmp® viral RNA mini kit (QIAGEN, Australia), as per manufacturer's protocol. The specificity of the RT-qPCR assay for MVEV nucleic acid extracts was verified by performing melt curve analysis and agarose gel electrophoresis. Viral RNA was reverse-transcribed using the MVEV16Rb primer (Table 3.2) and Superscript® III Reverse Transcriptase kit (Life Technologies, Australia), as described in Chapter 2 (Section 2.2.2). The resulting cDNA was used to template a 20µl reaction using KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, USA). The reaction contained 4µl of cDNA, 10µl KAPA SYBR® FAST qPCR Master Mix (2X), and 200nM of each forward (MVEV-10593F) and reverse primer (MVEV-10703R). The cycling conditions involved a denaturation step at 95°C for 3 min followed by 40 cycles of 95°C for 5 sec, 52°C for 20 sec, and 72°C for 30 sec. The reaction was then cooled to 55°C for 20 sec. The temperature was increased to 95°C in increments of 0.5°C for 10 sec with constant fluorescence reading. Results were analysed using CFX Manager™ Software (Bio-Rad, Australia). Representative PCR products of each genotype were also analysed on a 2% w/v agarose gel to confirm the absence of any secondary bands.

Table 3.1. *Sequences of flaviviruses used for the generation of MVEV specific primers and probe.*

Strains	Lineage/ Genotype *	Year of isolation	Location of isolation	Source of isolation	GenBank accession no.
MVE 1-51	1	1951	Australia	Human	NC000943
OR156	2	1973	Australia	Mosquito	KC852193
MK6684	3	1966	PNG	Mosquito	L48974
NG156	4	1956	PNG	Human	L48975
ALFV MRM3929	N/A	1966	Australia	Pheasant	AY898809
JEV K94P05	1	1994	Korea	Mosquito	AF045551
JEV FU	2	1995	Australia	Human	AF217620
JEV Nakayama	3	1935	Japan	Human	EF571853
JEV JKT6468	4	1981	Indonesia	Mosquito	AY184212
JEV Muar	5	1952	Malaysia	Human	HM596272
WNV NY99	1a	1999	USA	Human	AF196835
KUNV MRM61C	1b	1960	Australia	Mosquito	AY174504
WNV Sarafend	2	Unknown	unknown	unknown	AY688948
WNV Rabensburg	3	1997	Czech Republic	Mosquito	AY765264
WNV LEIV-Krnd88	4	1998	Russia	Tick	AY277251
WNV 804994	5	1980	India	Human	DQ256376
KUNV Sarawak	6	1966	Borneo	Mosquito	L49311
KOUV DakAaD 5443	7	1968	Senegal	Rodent	FVVNS5GAH
STRV 23759	N/A	1995	Australia	Mosquito	FVVNS5GAB
YFV 17D	IU	2011	USA	Human	JN628281
DENV-1	IU	2004	Venezuela	Human	FJ744701
DENV-2	IU	2001	Thailand	Human	FJ744724
DENV-3	IU	2004	USA	Human	FJ024470
DENV-4	IU	2007	Venezuela	Human	FJ882590

* **N/A:** Not applicable; **IU:** Information unavailable.

Table 3.2. *Primers and probe targeting the MVEV 3'UTR used in this study.*

Primers /Probe*	Sequence (5'→3')	Size (bp)	Tm (°C)
RT-qPCR primers			
MVEV-10593F	CGGTTGGAAAGCCTCCCAG	19	55.4
MVEV-10703R	CACAGATTGCACTCCTCGGC	20	55.9
Hydrolysis probe			
MVEV-10656	FAM-CCGTGTCAGATCGCGAAAGCGCCAC-MGBNFQ	25	64.2
Cloning primers			
MVEV-10563F	GAGGACTGGGTTACCAAAGC	20	53.8
MVEV-10734R	CGGCTTTGTTYACCCAGTCC	20	53.8
MVEV-16Rb	AGATCCTGTGGTCTTCTC	18	48.0

* Numbering is based on the genome of MVE-1-51 (NC000943)

3.2.4 Generating Plasmid Clones for Use as Quantification Calibrators

Quantification calibrators are used in the standard curve as reference molecules for quantifying unknown samples, and determining the efficiency of the assay for each genotype. Quantification calibrators were generated for each genotype of MVEV. For G1, an infectious cDNA clone of MVEV G1 [pMVE-1-51; (Hurrelbrink et al., 1999)] was kindly provided by Professor Peter McMinn, University of Sydney. pMVE-1-51 was transformed into DH10B™ Competent Cells (Life Technologies, Australia), and grown on LB plates containing 200µg/ml of ampicillin overnight. Briefly, this involved mixing 500ng of the full-length pMVE-1-51 with 50µl of DH10B™ electrocompetent cells on ice for 2 minutes. The mixture was then transferred into pre-chilled 0.1cm electroporation cuvettes (Life Technologies, Australia) and the electroporation was carried out in Bio-Rad GenePulser XCell™ (Bio-Rad, Australia), applying 200Ω resistance, 25µF capacitance, and 1800 volts. Five hundred microlitre of pre-warmed LB media was immediately added into the electroporation cuvette (Life Technologies, Australia), mixed and transferred into 1.5ml microcentrifuge tubes. After incubation at 37°C for 1 hour, with shaking, 100µl of the mixture was plated on to LB agar supplemented with ampicillin (200µg/ml), and incubated at 37°C for 18 hours. A single colony was selected and incubated in 4ml LB media supplemented with ampicillin for eight hours at 37°C. Next, 1ml of the grown culture was added to 150ml LB media supplemented with ampicillin and incubated at 37°C overnight.

The resulting plasmid was purified using the QIAGEN Plasmid Midi kit (QIAGEN, Australia), as per manufacturer's protocol. The purified plasmid was linearised with *Xba*I (New England Biolabs, USA) and purified by a phenol-chloroform extraction. *In vitro* transcription of the linearised product was carried out using the MEGAscript® T7 kit (Life Technologies, Australia), as per manufacturers' protocol. The reaction contained 1µg of purified pMVE-1-51 DNA, 7.5mM of each dATP, dCTP, dUTP, 2.5mM of dGTp, and 2µl of 10X Reaction Buffer to a final volume of 20µl. The reaction was incubated at 37°C for 4 hours. Full-length MVE-1-51 RNA transcripts were treated with 2U of TURBO DNase (Life Technologies, Australia) at 37°C for 30

min. The resulting RNA was purified with the QIAGEN RNeasy kit (QIAGEN, Australia), according to the manufacturer's protocol, to remove template DNA.

There are currently no full-length infectious clones for MVEV strains OR-156 (G2), MK6684 (G3) and NG156 (G4). Therefore, RNA for G2, G3 and G4 were generated by cloning a 172-nucleotide fragment of the 3'UTR encompassing primer and probe binding sites. Initially, the fragments were amplified using forward primer MVEV-10563F, reverse primer MVEV-10734R (Table 3.2), and the PCR SuperMix (Life Technologies, Australia), as described in Chapter 2 (Section 2.2.2). The products were purified using the ChargeSwitch Pro PCR kit (Life Technologies, Australia), and ligated into the pGEM®-T Easy vector (Promega, Australia), according to the manufacturer's protocol. Each ligation reaction contained 20ng of insert DNA, 5µl of 2X Rapid Ligation Buffer, 50ng of pGEM®-T Easy Vector, and 3 Weiss units of T4 DNA Ligase, to a final volume of 10µl. The reactions were incubated at 4°C for 18 hours, followed by inactivation at 65°C for 5 minutes. The inactivated ligated products were transformed into DH10B^{TM} competent cells (Life Technologies, Australia), and plasmids were purified using QIAGEN Plasmid Midi kit (QIAGEN, Australia). The purified plasmid was linearised using *Sal*I (Promega, Australia), and gel-purified using Wizard® SV Gel and PCR Clean-up System (Promega, Australia), according to the manufacturer's protocol. *In vitro* transcription of linearised products was performed as above, but for eight hours, to generate 264-nucleotide RNA products. The *in vitro* RNA transcripts were treated with 2U of TURBO DNase (Life Technologies, Australia) and purified using the QIAGEN RNeasy Kit (QIAGEN, Australia). The copy number of each plasmid was calculated according to the formula Xg/µl RNA / [transcript length in nucleotides x 340] x 6.022 x 10^{23}.

3.2.5 Reverse transcription real-time quantitative PCR (RT-qPCR)

Purified RNA was assayed in a 20µl reaction containing 8µl of RNA and 12µl of the assay master mix. The PCR mix consisted 0.3U of SuperScript® III Platinum® One-Step qRT-PCR (Life Technologies, Australia), 10U of RNaseOUTTM Recombinant Ribonuclease Inhibitor (Life Technologies, Australia), 0.5U of i-StarTaqTM DNA Polymerase (iNtRON Biotechnology, Korea), 1mM of DL-DTT (Sigma-Aldrich,

Australia) 0.3µM of MVEV-10593F and MVEV-10703R, and 0.1mM MVEV-10656-FAM. A one-step RT-qPCR was carried out in a CFX96TM Real-Time System (Bio-Rad, Australia). Thermocycling conditions were as follows: 50°C for 30 min, and 95°C for 5 min; followed by 50 cycles of 94°C for 15 sec, 55°C for 20 sec and 72°C for 30 sec with the fluorescence read at the end of each cycle. The assay was performed in HSP9601 96-well plates (Bio-Rad, Australia) sealed with microseal B (Bio-Rad, Australia). Each assay contained *in vitro*-transcribed MVE-1-51 RNA as the positive internal control and water as no template control (NTC). In addition, the sensitivity and specificity of previously reported MVEV-specific RT-qPCR assays (Harnett and Cattell, 2010 and Pyke et al., 2004) was assessed using MVEV RNA extracts (8µl). A modification of the Pyke et al. (2004) assay was employed whereby the same primers (400nM) and probe (120nM) were used in the AgPath-ID™ One-Step RT-PCR kit (Life Technologies, Australia), with thermocycling conditions as recommended by the manufacturer (45°C for 10 min, and 95°C for 10 min; followed by 45 cycles of 95°C for 15 sec, 60°C for 45 sec).

3.2.6 Efficiency, Linearity and Standard Curve

The PCR efficiency (the ability of the assay to double amplicons at the end of each cycle) and the co-efficient determination [(R^2) a measure of the linearity of the standard curve] of the assay were determined in triplicate by standard curve analysis using ten-fold serial dilutions of *in vitro*-transcribed RNA from each virus genotype. The efficiency, co-efficient determination (R^2) and slope values were calculated by CFX Manager™ Software (Bio-Rad, Australia). The efficiency was calculated from the slope as described previously (Pfaffl, 2001; Vandesompele et al., 2002).

3.2.7 Assay Performance

Assay performance was assessed following the guidelines for minimum information required for the publication of quantitative real-time PCR experiments (Bustin et al., 2009). A quantification cycle (*C*q; the cycle number at which fluorescence signal increases above the threshold value) of over 40 was considered as negative result.

3.2.7.1 Analytical Performance

The analytical sensitivity, which indicates the minimum copy number of the template in a sample that is detectable by the assay, was assessed by testing ten-fold serial dilutions (10^7 to 10^{-1} copies) of *in vitro* RNA transcripts from each genotype. The lower limit of detection (LOD), which signifies the lowest concentration of a template that the assay could detect, was determined for each genotype.

The analytical specificity of the assay, or the ability of the assay to correctly amplify and detect the desired target sequence, was evaluated by testing the *in vitro* RNA transcripts as well as viral RNA extracts from MVEV-positive tissue culture supernatants of all MVEV genotypes.

3.2.7.2 Sensitivity for Clinical and Environmental Samples

Clinical sensitivity of the assay was assessed using RNA extracted from mosquito homogenates, horse and duck brain homogenates and homogenates of duck tissues (lung, liver, kidney, spleen) that tested positive for MVEV. Clinical sensitivity was calculated as the ratio of the number of positive results/ (number of positive isolates tested + number of false negative results) as described previously (Bustin et al., 2009; Chien et al., 2006).

3.2.7.3 Diagnostic Specificity

The diagnostic specificity of the assay was determined by testing viral RNA extracted from tissue culture supernatants of cultures of related flaviviruses, including ALFV, JEV, WNV, KUNV, KOUV, KOKV, STRV, EHV, YFV, and DENV. In addition, viral RNA from environmental and clinical samples that tested positive for flaviviruses such as ALFV, KUNV and EHV were also tested. The specificity was calculated as the ratio of the number of negative results/ (the number negative isolates tested + the number of false positive results) as described previously (Chien et al., 2006).

The homogenates of mosquito pools were tested for the presence of MVEV in a fixed cell ELISA using the MVEV-specific mAb 10C6 (Hall et al., 1996), as part of a

panel of 12 mAbs against flaviviruses and alphaviruses (Broom et al., 1998). Testing of clinical samples involved a pan-flavivirus RT-qPCR (Chao et al., 2007) and an MVEV-specific RT-qPCR (Pyke et al., 2004).

3.2.8 Interlaboratory Comparisons

The interlaboratory tests were performed in three different laboratories: the research laboratory at Curtin University, PathWest Laboratory Medicine WA in Perth, and CSIRO, AAHL in Geelong, Victoria. The tests at Curtin University were performed as described above (Section 3.2.5). The tests at PathWest were performed with 0.2ml thin-walled clear PCR tubes (Axygen, Australia) in a Corbett RG6000 thermocycler (Corbett Life Science, Australia). Clinical samples were tested at AAHL using 0.2ml thin-walled clear PCR tubes in a Fast Real-time PCR System7500 (Applied Biosystems, Australia). Testing at all locations used the same RT-qPCR chemistry (Section 3.2.5); however, viral RNA templates were derived from the virus collections of each laboratory and were not standardised.

3.2.9 Assay Precision

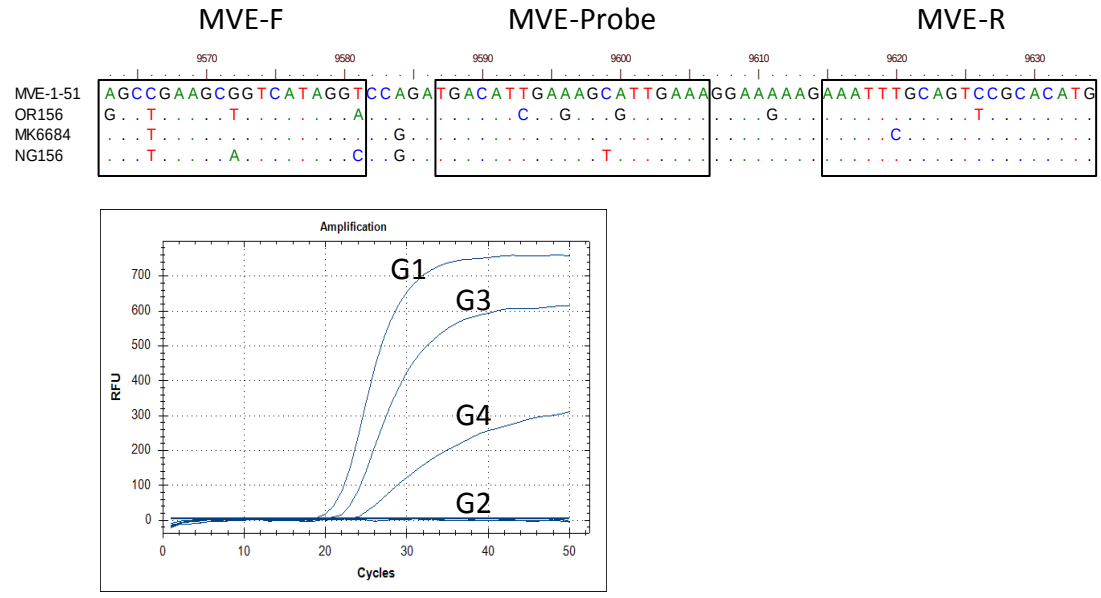
The precision of the assay was examined by applying the methods described elsewhere (Abdul-Careem et al., 2006; Bustin et al., 2009; Santhosh et al., 2007). Intra-assay repeatability (short-term precision, intra-assay variance) was assessed by testing ten-fold serial dilutions of MVE-1-51 *in vitro* RNA transcripts (10^7 to 10^1 copies) in triplicate in a single assay. For each dilution, mean *C*q values, their mean, standard deviation (SD) and coefficient of variation (CV) were calculated separately. Inter-assay reproducibility (long-term precision, inter-assay variance) was determined by running 10^6, 10^4 and 10^3 copies of MVE-1-51 *in vitro* RNA transcript in triplicates over four separate consecutive days. The mean *C*q, SD and CV of each concentration were calculated independently for each dilution.

3.3 Results

3.3.1 Assessment of Current RT-qPCR Assays for the Detection of MVEV

Alignment of the sequences of the primers and probes used in the reported MVEV-specific RT-qPCR assays (Harnett and Cattell, 2010; Pyke et al., 2004) with genomic

A.



B.

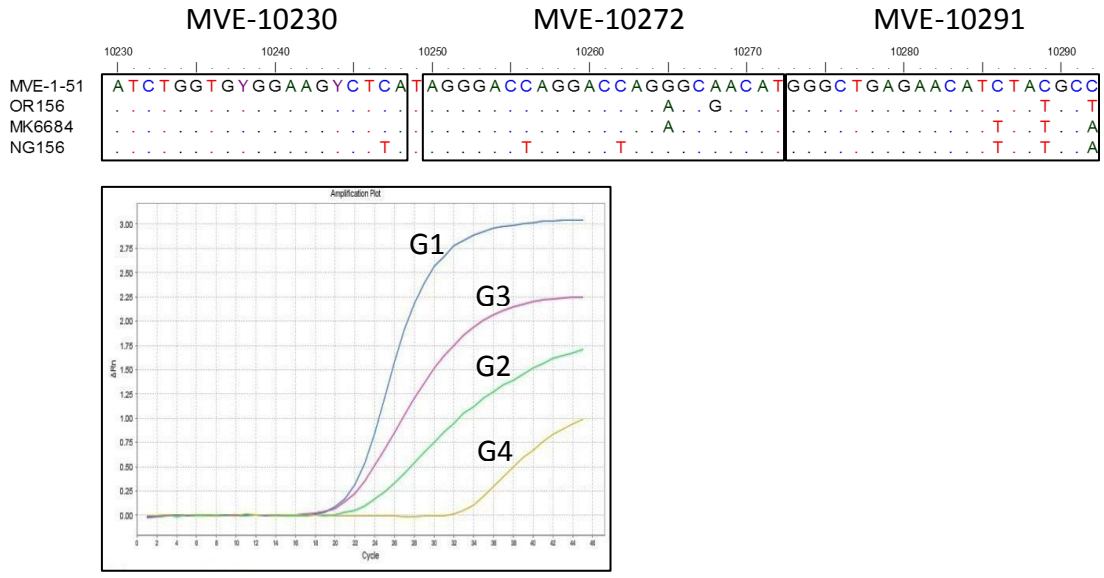


Fig. 3.1. *Multiple alignments and amplification curves of four genotypes of MVEV with the primers/probe used in previously reported MVEV RT-qPCR assays.*

A: Harnett and Cattell (2010); and **B:** Pyke et al. (2004). Boxes indicate the positions of forward and reverse primers at both ends and the probes in the middle. Numbers above the alignments indicate the nucleotide position according to the MVE-1-51 complete genome (NC000943). **RFU:** Relative fluorescence unit; **ΔRn:** Baseline subtracted normalised reporter signal. RT-qPCR cycle numbers are plotted against RFU in **(A)** or ΔRn in **(B).**

sequences of MVEV G1-G4 revealed mismatches in G2, G3 and G4 (Fig. 3.1). To compare the sensitivity and efficiency of this assay with previously reported MVEV-specific RT-qPCR assays, purified viral RNA from each genotype, standardised to a virus titre of 10^4 $TCID_{50}$/ml was tested by RT-qPCR as described by Harnett and Cattell (2010) and Pyke et al., (2004). A modification of the latter assay was employed, using the same primers and probe in a commercial RT-PCR kit. The mismatches in G2, G3 and G4 sequences considerably affected the amplification of these genotypes (Fig. 3.1). Amplification of G3 viral RNA resulted in higher *C*q values and comparatively lower fluorescence signal to G1 amplification for both assays. In the Harnett and Cattell (2010) assay, G2 was not detected, while G4 exhibited a considerably higher *C*q value to those of the G1 and G3 (Fig. 3.1). In the Pyke et al. (2004) assay, although both G2 and G4 viral RNA showed amplification, G4 exhibited a higher *C*q and lower RFU than G2 (Fig. 3.1).

3.3.2 Assay Design

The aim of this study was to develop an MVEV-specific RT-qPCR assay capable of detecting all genotypes with similar sensitivity and efficiency. Alignment of the full length genomes of all four genotypes of MVEV with other closely-related flaviviruses revealed that the 3'UTR was the most conserved region in the genome of MVEV that also demonstrates a high level of variation compared to this region of other flavivirus species (Fig. 3.2). Primers and probe were designed to target a 111-nucleotide fragment within this region that was fully conserved in all MVEV genotypes, except for two bases within the G2 strain (OR156; Table 3.2 and Fig. 3.2). Those mismatches, however, do not locate inside the primers and probe annealing sites. This region spanned nucleotide position 10593 to 10703 of the MVE-1-51 complete genome (NC000943). To ensure maximum efficiency of the assay, primers were selected outside the predicted 3' stem loop (3'SL) structure of the flavivirus genome (Brinton et al., 1986; Markoff, 2003; Thurner et al., 2004). BLAST search of the primers and hydrolysis probe revealed no significant matches to any available sequences of flaviviruses in the GenBank database, except to MVEV sequences (data not shown).

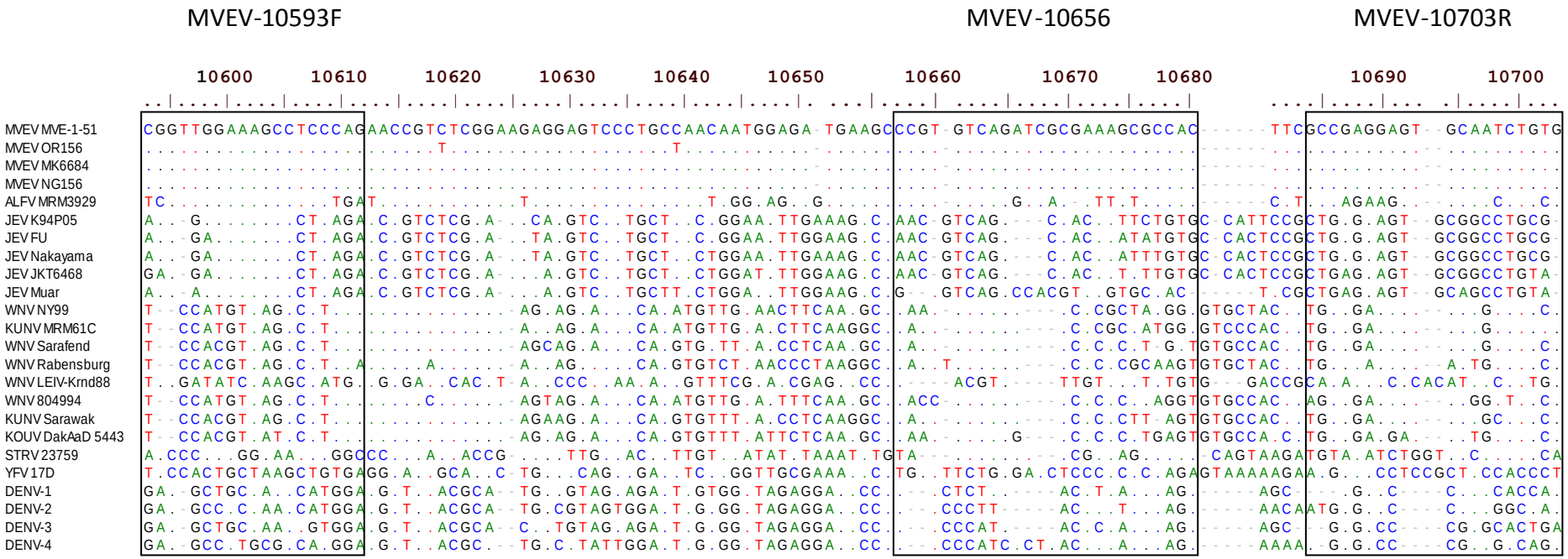


Fig. 3.2. *Alignment of MVEV isolates with representative strains of closely-related flaviviruses.*

The figure demonstrates a part of the 3'UTR of the flavivirus genome that is highly conserved among MVEV genotypes, but shows variation in other flaviviruses. Squares indicate the position of the assay primers at both ends and the probe in the middle. Dots indicates nucleotide identities, dashes indicate gaps in the alignment. Numbers on the top of the alignment indicates the nucleotide position based on MVE-151 complete genome (NC000943).

Table 3.3. *The lower limit of detection (LOD), efficiency, co-efficient determination (R^2) and the slope values of the assay for each genotype of MVEV.*

MVEV Genotypes	LOD (RNA copies)	Efficiency	R^2 †	Slope
G1	10	100	0.998	-3.321
G2	10	100.9	0.998	-3.301
G3	10	100	0.997	-3.323
G4	10	100.4	0.997	-3.313

† Co-efficient determination (R^2), the linearity of the standard curve of each assay. R^2 indicates how well data points (*C*q of all samples) fit on a standard curve.

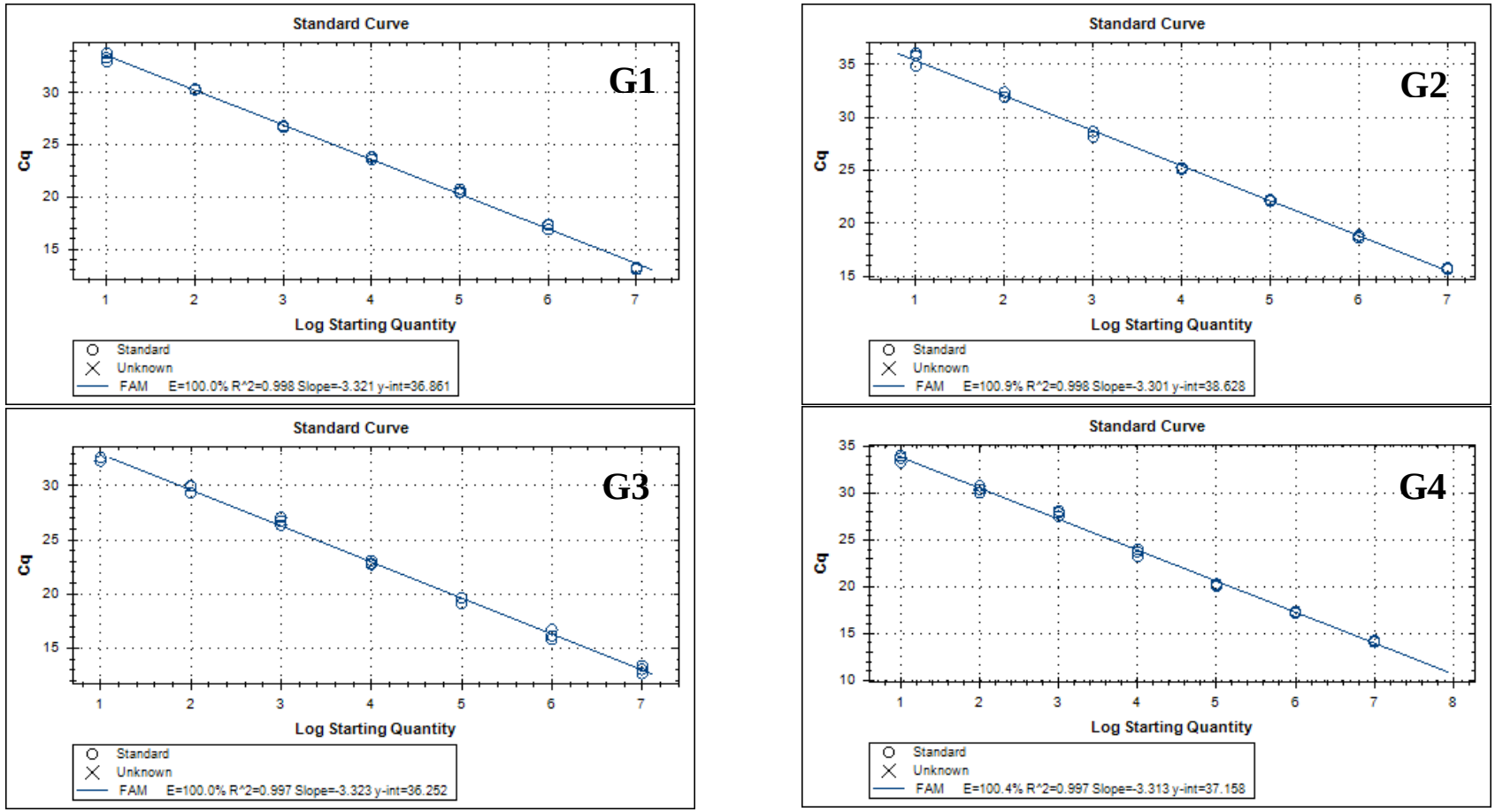


Fig. 3.3. *Standard curves for RT-qPCR amplification of RNA from MVEV genotypes G1, G2, G3 and G4 from which LOD, efficiency, slope and R^2 values for each genotype were calculated.*

Quantification cycle (*C*q) is plotted against $\log_{10}$ of the starting quantity of viral RNA.

3.3.3 Analytical Performance

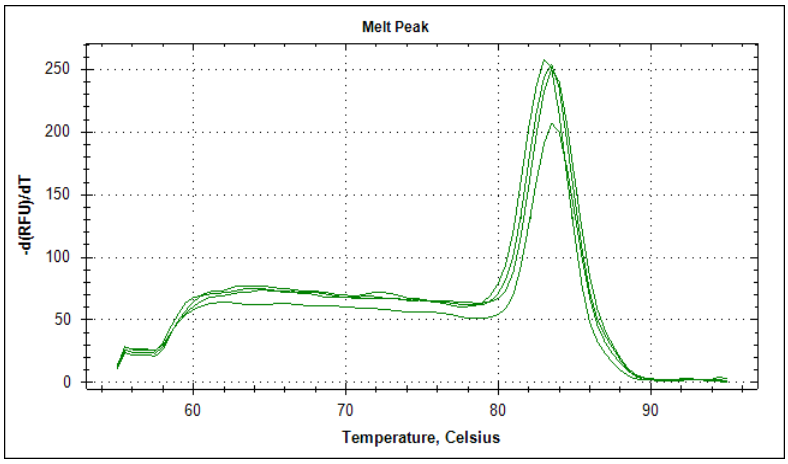
The lower LOD of the assay for each genotype was assessed by using ten-fold serial dilutions of *in vitro* RNA transcripts from each genotype in standard curve analyses. The lower LOD of the assay for all genotypes was 10 RNA copies (Table 3.3 and Fig. 3.3). The efficiency of the assay for all genotypes was 100% indicating that the amount of PCR product doubled at the end of each cycle. The slope of the standard curve, that provides an indication of the efficiency of the assay, was between -3.321 to -3.313 for all genotypes (Table 3.3, Fig 3.3). According to Kubista et al. (2006), a slope value between -3.3 to -3.8 is optimal. A slope of -3.3 to -3.8 is equivalent to an amplification efficiency of approximately 100% to 85%, respectively. The co-efficient determination (R^2) that indicates the linearity of the assay in a standard curve was ≥0.997 for RNA of all MVEV genotypes (Table 3.3 and Fig. 3.3). Thus, the assay could detect the target sequence in all MVEV-positive samples with high levels of efficiency and linearity.

3.3.4 Melt Curve Analysis

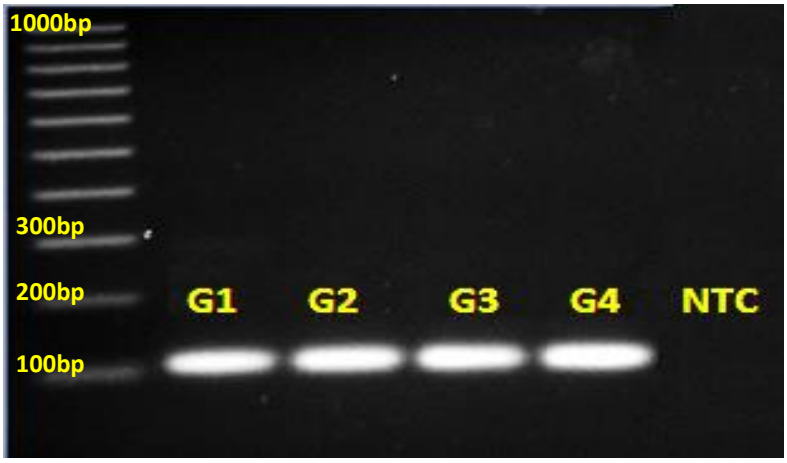
The melt curve analysis was carried out to assess primer-dimerisation, and any other non-specific amplification in the assay. A single peak was observed for each MVEV genotype, indicating the specificity of the assay only to MVEV RNA templates (Figure 3.4A). The melting temperature for all genotypes was 83.0 ± 0.5°C. In addition, agarose gel electrophoresis of the amplified products revealed no secondary bands, further confirming the absence of any secondary non-specific amplification (Figure 3.4B).

3.3.5 Assay Precision

Ten-fold serial dilutions of *in vitro*-transcribed MVE-1-51 RNA were used to test intra- and inter-assay variability (Appendix 3.1). The mean ± SD of CV values for intra-assay variability was 0.86 ± 0.62, while that of inter-assay variability was 1.22 ± 0.26 (Table 3.4). According to Islam et al. (2004), when inter- and intra-assay variability of an assay is <5.0, it is considered acceptable. Therefore, the experimental variability of the assay is in the acceptable range.



A



B.

Fig. 3.4. (A) *Melt curve analyses of RT-qPCR amplicons from viral RNA templates of all four MVEV genotypes.* **(B).** *Agarose gel electrophoresis of the RT-qPCR amplified products with 100bp ladder as a reference marker.*

NTC: No template control

Table 3.4. *Intra- and inter-assay variablities of Cq values following RT-qPCRs of serial dilutions of MVE-1-51 in vitro RNA transcripts.*

Variation	RNA Copies	*C*q		
		Mean	**SD**	**CV (%)**
Intra-assay	10^7	13.19	0.12	0.89%
	10^6	17.00	0.33	1.95%
	10^5	20.58	0.20	0.96%
	10^4	23.78	0.13	0.56%
	10^3	26.78	0.07	0.25%
	10^2	30.34	0.05	0.16%
	10^1	33.38	0.41	1.23%
Inter-assay	10^6	17.34	0.25	1.43%
	10^4	23.92	0.22	0.93%
	10^3	27.12	0.35	1.30%

SD: standard deviation; **CV:** coefficient of variation

3.3.6 Clinical Performance

The sensitivity and diagnostic specificity of the assay to detect MVEV in mosquito samples (environmental) and veterinary samples (clinical) were assessed using homogenates of mosquito pools and infected animal tissues that tested positive for MVEV and other related flaviviruses. The assay only amplified and detected MVEV-positive samples (Table 3.5). The assay demonstrated absolute sensitivity and specificity for MVEV isolates.

The clinical sensitivity and diagnostic specificity of the assay were also assessed by three interlaboratory comparisons. All interlaboratory testing detected only the MVEV-positive samples with comparable *C*q values. No non-MVEV flaviviruses or negative samples were detected by any of these analyses. Thus, all interlaboratory comparisons produced 100% sensitivity and specificity for the MVEV isolates.

3.4 Discussion

The detection of recent G2 strains of MVEV (Chapter 2) highlighted the need to develop a rapid and sensitive assay to detect all MVEV genotypes. The aim of the studies reported in this chapter was to design a sensitive and specific RT-qPCR assay capable of detecting and quantifying viral RNA from all four genotypes of MVEV, with a high level of efficiency in both laboratory and clinical samples. Primers and probes used in previously reported probe-based RT-qPCR assays for the detection of MVEV (Harnett and Cattell, 2010; Pyke et al., 2004) exhibit several mismatches to G2, G3 and G4 sequences, which significantly affect their analytical sensitivity in detecting viruses belonging to these genotypes (Fig. 3.1). Furthermore, no data on the efficiency, linearity, precision and interlaboratory validation has been reported for these assays (Harnett and Cattell, 2010; Pyke et al., 2004).

The originality of this assay is based on the comprehensive analysis of multiple full genome alignments of all four genotypes of MVEV with closely-related flaviviruses (Fig 3.2) to ensure the choice of specific primers and probe. Primers and hydrolysis probe were selected from the 3'UTR of MVEV genome that matched the sequence of all four MVEV genotypes (without any mismatches) but exhibited a high level of

Table 3.5. *Assessment of clinical sensitivity and diagnostic specificity of the RT-qPCR assay for the detection of viral RNA from flaviviruses extracted from tissue culture supernatants, environmental (mosquito) samples, and clinical samples.*

Samples	Source of isolate	Geographic origin	Year of collection/ isolation	*Cq*	Result
MVEV strains					
MVE-1-51 (G1) *	TC-SN	Australia	1951	15.18	Pos
OR156 (G2) *	TC-SN	Australia	1973	15.85	Pos
MK6684 (G3) *	TC-SN	PNG	1966	12.54	Pos
NG156 (G4) *	TC-SN	PNG	1956	13.94	Pos
Environmental samples (Mosquito homogenates)					
K59532 (G2)	*Cx. annulirostris*	Fitzroy Crossing, WA	2006	19.39	Pos
K59536 (G2)	*Cx. pullus*	Fitzroy Crossing, WA	2006	21.66	Pos
K62017 (G2)	*Cx. annulirostris*	Broome, WA	2006	35.07	Pos
K60555 (G1)	*Cx. annulirostris*	Kununurra, WA	2006	17.31	Pos
K68150 (G1)	*Cx. annulirostris*	Fitzroy Crossing, WA	2009	16.35	Pos
K68838 (G1)	*Cx. annulirostris*	Kununurra, WA	2009	17.11	Pos
K69612 (G1)	*Cx. pullus*	Kununurra, WA	2009	19.39	Pos
P8891 (G1)	*Ae. normanensis*	Karratha, WA	2006	19.37	Pos
K72445 (G1) †	*Cx. annulirostris*	Broome, WA	2011	20.20	Pos
K72682 (G1) †	*Cx. annulirostris*	Broome, WA	2011	16.60	Pos
K72974 (G1) †	*Cx. annulirostris*	Broome, WA	2011	16.10	Pos
K73041 (G1) †	*Cx. annulirostris*	Willare, WA	2011	17.50	Pos
K73051 (G1) †	*Cx. annulirostris*	Willare, WA	2011	19.20	Pos
K73072 (G1) †	*Cx. annulirostris*	Willare, WA	2011	20.10	Pos
K73080 (G1) †	*Cx. annulirostris*	Willare, WA	2011	17.40	Pos
K73096 (G1) †	*Cx. annulirostris*	Willare, WA	2011	16.50	Pos
K73146 (G1) †	*Cx*. species	Derby, WA	2011	18.60	Pos

Table 3.5. ***continued***

Samples	Source of isolate	Geographic origin	Year of collection/ isolation	*Cq*	Result
K73164 (G1) †	*Cx. annulirostris*	Derby, WA	2011	30.90	Pos
K73251 (G1) †	*Cx. annulirostris*	Derby, WA	2011	15.30	Pos
K73350 (G1) †	*Cx. annulirostris*	Derby, WA	2011	16.40	Pos
K73350 (G1) †	*Cx. annulirostris*	Derby, WA	2011	16.40	Pos
K73384 (G1) †	*Cx. annulirostris*	Derby, WA	2011	17.60	Pos
K73445 (G1) †	*Cx. annulirostris*	Derby, WA	2011	17.00	Pos
K73475 (G1) †	*Cx. annulirostris*	Derby, WA	2011	15.30	Pos
K73607 (G1) †	*Cx. annulirostris*	Fitzroy Crossing, WA	2011	18.80	Pos
K73846 (G1) †	*Cx. annulirostris*	Geikie Gorge, WA	2011	17.80	Pos
K73996 (G1) †	*Cx*. species	Halls Creek, WA	2011	17.60	Pos
K74130 (G1) †	*Cx*. species	Halls Creek, WA	2011	16.30	Pos
K74283 (G1) †	*Cx. annulirostris*	Kununurra, WA	2011	14.40	Pos
K74400 (G1) †	*Cx. annulirostris*	Kununurra, WA	2011	19.20	Pos
Clinical samples					
11-00937-0001 ‡	Duck brain	SA, Australia	2011	21.8	Pos
11-00937-0002 ‡	Duck lung	SA, Australia	2011	15.1	Pos
11-00937-0003 ‡	Duck liver	SA, Australia	2011	19.0	Pos
11-00937-0004 ‡	Duck kidney	SA, Australia	2011	19.9	Pos
11-00937-0006 ‡	Duck spleen	SA, Australia	2011	16.8	Pos
11-01572-0001 ‡	Horse brain	VIC, Australia	2011	36.8	Pos
11-01572-0003 ‡	Horse brain	VIC, Australia	2011	31.6	Pos
11-01572-0004 ‡	Horse brain	VIC, Australia	2011	30.1	Pos
11-01572-0008 ‡	Horse brain	VIC, Australia	2011	34.0	Pos

Table 3.5. ***continued***

Samples	Source of isolate	Geographic origin	Year of collection/ isolation	*Cq*	Result
Non-MVEV					
ALFV *	TC-SN	Australia	1966	>40	Neg
JEV TS00 *	TC-SN	Australia	2000	>40	Neg
JEV FU *	TC-SN	Australia	1995	>40	Neg
JEV Nakayama *	TC-SN	Japan	1935	>40	Neg
JEV JKT6468 *	TC-SN	Indonesia	1981	>40	Neg
WNV NY99 *	TC-SN	USA	1999	>40	Neg
KUNV MRM16C *	TC-SN	Australia	1960	>40	Neg
WNV G22886 *	TC-SN	India	1958	>40	Neg
KUNV Sarawak *	TC-SN	Borneo	1966	>40	Neg
KOUV *	TC-SN	Senegal	1968	>40	Neg
KOKV MRM32 *	TC-SN	Australia	1960	>40	Neg
STRV *	TC-SN	Australia	1995	>40	Neg
EHV *	TC-SN	Australia	1961	>40	Neg
YFV 17D *	TC-SN	USA	1937	>40	Neg
DENV-1 *	TC-SN	Venezuela	2004	>40	Neg
DENV-2 *	TC-SN	Thailand	2001	>40	Neg
DENV-3 *	TC-SN	USA	2004	>40	Neg
DENV-4 *	TC-SN	Venezuela	2007	>40	Neg
Environmental samples (Mosquito homogenates)					
K72837 (ALFV) †	*Cx. annulirostris*	Broome, WA	2011	>40	Neg
K72910 (ALFV) †	*Cx. annulirostris*	Broome, WA	2011	>40	Neg
K72944 (ALFV) †	*Cx. annulirostris*	Broome, WA	2011	>40	Neg
K74154 (ALFV) †	*Cx. pullus*	Kununurra, WA	2011	>40	Neg
K74157 (ALFV) †	*Cx. pullus*	Kununurra, WA	2011	>40	Neg
K74390 (ALFV) †	*Cx. annulirostris*	Kununurra, WA	2011	>40	Neg

Table 3.5. ***continued***

Samples	Source of isolate	Geographic origin	Year of collection/ isolation	*Cq*	Result
K73822 (KUNV) †	*Cx. annulirostris*	Halls Creek, WA	2011	>40	Neg
K74015 (KUNV) †	*Cx. annulirostris*	Geikie Gorge, WA	2011	>40	Neg
K74003 (EHV) †	*Ae. normanensis*	Halls Creek, WA	2011	>40	Neg
Clinical samples					
11-00937-0005 ‡	Duck kidney	SA, Australia	2011	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-00186-0028 ‡	Duck pooled tissues	SA, Australia	2013	>40	Neg
13-01336-0001 ‡	Horse brain	QLD, Australia	2013	>40	Neg
13-01440-0003 ‡	Horse CSF	NSW, Australia	2013	>40	Neg
13-01645-0001 ‡	Horse brain	WA, Australia	2013	>40	Neg

WA: Western Australia, **SA:** South Australia, **VIC:** Victoria. **TC-SN:** Tissue culture supernatants; **Cx:** *Culex*; **Ae:** *Aedes;* ***Cq*:** quantification cycle, **G1:** Genotype 1, **G2:** Genotype 2.

* Standardised at 10^4 $TCID_{50}$/ml.

† Tested at PathWest Laboratory Medicine, WA. For methodology refer to Section 3.2.8.

‡ Tested at CSIRO, AAHL, Geelong, Victoria. These specimens submitted to AAHL for routine diagnostic testing. For methodology refer to Section 3.2.8.

variation in other flaviviruses. This region was previously selected for designing RT-qPCR assays for other flaviviruses such as JEV (Yang et al., 2004) and DENV (Chien et al., 2006; Gurukumar et al., 2009; Houng et al., 2001). This region is functionally important because the highly conserved RNA sequences at the beginning of the 3'UTR offer genomic RNA resistance to nucleases (Pijlman et al., 2008). The incomplete degradation of flavivirus genomic RNA by the host cell nucleases produces subgenomic flavivirus RNA (sfRNA) which is critical for the pathogenicity and infectivity of the virus (Pijlman et al., 2008).

This is the first MVEV RT-qPCR assay, to date, that has undergone an exhaustive examination of reproducibility, efficiency, linearity, sensitivity and specificity for all MVEV genotypes. The applicability of the assay for laboratory diagnosis of MVEV was demonstrated by testing flavivirus-positive environmental and clinical samples. These samples included homogenates of mosquito pools and infected horse and duck tissues. The assay could detect all MVEV-positive samples and no other flaviviruses or negative samples were amplified by the assay. An interlaboratory comparison was performed to confirm the sensitivity, and specificity of the assay. These interlaboratory tests were carried out using the RT-PCR chemistry and thermocycling protocol developed in this study, by different operators and different real-time instruments. In each interlaboratory test, the assay only detected MVEV samples with highly reproducible and repeatable results, making it suitable for laboratory diagnosis.

RT-qPCR assay quantification requires standardisation of the assay with a reference molecule (Hugget et al., 2005). In previous studies, the lower LOD of MVEV specific RT-qPCR assay was determined by two different methods. Studies tested RNA that was purified from the ten-fold serial dilutions of viruses that were quantitated by plaque assay (Dash et al., 2012; Kwallah et al., 2013; Pyke et al., 2004; Shu et al., 2003), or using ten-fold serial dilutions of *in vitro*-transcribed RNA (Eiden et al., 2010; Leparc-Goffart et al., 2009; Santhosh et al., 2007; Schwaiger and Cassinotti, 2003). According to Mantel et al. (2008), several factors, including the methods and kits used for RNA purification, the number of samples that undergo purification at the same time, and inter-operator variability, substantially affect the accuracy of

the lower LOD using RNA purified from serial dilutions of seeded viruses. Hence, the synthetic RNA obtained from *in vitro* transcription is more suitable for characterisation of the LOD. From the previously reported RT-qPCR assays for the detection of MVEV, the assay designed by Harnett and Cattell (2010) does not report the lower LOD, while Pyke et al. (2004) reported a lower LOD of <1 PFU, which corresponded to *C*q values between 31 and 34. In this study, *in vitro*-transcribed RNAs encompassing RT-qPCR amplicons were constructed and used to determine the LOD of the assay for each genotype. The lower LOD of this assay is 10 RNA copies, which is comparable or better than the LOD reported in the other flavivirus RT-qPCR assays [e.g., 3 RNA copies in Eiden et al. (2010), 10 RNA copies in Schwaiger and Cassinotti (2003), 20 RNA copies in Santhosh et al. (2007) and 100-5000 RNA copies in Leparc-Goffart et al. (2009)]. The ability of the assay to detect very low RNA copies from these transcripts (Table 3.3 and Fig. 3.3), coupled with sensitive and specific detection of all MVEV genotypes, demonstrates its suitability for the detection and quantification of viral RNA in both clinical and laboratory settings.

In conclusion, the MVEV one-step RT-qPCR assay reported here is highly sensitive and specific for the detection of all MVEV genotypes. This assay may have application for virus surveillance of MVEV in the Australasian region. In addition, this assay may also be suitable for the molecular diagnosis of MVEV infection in humans and animals, with the potential for a significant impact on disease management. Further, this assay may be used in overseas diagnostic laboratories where imported MVEV infections need to be considered.

In this thesis, the MVEV-specific RT-qPCR assay was used to determine viral RNA copy number in MVEV environmental samples, before undergoing NGS for characterising the depth of genetic diversity (Chapter 4). Moreover, this assay was used to detect MVEV in the brains of mice as part of characterising virulence phenotypes of different strains of MVEV from the two co-circulating Australian genotypes (G1 and G2; Chapters 5 and 6).

CHAPTER 4

CHARACTERISATION OF GENETIC DIVERSITY AND MUTATION SPECTRA OF MURRAY VALLEY ENCEPHALITIS VIRUS POPULATIONS WITHIN INFECTED MOSQUITOES

4.1 Introduction

In Chapter 2, genetic diversity between MVEV populations at the genotype level was characterised using consensus sequences of virus isolates obtained by Sanger capillary sequencing technology. It was reported that G2 viruses of MVEV still circulated in the Kimberley region of WA, and G1 viruses were divided into two subgenotypes (G1a and G1b). One of the limitations of Sanger sequencing is its inability to detect low frequency variants, only being able to detect the majority of nucleotides at a given position. However, due to a large population size and rapid replication rate, driven by an error-prone and low fidelity RdRp, RNA viruses, including flaviviruses, circulate in nature as highly genetically diverse and interactive populations (Ciota et al., 2012; Ciota and Kramer, 2010; Drake and Holland, 1999; Jerzak et al., 2005; Jerzak et al., 2007; Steinhauer et al., 1992). This genetically-linked ensemble of variants within a viral population is called a quasispecies (Eigen and Schuster, 1977). These variants collectively interact on a functional level to represent the complex behaviour of viruses, such as providing varying fitness phenotypes that benefit virus population when presented with selection pressure and bottlenecks (Domingo et al., 2001; Lauring and Andino, 2010). Many studies have investigated the importance of quasispecies and their effect on viral phenotypes, some of which have been reviewed in Chapter 1 (Section 1.3.3). These studies revealed that the complexity (the number of various genomic sequences and the mean number of mutations in the viral population) of a quasispecies population affects viral fitness, adaptability, pathogenesis, virulence and response to antiviral drugs (Ciota et al., 2007a; Elena and Sanjuan, 2005; Jerzak et al., 2007; Perales et al., 2010; Vignuzzi et al., 2006).

Traditionally, the extent of genetic diversity within a flavivirus population has been determined by biological and molecular cloning and subsequent Sanger sequencing (Ciota et al., 2007c; Jerzak et al., 2005). Sanger sequencing only characterises the consensus sequence of each virus population, which represents the most frequent nucleotide at each position; minority sequences are not characterised in these studies. It is now evident that minority but phenotypically-important variants can

persist in a virus population without ever being fixed into the consensus sequence (Coffey and Vignuzzi, 2010; Sanz-Ramos et al., 2008). Therefore, to relate the genetic diversity of a flavivirus population to a particular phenotype, the consensus sequence provides an imperfect snapshot of the underlying genome. Moreover, due to their time-consuming and labour-intensive nature, the accurate estimation of the level of genetic diversity within a flavivirus population with adequate detail using cloning and Sanger sequencing is challenging.

More recently, NGS has been used to characterise the genetic diversity within a viral population at an extraordinary level of detail. NGS can detect low frequency mutations by massively paralleled sequencing of a sample with coverage of up to 10,000 reads for each base (Beerenwinkel and Zagordi, 2011; Su et al., 2011). Therefore, it can provide valuable information on the composition of the mutation spectra and genetic diversity within viral quasispecies populations (Beerenwinkel and Zagordi, 2011; Leamon et al., 2007; Mardis, 2008; Margulies et al., 2005; Wang et al., 2007).

So far, for flaviviruses, the use of NGS technology has been reported only for the detection of YFV in clinical specimens (McMullan et al., 2012) and DENV in mosquitoes (Bishop-Lilly et al., 2010) and for whole genome sequencing of MVEV, WNV and DENV (Henn et al., 2010; Mann et al., 2013). To date, the use of NGS for characterisation of intra-population genetic diversity and mutation spectra in a flavivirus population has not been reported. However, this technique has been used extensively to detect and characterise the complexity of quasispecies and mutant swarms of other RNA viruses (Fleury et al., 2013; Hedskog et al., 2010; Kuroda et al., 2010; Lauck et al., 2012; Nishijima et al., 2012; Solmone et al., 2009; Tsibris et al., 2009). These studies have demonstrated that NGS has been able to detect minor variants that were not detectable with standard Sanger sequencing. For example, Tsibris et al. (2009) and Görzer et al. (2010a) revealed that NGS was able to detect and quantify low-frequency variants (as low as 1%) of HIV-1 and human cytomegalovirus, respectively.

However, this technique comes with a few fundamental short-comings. All steps involved in this process are subject to errors. NGS technology has a higher error rate (~4-10 errors/kb) than conventional Sanger sequencing [~0.01 error/kb; (Huse et al., 2007; Wang et al., 2007)]. Point mutations, indels, and recombinant chimeric sequences can be generated during the RT-PCR and NGS steps, providing imperfect, incomplete and/or unreliable information on a viral population structure (Beerenwinkel et al., 2012; Görzer et al., 2010b; Malet et al., 2003). Furthermore, selective amplification bias during primary rounds of PCR can affect the relative frequency of genetic variants (Beerenwinkel and Zagordi, 2011). Therefore, NGS data needs to be further processed to adjust for confounding factors and provide more reliable estimation of the genetic diversity in a virus population (Beerenwinkel and Zagordi, 2011; Chen-Harris et al., 2013; Yang et al., 2012). Post-sequencing processing of NGS data involves multiple steps, including filtering to remove low quality reads, alignment of the filtered reads with a reference sequence, error correction of the reads, and frequency estimation of variants (Beerenwinkel and Zagordi, 2011; Chen-Harris et al., 2013).

This chapter reports the use of NGS technology to test the hypothesis that MVEV exists in nature as a highly genetically diverse complex of variants. Homogenates of mosquitoes were used in this study to avoid any artefacts associated with isolation in cell culture. Results obtained from this analysis reveal the complexity of the mutation spectrum and genetic diversity within MVEV populations in the two co-circulating Australian genotypes (G1 and G2) and show that differences exist in genetic diversity between viruses belonging to different genotypes and subgenotypes.

4.2 Materials and Methods

4.2.1 Viruses

Virus samples used in this study were kindly provided by the Arbovirus Surveillance and Research Laboratory, University of Western Australia, and consisted of eight MVEV-positive homogenates of mosquito pools that were collected as part of the

annual survey of mosquito fauna and arbovirus activities in WA. Each sample comprised 20 mosquitoes of a single species that were processed as described previously (Johansen et al., 2009).

4.2.2 Viral Concentration in Mosquito Homogenates

The concentration of MVEV in each mosquito homogenate was quantified using the MVEV-specific RT-qPCR assay, as described in Chapter 3 (Section 3.2.5). For each homogenate, the viral RNA copy number was calculated based on the standard curve that was generated using the *in vitro*- transcribed MVE-1-51 RNA.

4.2.3 RNA Purification, and RT-PCR

Total RNA was extracted from 140µl of mosquito homogenates using a QIAamp® Viral RNA mini Kit (QIAGEN, Australia), according to the manufacturer's protocol. Purified RNA was reverse transcribed using MVE-E1990R (Table 2.1) and the high fidelity SuperScript® III Reverse Transcriptase Kit (Life Technologies, Australia), as described in in Chapter 2 (Section 2.2.2).

A 675bp region was amplified using the forward primer (MVE-E1269F), the reverse primer (MVE-E1990R; Table 2.1) and the KAPA HiFi™ DNA Polymerase (KAPA Biosystems, USA), as per the manufacturer's protocol. Each reaction contained 10µl of cDNA, 0.3µM of each forward and reverse primers, 0.3µM of dNTP mix, 5µl of 1x KAPA HiFi Fidelity buffer, and 0.5U of KAPA HiFi DNA Polymerase to a final volume of 25µl. high-fidelity enzymes were used for reverse-transcription and amplification. The amplification was performed with the following thermocycling conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 98°C for 20 sec, 58°C for 15 sec and 72°C for 30 sec, and a final extension at 72°C for 5 min. The amplification was carried out in thin-walled 0.2ml PCR tubes using an Eppendorf AG thermal cycler. The PCR products were visualised on a 2% w/v agarose gel stained with ethidium bromide and purified using the QIAquick® PCR purification Kit (QIAGEN, Australia), according to the manufacturer's protocol. The concentration and purity of each DNA template was determined using a NanoDrop 2000 Spectrophotometer (Thermoscientific, Australia).

4.2.4 Quality Control

Prior to library preparation, an additional purification step was carried out using Agencourt® AMPure® XP Kit (Beckman Coulter, Australia), according to the manufacturer's protocol. The quality, quantity and length of each purified DNA fragment were determined using the Agilent 2100 bioanalyzer high sensitivity DNA chip (Agilent Technologies, Australia), according to the manufacturer's protocol. This step and subsequent DNA library preparation, emulsion PCR (emPCR), and NGS were performed at the Institute for Immunology & Infectious Diseases at Murdoch University, Perth, WA.

4.2.5 DNA Library Preparation

The DNA library was prepared using long-read GS FLX Titanium Rapid Library Preparation Kit XL+ (Roche/454, Australia), according to the manufacturer's protocol. The rapid library (RL) preparation was carried out as outlined below (steps 1-5 in Fig. 4.1).

Approximately 1µg of the purified DNA template from each sample (except K59536 for which only 0.55µg was available) was nebulised as per standard Roche/454 protocol, applying 15 psi of nitrogen for 1 min. The nebulised DNA fragments were purified using the QIAGEN MinElute PCR Purification Kit (QIAGEN, Australia), according to the manufacturer's protocol. Sixteen microlitres of purified products were end-repaired in a 25µl reaction containing 2.5µl of RL 10x polynucleotide kinase (PNK) buffer, 2.5µl of RL ATP, 1µl of RL dNTP, 1µl of RL T4 polymerase, 1µl of RL PNK, and 1µl of RL Taq polymerase. The reactions were mixed, centrifuged for 5 sec and incubated at 25°C for 20 min, followed by 72°C for 20 min. To ligate the unique RL multiplex identifier (MID) adaptors to each end-repaired product, 1µl of RL ligase was added to each reaction, mixed, centrifuged for 5 sec and incubated at 25°C for 10 min. The end-repaired-adaptor-ligated products were purified using Agencourt AMPure beads (Beckman Coulter, Australia), as per the manufacturer's protocol. The concentration of each sample was quantified in single use cuvettes using a TBS 380 Fluorometer (Promega, Australia). The quality of the DNA products was assessed using an Agilent 2100 Bioanalyser high sensitivity DNA chip (Agilent

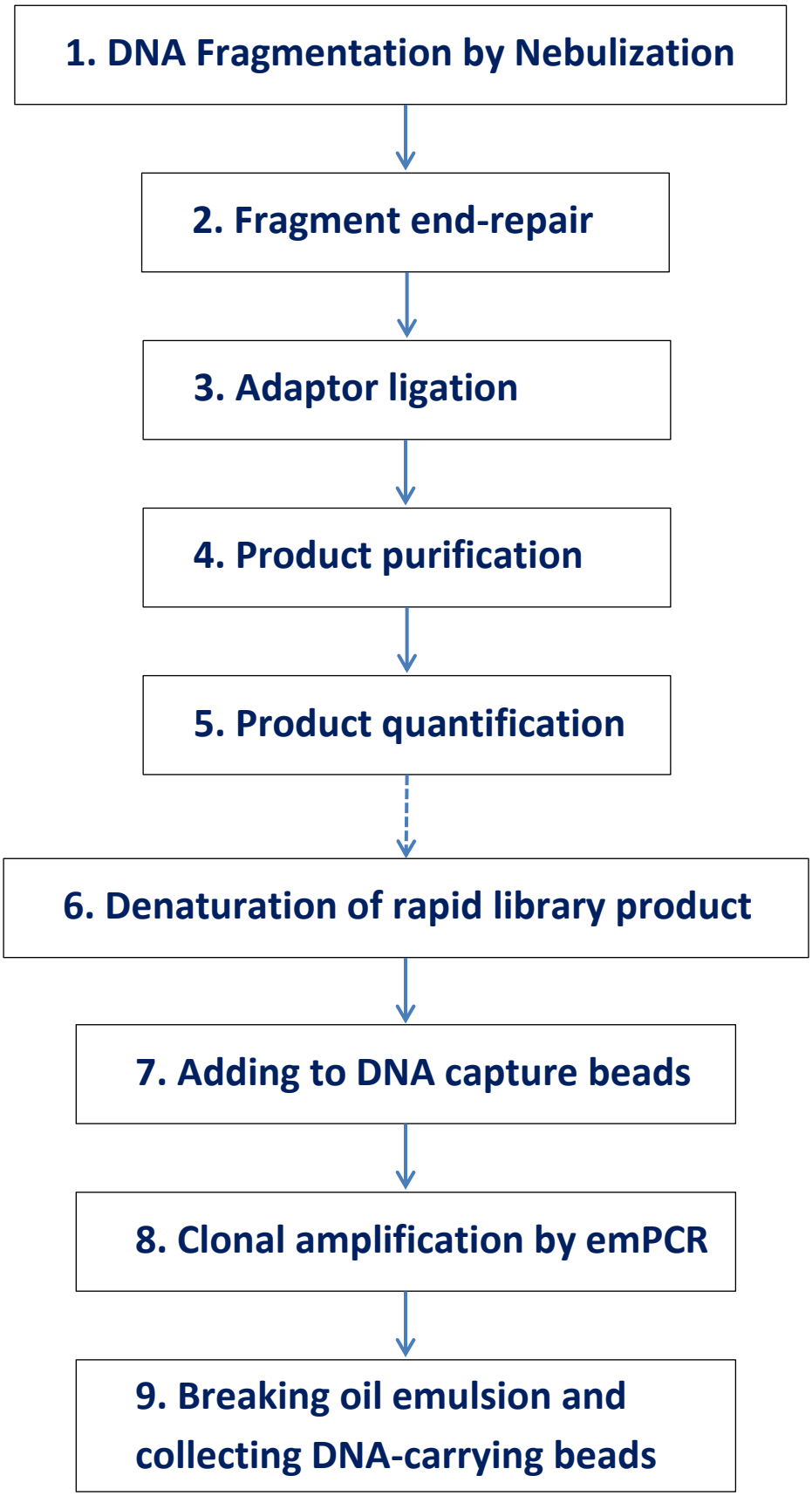


Fig. 4.1. *Schematic representation of the steps involved in the rapid library preparation and clonal amplification of DNA products by emulsion PCR.*

Technologies, Australia). The adaptor-ligated purified products were then pooled in equimolar ratios before undergoing the emPCR.

4.2.6 Clonal Amplification of Products by Emulsion PCR

The emPCR of DNA products was carried out using GS FLX Titanium SV emPCR Kit (Lib-L; Roche/454, Australia) and the GS Titanium emPCR Filters SV (Roche/454, Australia), according to the manufacturer's protocols. EmPCR was performed in four main steps outlined below (steps 6-9 in Fig. 4.1).

First, amplification and emulsion mixes were prepared by adding 290µl 1x Mock Amplification Mix to each tube of emulsion oil, mixed by inverting 3 times, and shaken at 25Hz for 5 min in a TissueLyser (QIAGEN, Australia). For each reaction, 80µl of DNA capture beads were removed and beads were washed twice with 1ml of 1x capture bead wash buffer TW. Purified DNA products (10µl) were denatured at 95°C for 2 min, added to DNA capture beads (approximately 2.4 x 10^6 beads for each reaction) and mixed. Next, 215µl of Live Amplification Mix was added to each reaction. The content of each tube was added to the emulsion solution (prepared above), mixed by inverting 3 times, and shaken at 15Hz for 5 min on TissueLyser (QIAGEN, Australia). After emulsification, 100µl of the emPCR amplification mix was added to each well of 96-well thermocyler plates (Bio-Rad, Australia); plates were sealed with microseal B (Bio-Rad, Australia), and subjected to thermocycling conditions of 94°C for 4 min, and 50 cycles of 94°C for 30 sec, and 60°C for 10 min.

At the termination of emPCR amplification, each sample containing the micro-reactor emulsions of clonally-amplified DNA molecules was drawn into a 10ml syringe using a 16 gauge blunt, flat tip needle. To disrupt emulsions, 100µl of isopropanol was then added to each well and mixed by pipetting up and down. The isopropanol rinse of each well was filtered using GS Titanium emPCR Filters SV (Roche/454, Australia), according to the manufacturer's protocol. The beads were then recovered from the filter, using GS FLX Titanium SV emPCR Kit (Lib-L; Roche/454, Australia), according to the manufacture's protocol. The DNA-positive beads were then purified and collected using Magnetic Enrichment Beads and a Magnetic Particle Concentrator (Roche/454, Australia), as per the manufacturer's

protocol and resuspended in 30µl of Annealing Buffer XT. Six microlitres of sequencing primer was added to each sample, incubated at 65°C for 5 min, and placed on ice for 2 min. Next, 500µl of Annealing Buffer XT was added to each tube, mixed, centrifuged and supernatants were discarded. This step was repeated two more times. The percentage of bead enrichment was determined according to the standard Roche/454 protocol. Finally, 100µl of Annealing Buffer XT was added to each tube before sequencing.

4.2.7 Next Generation Sequencing and Initial Filtering

Next generation sequencing in this study was performed on one of the eight lanes of the GS FLX+ instrument (Roche/454, Australia) using GS FLX Titanium Sequencing Kit XL+ (Roche/454, Australia) and 70x75 PicoTitrePlate (Roche/454, Australia), according to the manufacturers' protocols. The raw 454 data were processed, analysed and filtered by default Roche/454 GS FLX+ system software. Low quality reads were filtered out and the sequences were assigned to the original samples via 12-nucleotide barcodes. For each sequence, a .sff file was obtained, from which nucleotide sequence data (.fna file) and a *phred*-like quality score (.qual file) were extracted.

4.2.8 Sanger Sequencing

To facilitate NGS data alignment, filtering and variant analysis, all samples were subjected to conventional RT-PCR and capillary sequencing using MVE-E1269F and MVE-E1990R primers (Table 2.1), as described in Chapter 2 (Section 2.2.3).

4.2.9 Control Experiments

PCR and NGS errors: To accommodate for errors introduced during the RT-PCR, emPCR and NGS, the MVEV-1-51 plasmid (Chapter 3, Section 3.2.4) was directly sequenced by conventional Sanger sequencing. This was performed to provide a comparative sequence used to identify the level of mutations introduced over the course of the RT-PCR, emPCR and NGS. Similar to the RNA purified from the mosquito homogenates, the *in vitro*-transcribed MVE-1-51 RNA (Chapter 3, Section

3.2.4) underwent the RT-PCR, emPCR and NGS steps as mentioned above. Any differences between the plasmid sequence and the NGS sequences derived from *in vitro*-transcribed RNA of pMVE-1-51 were assumed to be due to PCR and NGS errors. Per-nucleotide variance of the MVE-1-51 was calculated after computation and statistical analysis (detailed below) and served as the baseline error rate of the PCR and NGS protocol in this study.

Effects of experimental protocol on relative amplification and quantification of minority genotypes: To ensure an equal and impartial sensitivity of experimental protocols used in this study on the amplification and quantification of minority sequences, an additional control was employed comprising a defined ratio of 1:10:89 of RNA from MVE-1-51 (G1), NG156 (G4) and OR156 (G2), respectively. Genome copy numbers of the three control RNAs were determined by the MVEV-specific RT-qPCR assay described in Chapter 3, then RNA from each virus strain was pooled prior to RT-PCR amplification.

4.2.10 Post-Sequencing Data Processing

A series of computational tools were utilised for the alignment, filtering, analysis and interpretation of each dataset. First, all sequences of each dataset were mapped with the sample-specific consensus sequence using Roche/454 FLX Newbler software, and a 400-nucleotide sequence window was identified. The sequence window spanned nucleotide positions 487-886 corresponding to amino acid positions 163-295 of the E protein of MVE-1-51 (GenBank accession No. NC000943). In the initial filtering step, sequences that did not span the entire sequence window and those with ambiguous nucleotides (Ns) were filtered out. Using ClustalW and Muscle softwares, the remaining sequences were aligned to the sequence window and the overhang nucleotides at both ends were trimmed. Since there were multiple counts of unique sequences present in each dataset, a binning process was performed, in which unique sequences were identified and counted. The nomenclature of each sequence comprised a unique identifier followed by the number of times it was found in the dataset. Then, nucleotide sequences were translated into amino acid sequences and aligned with that of the consensus

sequence. Homopolymer errors and frameshifts introduced by the NGS protocols were corrected and the reads were re-binned as above. The per-nucleotide variance of each processed and final dataset was calculated as the total number of nucleotide mutations observed divided by the total number of nucleotides in the dataset. For example, if at the termination of the filtering step there were 1000 sequences in a dataset 400-nucleotide long, and the total numbers of nucleotide mutations in all sequences were 2000, per-nucleotide variance was calculated as: 2000/(1000x400)=0.005.

4.2.11 Determination of Quasispecies and Mutant Swarm in MVEV Samples

The NGS sequence composition of the processed sequence dataset for each sample was compared to the consensus sequence. Genetic diversity and mutation spectra of each sample was determined by the proportion of sequences that differed from the sample-specific consensus sequence as well as the corrected per-nucleotide variance of each sample (calculated as the subtraction of per-nucleotide variance of the control dataset from the sample-specific per-nucleotide variance; see above). Two-tailed *t*-tests were performed to assess whether there were significant differences between the genetic diversity and complexities of mutation spectra of MVEV samples belonging to different genotypes/subgenotypes. Genetic diversity and single nucleotide polymorphism (SNP) was determined by determining the proportion of nucleotides that differed from consensus sequence at each variable site. To ensure true polymorphism, a nucleotide change in a sequence was considered as SNP only when it occurred at a frequency of 0.1% per site (approximately double the baseline error) and when there was at least two nucleotide mutations in that sequence. Mutations with a frequency of <0.1% could not reliably be classified as either a sequencing error or true SNP, and therefore were not accounted for.

4.2.12 Assessment of Selection Pressures

The operation of selection pressure was tested on each dataset separately in MEGA 5.2.1 as described in Chapter 2 (Section 2.2.6). Briefly, the hypotheses of neutral, purifying and positive selection were tested using the Z-test of selection averaged

over and between sequence pairs. In addition, a HyPhy analysis (Pond et al., 2005) was performed on each dataset to determine specific codons under adaptive evolution.

4.2.13 Phylogenetic Analysis

For each filtered dataset, an ML tree was constructed using MEGA 5.2.1 software. Initially, all sequences in each dataset were subject to a model test to find the best-fit substitution model, which was then used to construct the ML tree. The reliability of ML trees was estimated by applying bootstrapped support analysis of 1000 replicates.

4.2.14 Statistical Analysis

Two-tailed *t*-tests were performed to determine significant differences in measures of genetic diversity between the filtered and corrected datasets belonging to different genotypes/subgenotypes. Specifically, the per-nucleotide variance and the number of sequences that differ from the sample-specific consensus sequence for each sample were compared. The statistical analyses were performed using StatistiXL 1.10 software (Roberts and Withers, 2007). A *p*-vale of <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Sample Selection

Samples were selected to represent recent isolates of the two co-circulating Australian genotypes (G1 and G2), different mosquitoes vector species, geographical regions and time of collection (Table 4.1). In addition, attempts were made to select samples with a comparable viral genome copy number determined by RT-qPCR (Chapter 3). The latter was performed to ensure that population sizes in all samples were comparable. Attempts to select mosquito samples infected with G2 MVEV from different geographic areas could not be achieved, since all G2 infected specimens included in this study were collected from Fitzroy Crossing. The only infected mosquito pool collected from Broome (K62017) could not be included

Table 4.1. *Details of Murray Valley encephalitis viruses used in this study.*

Samples	Genotype	Year of collection	Location of collection	Species of origin *	Viral copy No.†
K68320	G1a	2009	Billiluna (SE Kimberley)	*Cx. annulirostris*	7.11
K69612	G1a	2009	Kununurra (NE Kimberley)	*Cx. pullus*	7.37
K68150	G1b	2009	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	7.18
K60555	G1b	2006	Kununurra (NE Kimberley)	*Cx. annulirostris*	7.05
P8891	G1b	2006	Karratha (W Pilbara)	*Ae.normanensis*	6.70
K59532	G2	2006	Fitzroy Crossing (W Kimberley)	*Cx. annulirostris*	5.85
K59536	G2	2006	Fitzroy Crossing (W Kimberley)	*Cx. pullus*	4.36
K68196	G2	2009	Fitzroy Crossing (W Kimberley)	*Cx.annulirostris*	6.25

**** Cx:*** *Culex;* ***Ae:*** *Aedes*

† Numbers represent the Log_{10} viral copy number in 140µl of the mosquito homogenates used for RNA purification in this study.

in this study because it did not yield high concentration of viral RNA. The genome copy numbers of G1 samples included in this study were between 6.70 to 7.37 $\log_{10}$, whereas G2 samples contained between 4.36 to 6.25 $\log_{10}$ viral genome copies (Table 4.1).

4.3.2 Assessment of the Accuracy of the Experimental Design

To determine the extent of RT-PCR and NGS error, and to differentiate naturally occurring mutations from amplification and sequencing errors, a clonal population of MVEV RNA was included in this study. This was derived from the pMVE-1-51 infectious clone by *in vitro* transcription, and underwent the same experimental procedures as test samples. The target sequence region for NGS was also determined for the pMVE-1-51 by direct sequencing of the plasmid by conventional sequencing. This sequence was used as a reference for the computational analyses of the NGS of pMVE-1-51-derived RNA. Thus, observed nucleotide differences between the NGS-derived sequences to the reference sequence of pMVE-1-51 provided an indication of experimental error. The overall per-nucleotide variance of the plasmid sequence dataset was 0.057%. This empirically observed per-nucleotide variance was used as the baseline reference to distinguish authentic low-frequency variants from sequencing errors. Corrected per-nucleotide variance measures were calculated for each test sample by subtraction of plasmid baseline error (0.057%) from the sample-specific per-nucleotide variance. The corrected per-nucleotide variance more accurately demonstrates the complexity of the quasispecies of each sample.

A second control sample was included in this study to ensure the experimental and computational procedures applied were not biased towards specific or low frequency MVEV genotypes or sequences. For this purpose, a defined input ratio of 1:10:89 of viral genome copies of G1 (MVE-1-51), G4 (NG156) and G2 (OR156) viruses, respectively, were pooled and underwent the same experimental procedure as the test samples. After computational analysis an output ratio of 80.9%, 15.2% and 3.9% were obtained, indicating that the ratio of the input RNA was relatively well preserved throughout the RT-PCR and sequencing steps and that

significant sequencing bias did not occur. Therefore, any differences in the level of genetic diversity and the complexity of mutation spectra observed between different genotypes and subgenotypes in this study were considered a genuine quasispecies phenomenon.

4.3.3 RT-PCR and Sequencing

Purified viral RNA from mosquito homogenates and controls were used to template reverse transcription and PCR using high fidelity enzymes to minimise the introduction of nucleotide errors. Purified amplicons were sequenced by both Sanger sequencing and NGS, in order that consensus (Sanger) sequences could be used as references in subsequent bioinformatics filtering steps.

4.3.4 Data Filtering

NGS raw sequencing datasets were initially processed by the instrument's default software. A total of 31,167,084 nucleotides were obtained for all samples tested, with a read length of 530 ± 215 nucleotides. The maximum read length obtained was 791 nucleotides while the shortest was 40. Due to a large number of sequences with variable lengths (Appendices 4.1 and 4.2), start and end positions, a series of bioinformatics tools were developed to align, filter and analyse the NGS data. By assessing the coverage plots of all datasets, a 400-nucleotide sequence window was determined to achieve optimal coverage of the majority of sequences. The sequence window contains the glycosylation site and flexible hinge region of the E protein. For each dataset, the sample-specific Sanger sequence was used as a reference for aligning and filtering the NGS data. In the initial step of data filtering, 47.5 ± 3.6% of the reads with ambiguous nucleotides (Ns) and those that did not span the entire window sequence were removed (Table 4.2). Next, sequences that contained frameshifts and indels were filtered out (Table 4.2). The remaining sequences were re-aligned to the reference sequence for each sample and the number of unique sequences determined (Section 4.2.10). Six samples contained sequences with a single stop codon (Table 4.2), ranging in frequency between one sequence (K69612 and K59532) and 13 sequences (P8891). At the end of data processing and filtering steps, 31.1 to 41.0% of G1 and 39.0 to 44.6% of G2

sequences remained of the original sequence datasets (Table 4.2). The final filtered sequence alignment for each sample contained unique sequences comprising singletons and redundant clones.

4.3.5 Intra-Population Genetic Diversity and Phylogenetic Analyses

Despite filtering out a large proportion of reads from the raw datasets, a large number of high quality reads were retained and used for the characterisation of mutation spectra and quasispecies within each MVEV sample. Genetic diversity was assessed by considering the per-nucleotide variance within each sample dataset, as well as the proportion of sequences that differed from the sample-specific consensus sequence. To determine the complexity and phylogenetic relationship of the variants, an ML tree was constructed using sequence sets for each sample. Genetic diversity of the samples within each genotype/subgenotypes is explained separately below.

4.3.5.1 Genotype 1a

For the K68320, 466 reads were obtained after filtration, 262 (56.2%) of which matched the consensus nucleotide sequence with 100% identity and represent the dominant clonal sequence in this virus sample (Fig 4.2). The remaining 204 (43.8%) sequences differ from the consensus sequence by one to 30 nucleotides. Of these, 110 (23.6%) are identical to each other (Fig. 4.2), but differ from the consensus sequence by 27 nucleotides (four amino acids). The remaining sequences are minority variants, most of which (45) are singletons. Four of the singletons exhibited a single stop codon in their encoded amino acid sequence (Table 4.2). The corrected per-nucleotide variance of this isolate is 2.285%. The ML phylogenetic tree clearly showed two distinct clades for this sample (Fig. 4.2). Interestingly, the two most-frequent sequences are located on two separate clades, indicating a distinct phylogenetic relationship between the major variants in this sample.

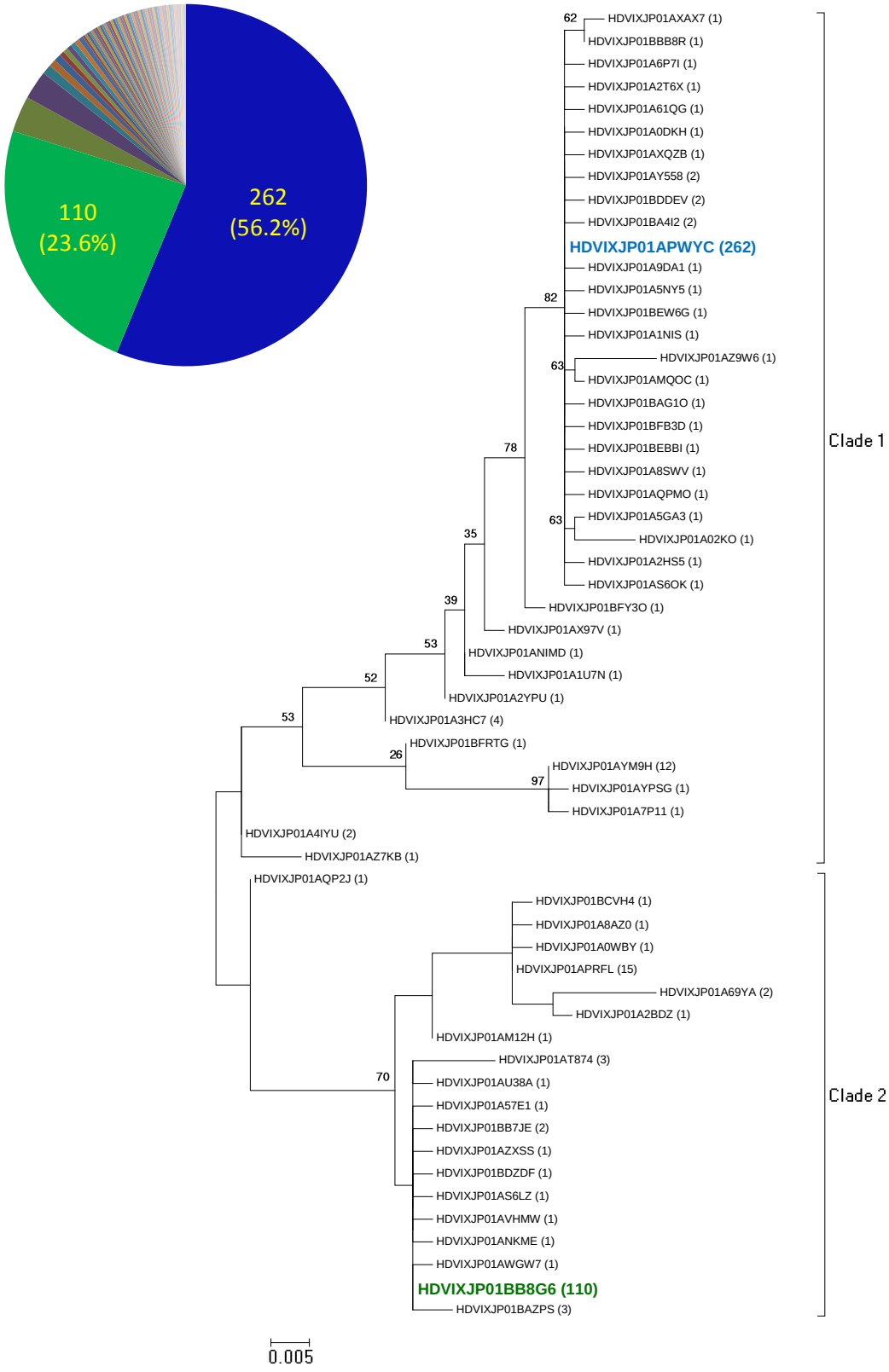


Fig 4.2. *Maximum likelihood phylogenetic tree constructed using K68320 (genotype 1a) sequences. The pie chart demonstrates the relative abundance of the variants.*
The dominant sequence clones are highlighted as **blue**: consensus; and **green**: the most frequent variant that differ from consensus sequence. Variants that occur ≤5% are not indicated.

Table 4.2. *Viral genome copy numbers, read metrics of steps in the sequence filtering pipeline, and estimates of genetic variation for each sample of Murray Valley encephalitis virus tested.*

Isolates	Genotype	Viral copy No.	Raw reads	No. of reads remaining after filtering							
				<400-nt window, Ambiguous nt.	Frameshifts, indels	Unique sequences	Singletons	Redundant sequences	sequences with stop codon	Sample-specific Per-nucleotide Variance (%)*	Corrected Per-nucleotide variance (%) †
K68320	G1a	7.11	1,449	710	466	58	45	13	4	2.342	2.285
K69612	G1a	7.37	357	188	111	24	16	8	1	2.887	2.830
K68150	G1b	7.18	10,204	5,160	3,689	284	190	94	8	0.275	0.218
K60555	G1b	7.05	3,145	1,834	1,288	100	73	27	3	0.149	0.092
P8891	G1b	6.70	21,559	11,599	8,153	434	260	174	13	0.213	0.156
K59532	G2	5.85	1,704	860	726	54	44	10	1	0.098	0.041
K59536	G2	4.36	270	131	105	4	3	1	0	0.012	-0.045
K68196	G2	6.25	65	37	29	4	3	1	0	0.034	-0.023
							Control Dataset per-nucleotide variance			**0.057**	

* Sample-specific per-nucleotide variance for each sample was calculated as the number of nucleotide mutations observed in a dataset divided by the total number of nucleotides in the dataset.

† Corrected per-nucleotide variance was calculated as the subtraction of control dataset per-nucleotide variance (baseline error) from the sample-specific per-nucleotide variance.

For K69612, a total of 111 sequences were obtained after filtration, 54 (48.6%) of which exactly matched the consensus sequence. The remaining 57 (51.4%) sequences differed from the consensus sequence; 23 (20.7%) are identical to each other (Fig. 4.3), but differ to the consensus sequence by 27 nucleotides and four amino acids. There are 16 singletons in the K69612 sequence set, only one of which contains a (single) stop codon (Table 4.2). The corrected per-nucleotide variance of this sample was 2.830%, comparable to that of K68320 (above). As for K68320, in the ML phylogenetic tree, two clearly distinct clades were observed for this isolate (Fig. 4.3). Interestingly, the two major variants also belonged to two separate clades, again indicating a distinct phylogenetic relationship between the most frequent variants in this virus sample.

Overall, G1 isolates (K68320 and K69612) demonstrated a complex mutation spectrum in their sample population, with a comparable level of genetic diversity as measured by per-nucleotide variance, the proportion of mutant sequences that differ from consensus sequence as well as phylogenetic analysis. Furthermore, the results demonstrate no substantial differences in the genetic diversity between viruses vectored by different mosquito species (*Cx. annulirostris* for K68320 and *Cx. pullus* for K69612), suggesting that different species of mosquitoes do not considerably affect the genetic diversity in G1a MVEV populations.

4.3.5.2 Genotype 1b

In the case of K68150, at the completion of filtering steps, 3,689 reads remained, of which 2,651 (71.9%) exactly matched to the consensus nucleotide sequence. The remaining 1,038 (28.1%) reads differed from the sample-specific consensus sequence, 372 (10.1%) of which only differed by four nucleotides and one amino acid residue. In the final sequence dataset, there are 190 singletons. Eight sequence clones contained a single stop codon, four of which are singletons. The corrected per-nucleotide variance of this sample was 0.218%, at least 10-fold lower than the comparative values of the G1a viruses analysed, indicating a much lower level of genetic complexity in the K68150 virus population. The ML phylogenetic tree

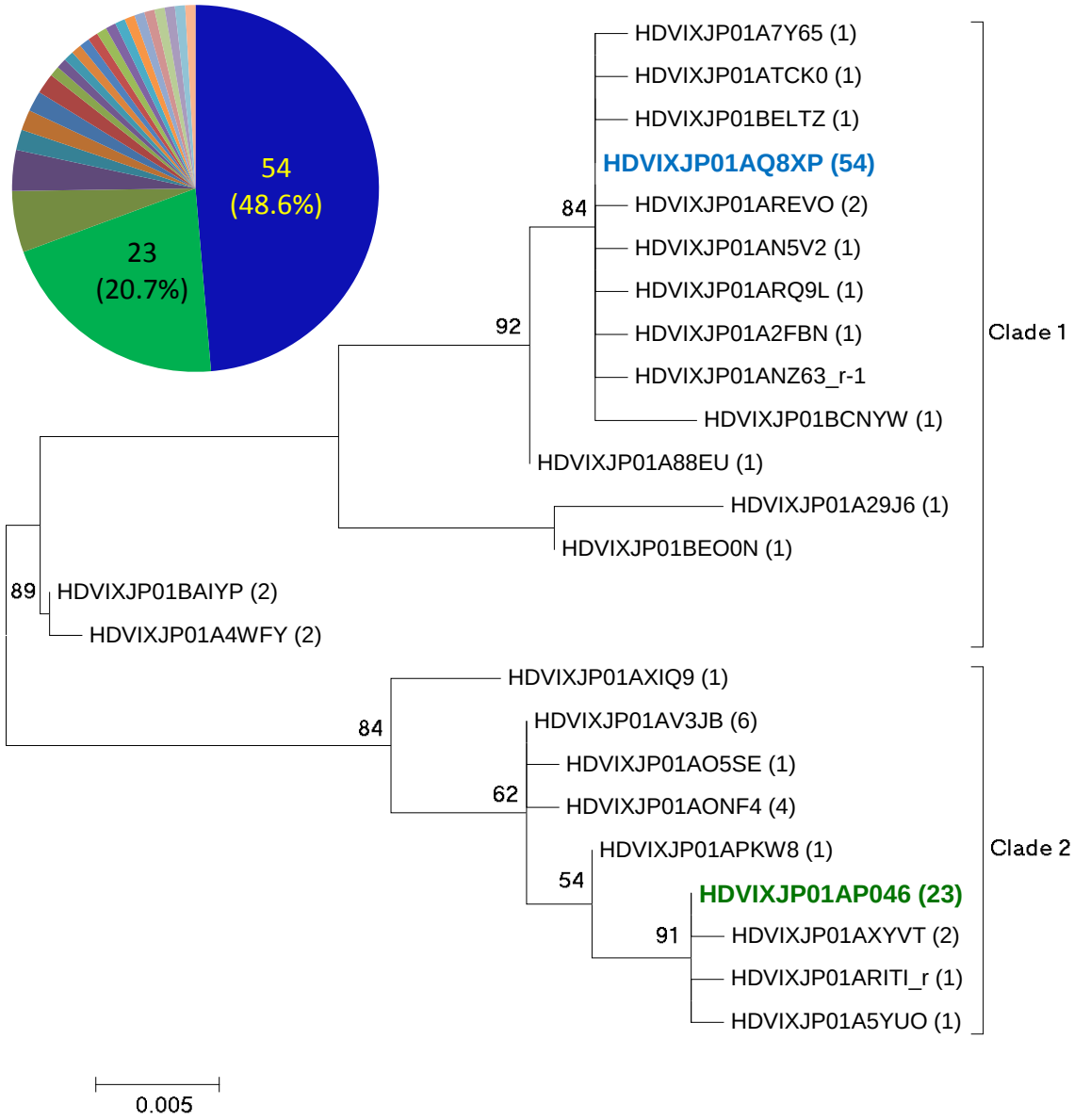


Fig 4.3. *Maximum likelihood phylogenetic tree constructed using K69612 (genotype 1a) sequences. The pie chart demonstrates the relative abundance of the variants.*

For legend, please refer to page 17.

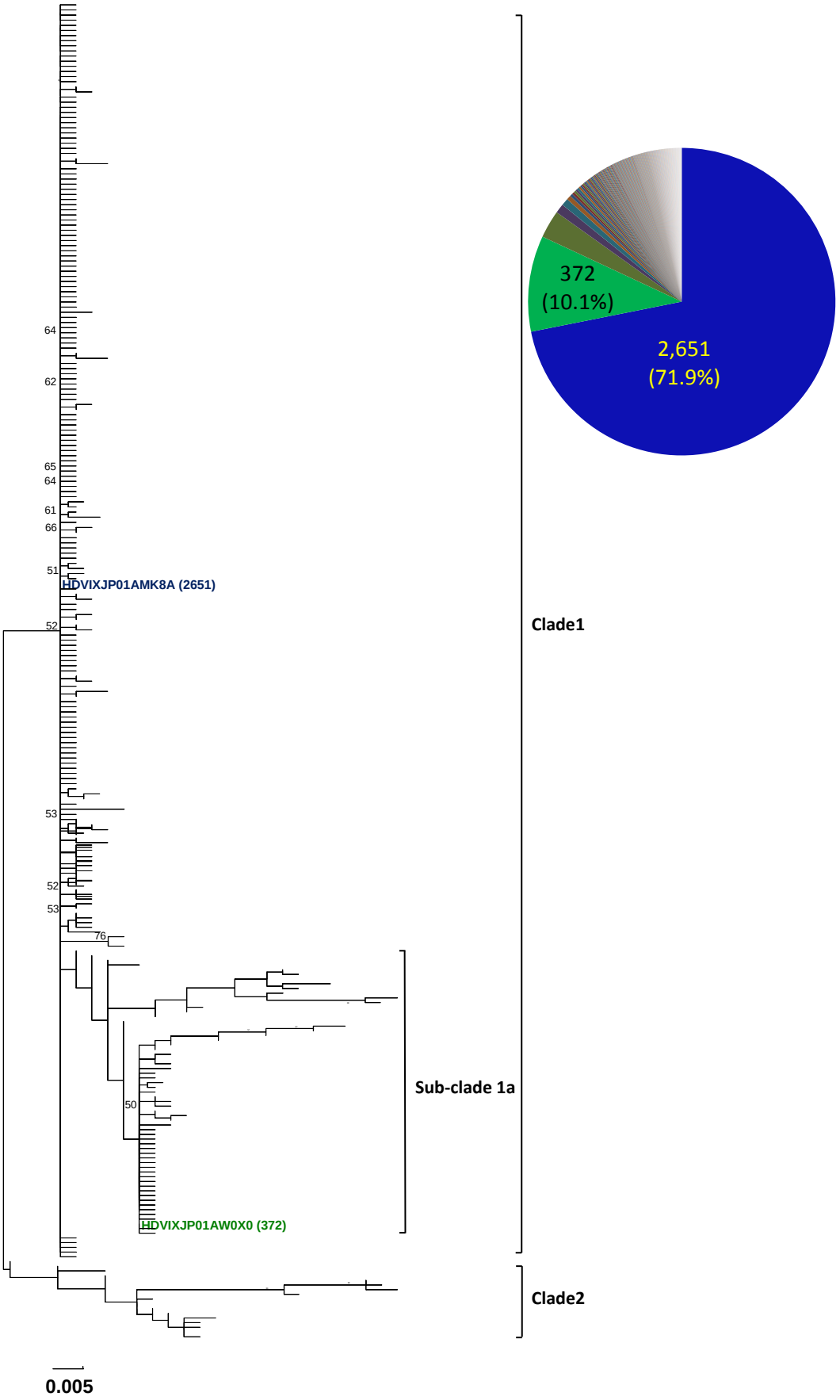


Fig. 4.4. *Maximum likelihood phylogenetic tree constructed using K68150 (genotype 1b) sequences. The pie chart demonstrates the relative abundance of the variants.*

For legend, please refer to page 17.

showed two distinct clades for this sample (Fig. 4.4). Furthermore, clade 1 also contained a sub-clade 1a. The two most-frequent variants are located on the main branch of clade 1 and in sub-clade 1a. Clade 2 only contains 58 (1.57%) counts of all sequences within this virus population, and therefore represents a minority lineage. Variants within sub-clade 1a and clade 2 showed a higher level of heterogeneity, with higher levels of nucleotide distance occurring between sequences within each of these groups (Fig. 4.4). The remaining sequences of clade 1 appear to be more homogeneous.

For K60555, 1,288 sequences remained after the filtering steps, of which 1,109 (86.1%) had 100% nucleotide identity to the consensus sequence (Fig. 4.5), and made up the dominant sequence clone in this virus sample. The remaining 179 (13.9%) reads consisted of minority variants that differed by one to 19 nucleotides from the consensus sequence. There were 27 singletons in K60555, three of which contain a single stop codon (Table 4.2). The corrected per-nucleotide variance of this sample was 0.092%, indicating a much lower level of genetic diversity and complexity within this virus population compared to K68150. The ML phylogenetic tree demonstrates two distinct clades. The most frequent variant belongs to clade 1, which also contains the majority (97.98%) of the total sequences of this sample. Clade 2 only contains 26 variants of K60555 (Fig. 4.5), representing a minority lineage.

For the final G1b isolate (P8891), 8,153 sequences remained following the filtering steps, of which 6,711 (82.3%) were identical to consensus nucleotide sequence. The remaining 1,442 (17.7%) sequences were minority variants that differed from the consensus sequence by 1-31 nucleotides (1-4 amino acids), and 260 of these were singletons. Thirteen sequence clones from this sample contained a single stop codon, nine of which were singletons. The corrected per-nucleotide variance of this isolate was 0.156%, higher than K60555, but lower than K68150. Phylogenetic analyses of P8891 sequences demonstrated three separate clades (Fig. 4.6). Clade 1 is further divided into several sub-clades (1a-1f; Fig. 4.6) each containing variable level of genetic diversity. The most frequent variant of this sample belongs to clade

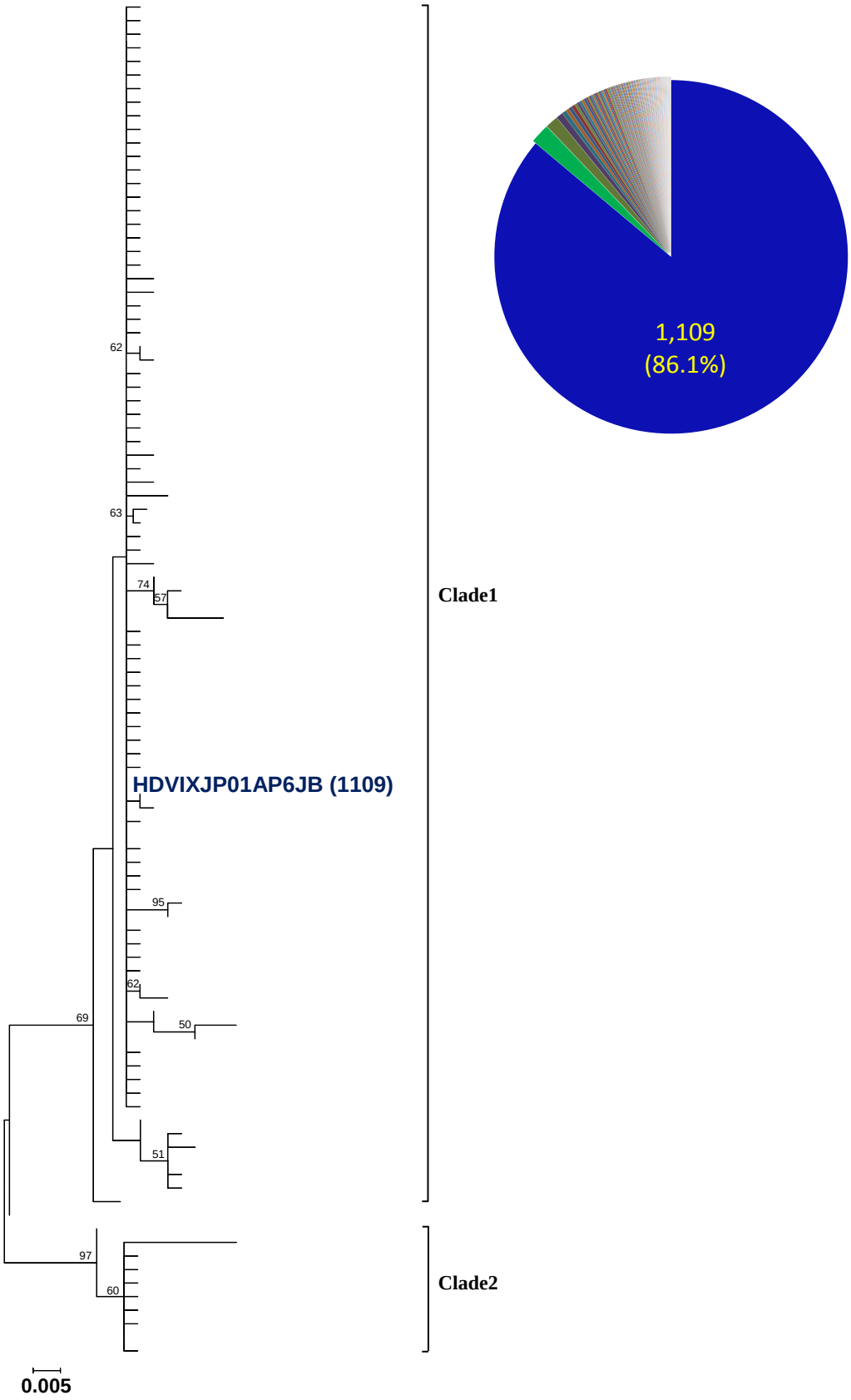


Fig. 4.5. *Maximum likelihood phylogenetic tree constructed using K60555 (genotype 1b) sequences. The pie chart demonstrates the relative abundance of the variants.*

The dominant sequence clones are highlighted **blue.** Variants that occur ≤5% are not indicated.

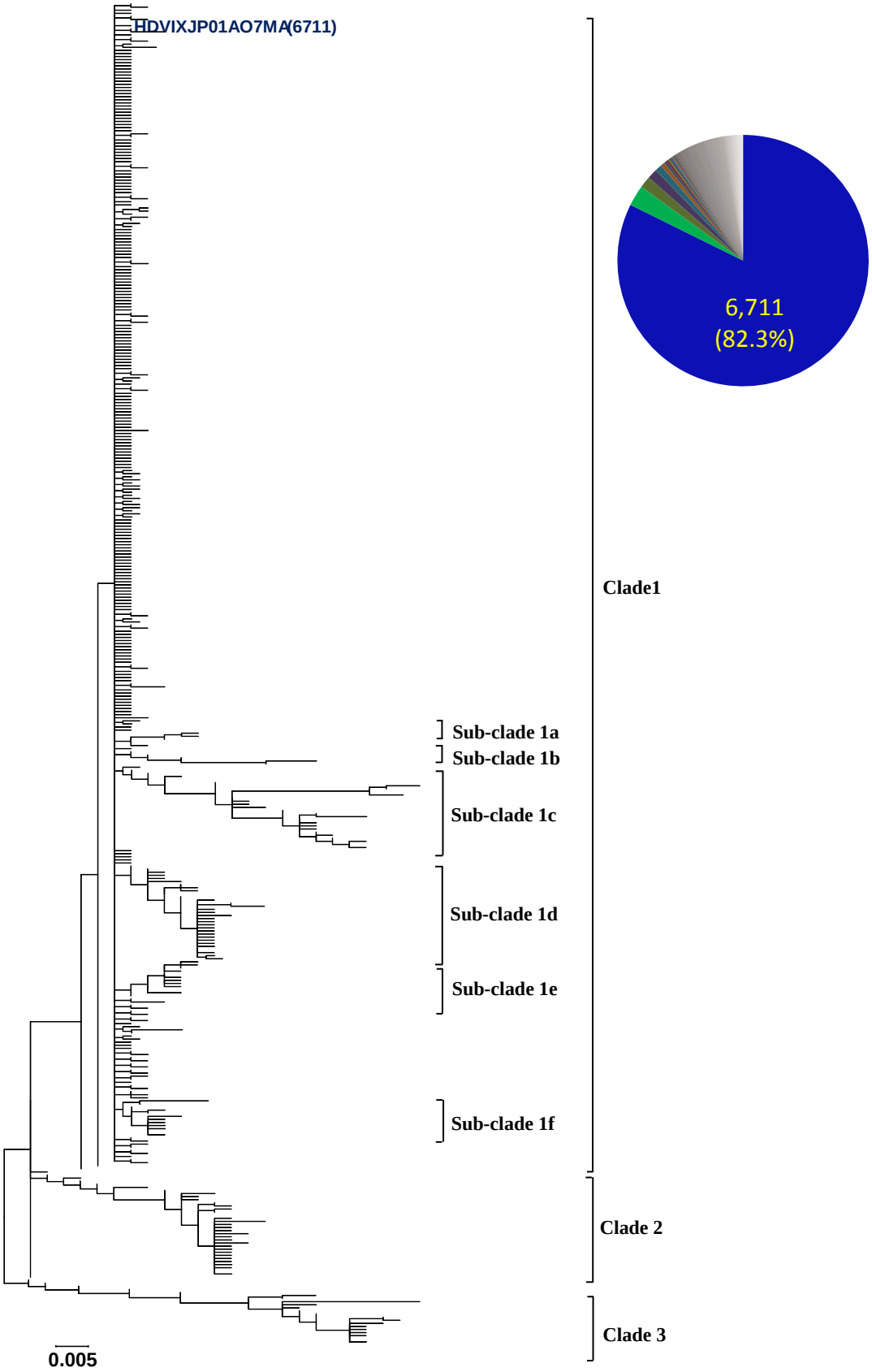


Fig. 4.6. *Maximum likelihood phylogenetic tree constructed using P8891 (genotype 1b) sequences. The pie chart demonstrates the relative abundance of the variants.*
The dominant sequence clone are highlighted **blue.** Variants that occur ≤5% are not indicated.

1. Clade 2 and 3 were minority subsets of sequences that demonstrated a considerable level of intra-clade genetic diversity. Clade 2 contained 166 (2.0%) sequences, whereas clade 3 only contained 56 (0.7%) of the total sequences of this virus sample (data not shown).

Altogether, consistent with the high numbers of raw reads obtained for G1b samples, these also contained higher numbers of unique sequence clones compared to G1a. However, the proportion of sequence clones that differed from sample-specific consensus sequence is significantly lower in G1b isolates when compared to G1a (p=0.021; Appendix 4.3). Similarly, the per-nucleotide variance of G1b virus populations was significantly less than those of G1a (p=0.001; Appendix 4.4). These two measures indicate that the complexity of mutation spectra is lower in G1b quasispecies populations than G1a.

As per G1a samples, there is no substantial differences between the genetic diversity of G1b samples from different mosquito species (*Cx. annulirostris* for K68150 and K60555, and *Ae. normanensis* for P8891), indicating that the species of mosquito vector also does not impact on the level of genetic diversity of G1b virus populations.

4.3.5.3 Genotype 2

Initially, significantly lower concentrations of G2 viruses compared to G1 viruses were obtained from homogenates of G2-infected mosquito pools (Table 4.2). For K59532, a total of 726 sequences were obtained following the filtration steps, of which 653 (89.9%) exactly matched the nucleotide consensus sequence. The 73 remaining sequences (10.1%) consisted of low frequent variants, including 44 singletons that differed from the consensus sequence by 1 to 14 nucleotides (two amino acids). Only one sequence with a single stop codon was found in this G2 sample. The corrected per-nucleotide variance of K59532 was 0.041% lower than that of G1 samples, indicating the presence of a less diverse quasispecies population. The phylogenetic relationship of the sequences within the K59532 population is depicted in Fig. 4.7. Two distinct clades could be observed. The most

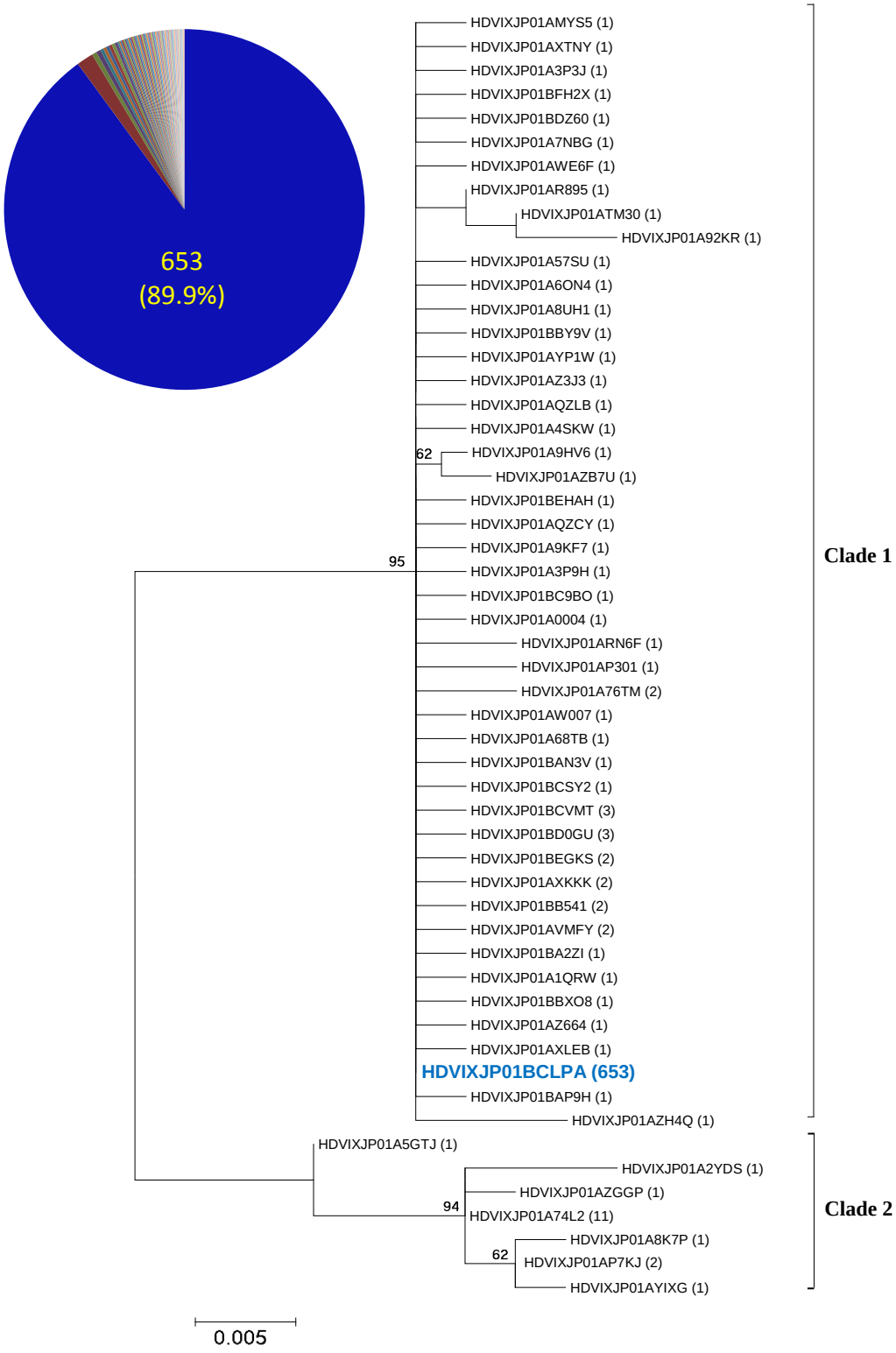


Fig. 4.7. *Maximum likelihood phylogenetic tree constructed using K59532 (genotype 2) sequences. The pie chart demonstrates the relative abundance of the variants.*
The dominant sequence clone is highlighted **blue.** Variants that occur ≤5% are not indicated.

frequent variant in this sample belonged to the clade 1, which contained the majority of sequence clones in this sample. Clade 2 is minority lineage, consisting of only 18 (2.48%) sequences.

For K59536, a total of only 105 sequences were obtained after filtering; 102 (97.1%) of these were 100% identical to the consensus nucleotide sequence. Three (2.9%) remaining sequences were singletons showing one to three nucleotides mutations (zero to one amino acid change). No sequences with a stop codon were observed in this sample. The corrected per-nucleotide variance of K59536 was -0.012% below the baseline variance of the control dataset (Table 4.2). This indicates a very high level of sequence homogeneity in this sample. The ML phylogenetic tree demonstrating the relationships between the K59536 sequences is displayed in Fig. 4.8.

For the K68196 sample, a total of only 29 filtered sequences were obtained, of which 26 (89.7%) exactly matched the consensus sequence. Three remaining sequences (10.4%) were singletons (Table 4.2 and Fig. 4.9), differing from the consensus sequence by one to two nucleotides and only one amino acid. No stop codon was observed in any of the K68196 sequences. As for K59536, the per-nucleotide variance of this sample was lower than the baseline error rate (0.034%), and also indicates a high level of genetic homogeneity between the sequences of this sample. The phylogenetic relationships between the sequences of this sample are displayed in Fig. 4.9.

As for G1 samples, a similar level of genetic diversity was observed between G2 viruses found in different mosquito species (*Cx. annulirostris* for K59532 and K68196, and *Cx. pullus* for K59536). This indicates that the depth of genetic diversity of G1 and G2 MVEV viral populations is not substantially affected by different species of mosquito vectors.

Altogether, the G2 samples demonstrated a much lower level of genetic diversity than the G1 samples, indicating that the populations of the G2 viruses are highly homogeneous. It should also be emphasised that the raw sequence reads and subsequent filtered sequence clones obtained for K59536 and K68196 were very much lower than for other viruses (Table 4.2), which may have affected sampling of minority variants.

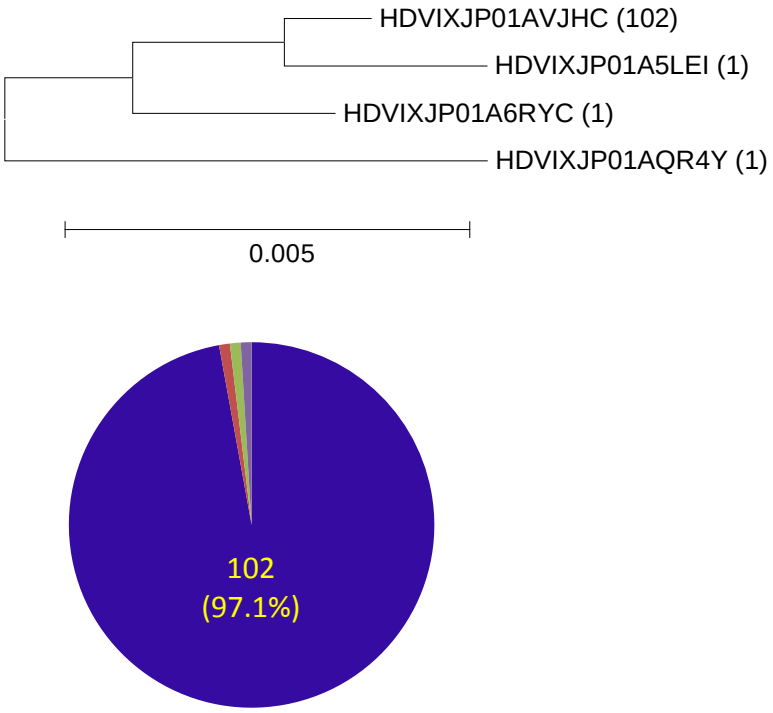


Fig. 4.8. *Maximum likelihood phylogenetic tree constructed using K59536 (genotype 2) sequences. The pie chart demonstrates the relative abundance of the variants.*
The dominant sequence clone is highlighted **blue.** Variants that occur ≤5% are not indicated.

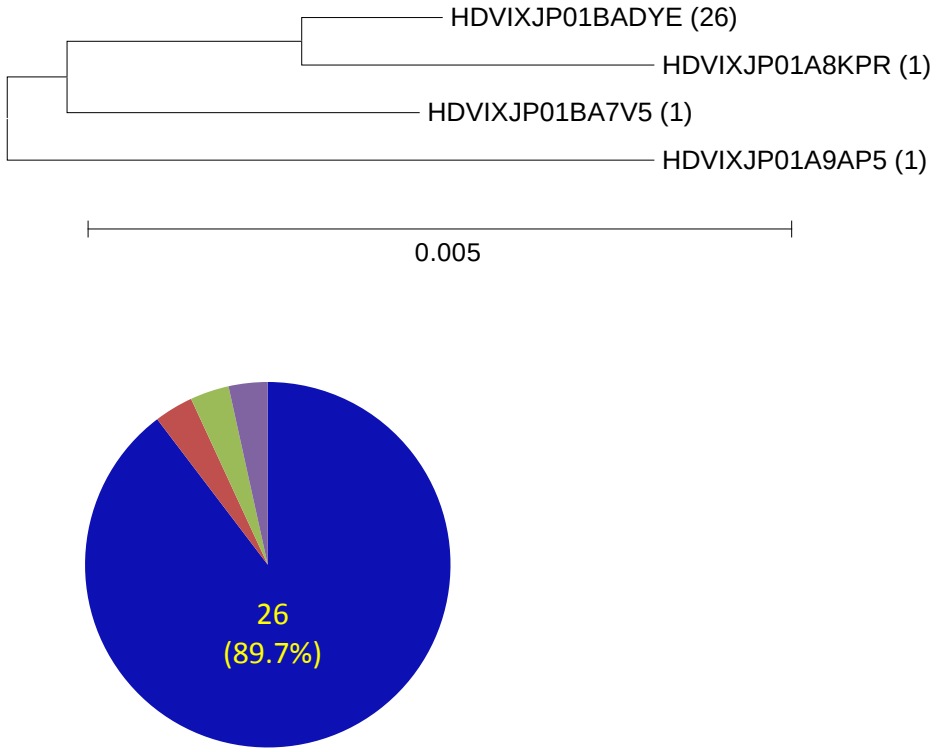


Fig. 4.9. *Maximum likelihood phylogenetic tree constructed using K68196 (genotype 2) sequences. The pie chart demonstrates the relative abundance of the variants.*
The dominant sequence clone is highlighted **blue.** Variants that occur ≤5% are not indicated.

4.3.6 Analysis of Single Nucleotide Polymorphism

In order to assess the level of diversity and polymorphism at each variable site in the filtered sequence alignment of each virus sample, the proportion of mutations at each site was calculated (Tables 4.3 and 4.4). Interestingly, samples within G1a and G1b shared non-consensus changes at identical positions. G1a viruses shared nucleotide polymorphisms at 30 different positions scattered throughout the 400-nucleotide region of the E gene analysed (Table 4.3). These nucleotide changes resulted in only four substitutions in the encoded amino acid sequences of the minority variants, including $L^{180}M$, $I^{238}V$, $S^{275}P$ and $S^{277}N$. The $L^{180}M$ and $I^{238}V$ substitutions are identical with G2 viruses, whereas $S^{275}P$ and $S^{277}N$ substitutions were not observed in any G2 viruses. The latter substitutions were located on the flexible hinge region (Fig. 2.4), which acts as a hinge between domain I and domain II of the E protein. G1b viruses displayed polymorphism at 31 nucleotide positions (Table 4.3), which resulted in only two amino acid substitutions ($P^{275}S$ and $S^{277}N$) in the flexible hinge region of the minority variants. These two amino acid substitutions were not seen in any G2 virus samples.

In G2 viruses, a lower level of polymorphism was observed compared to G1 viruses. In K59532, a total of 12 nucleotide positions demonstrated SNPs (Table 4.4), only one of which encoded an amino acid substitution at position 222 ($L^{222}P$) in minority variants. SNPs were also observed in three nucleotide positions in the K59536 virus population, none of which changed the amino acid sequence. For the K68196, only two SNPs were observed, one encoding an amino acid substitution at position 240 ($M^{240}I$) in minority variants. None of the amino acid substitutions in the G2 viruses were identical to amino acids encoded by G1 viruses at corresponding positions.

4.3.7 Assessment of Selection Pressures

The operation of selection pressure on each dataset was assessed by employing the Z-test of selection. Table 4.5 summarises the results of testing positive, purifying and neutral selection averaged over all sequences in each dataset. As the *p*-value is more than the significance level (0.05) for the test of positive selection, these

Table 4.3. *Single nucleotide polymorphisms between nucleotides 487-886 of the E gene of genotype 1a and genotype 1b MVEV viruses and their relative proportion (%) within their respective sequence datasets.*

Nucleotide			Genotype 1a				Genotype 1b		
Position	Cons*	Var†	K68320	K69612	Cons*	Var†	K68150	K60555	P8891
489	A	G	34.5	36.0	G	A	2.0	2.2	3.1
513	G	A	37.6	40.5	A	G	0.4	0.2	0.6
519	C	T	37.6	40.5	T	C	0.4	–	0.6
538	T	A ‡	37.6	40.5	A	T	0.3	–	0.7
561	T	C	34.3	36.9	C	T	2.0	2.3	3.1
570	T	T	5.2	10.8	C	T	17.9	2.3	3.7
586	C	A	4.9	10.8	T	C	14.7	2.3	3.7
594	G	C	36.9	41.4	A	G	0.4	–	0.7
600	T	C	36.7	41.4	C	T	0.4	–	0.6
606	C	T	33.7	37.8	T	C	1.8	2.2	3.0
609	C	T	29.0	17.1	C	T	12.8	4.7	6.7
630	C	T	32.8	37.8	T	C	1.8	2.3	3.1
639	G	A	32.8	37.8	A	G	1.8	2.2	3.1
657	C	T	35.8	40.5	T	C	0.3	–	0.7
660	C	T	35.8	40.5	T	C	0.3	–	0.7
693	G	A	33.0	36.9	A	G	1.9	2.0	3.0
711	A	G	32.6	36.9	G	A	1.9	2.0	3.0
712	A	G ‡	32.6	36.9	G	A	1.7	2.0	3.0
756	G	G	27.5	25.2	G	A	12.6	4.3	6.6
763	C	T	35.2	39.6	T	C	0.4	–	0.7
789	T	C	35.2	39.6	C	T	0.3	–	0.7
816	T	C	–	47.7	C	T	0.1	–	0.1
822	T	C	31.8	36.0	C	T	1.8	2.2	2.9
823	T	C ‡	31.8	36.0	C	T ‡	1.7	2.2	2.8
830	G	A ‡	27.3	36.9	G	A ‡	12.3	4.4	6.4
831	T	C	35.0	16.2	C	T	0.3	–	0.7
840	G	A	35.0	39.6	A	G	0.3	–	0.7
852	G	A	35.0	39.6	A	G	0.4	–	0.7
861	G	A	31.1	36.0	A	G	1.8	2.3	2.8
870	G	A	31.1	32.4	A	G	1.8	2.3	2.8
873			–	–	A	G	–	0.4	0.4

* **Cons:** consensus sequence nucleotide; **†Var:** nucleotide variation in minority sequences

‡ Indicate nucleotides that encode amino acid changes. **─** Indicate positions where no SNP observed.

Table 4.4. *Single nucleotide polymorphisms between nucleotides 487-886 of the E gene of genotype 2 MVEV viruses and their relative proportion (%) within their respective sequence datasets.*

Nucleotide Positions	Consensus	Variations	K59532	K59536	K68196
501	C	T	2.5	–	–
525	C	T	2.5	–	–
564	G	A	–	–	3.5
588	G	A	2.5	–	–
594	G	A	2.5	–	–
618	T	C	2.5	–	–
624	A	G	2.5	–	–
627	G	A	2.5	–	–
666 *	T	C	2.5	–	–
690	C	T	2.5	–	–
696	T	C	–	0.95	–
720	G	A	–	–	3.5 *
732	A	G	–	0.95	–
783	G	A	–	0.95	–
784	T	C	2.5	–	–
825	A	G	2.5	–	–
828	T	C	2.5	–	–

* Indicates nucleotides that encode amino acid changes.

– Indicate positions where no SNP observed.

Table 4.5. *Identification of selection acting on the nucleotides 487-886 region of the E gene of MVEV strains included in this study.*

		***p*-value* (Z-test of selection)†**		
Samples	**Genotype**	**Positive selection**	**Purifying selection**	**Neutral selection**
K68320	G1a	1.000 (-5.287)	0.000 (5.377)	0.000 (-5.241)
K69612	G1a	1.000 (-5.385)	0.000 (5.266)	0.000 (-5.283)
K68150	G1b	1.000 (-4.078)	0.000 (4.124)	0.000 (-3.993)
K60555	G1b	1.000 (-4.074)	0.000 (4.012)	0.000 (-3.966)
P8891	G1b	1.000 (-4.759)	0.000 (4.739)	0.000 (-4.980)
K59532	G2	1.000 (-4.302)	0.000 (4.531)	0.000 (-4.382)
K59536	G2	1.000 (-1.350)	0.094 (1.325)	0.182 (-1.342)
K68196	G2	1.000 (-0.081)	0.468 (0.081)	0.938 (-0.078)

Values in selection column denote p-value. Numbers in the parentheses **( )** denote the Z-test of selection.

***** A *p*-value of >0.05 was considered not to be significant.

† dN – dS (Z test of selection).

analyses revealed no evidence of positive selection in the evolutionary history of these MVEV samples. Conversely, a *p*-value of <0.05 was obtained when both purifying and neutral selection were tested in all G1 and one of the G2 samples (K59532), indicating the operation of purifying and neutral selection on these virus populations. When positive and neutral selection were assessed by Z-test analysis, all dN – dS values were negative, indicating the operation of purifying selection. In contrast, Z-test analysis of adaptive evolution in two G2 samples (K59536 and K68196) produced a *p*-value of >0.05 for all positive, purifying and neutral selection pressures; therefore, a significant conclusion about selection averaged over all sequences of each of these two virus populations could not be made.

When the above hypotheses were tested between sequence pairs of each dataset, the majority of sequence pairs (>99.9%) appeared to be under purifying and neutral selection. A minor number of sequences, mostly singletons belonging to G1 samples (except K69612), and one G2 sample (K59532) demonstrated a positive dN – dS value with a *p*>0.05, indicating positive selection (data not shown).

The operation of selection pressure was also assessed at the codon level via HyPhy using an ML analysis. This analysis revealed no significant evidence for the operation of positive selection at any of the codons of the partial E gene sequences in the dataset of each tested samples (data not shown). Therefore, based on the three analyses described above, purifying and neutral selections have been the most dominant driving forces of MVEV evolution over time.

4.4 Discussion

The primary aim of this chapter was to characterise and compare the level of genetic diversity within G1 and G2 MVEV populations circulating in the environment. This study revealed that MVEV exists in nature as a highly genetically diverse ensemble of variants and that the diversity is more pronounced within the populations of G1 virus populations compared to those of G2.

In this study, viral RNA transcribed from the genetically defined pMVE-1-51 plasmid (Hurrelbrink et al., 1999) was included to estimate the inherent error rate of RT-PCR and NGS. The per-nucleotide variance of the MVE-1-51 control (implying the inherent error rate), after the data filtration steps, was 0.057%. This is substantially lower than that obtained in previous NGS studies: 0.98% (Wang et al., 2007), 0.27% (Solmone et al., 2009), 0.13% (Nishijima et al., 2012) and 0.11% (Tsibris et al., 2009). However, it is comparable to the 0.05% error rate obtained in the conventional molecular cloning approach used by Ciota et al. (2007c), but substantially higher than the 0.004% reported by Jerzak et al. (2005), using the same methodology. This indicates that the experimental design employed in this study produced final sequence datasets containing a minimum nucleotide error rate. It should be noted that only a single control replicate was employed. For rigorous validation of error rate, ideally control replicates should be included. However, the current cost of NGS is a limiting factor in terms of numbers of both control and test samples replicates that can be included in a given study.

The impartial and unbiased efficiency of the experimental and computational processes on detecting different genotypes or minority variants in this study was assessed by including a second control containing defined ratios of different strains of MVEV belonging to: 1% MVE-1-51 (G1), 10% NG156 (G4) and 89% OR156 (G2). Following NGS, the ratio was reasonably preserved showing 3.9% MVE-1-51, 15.2% NG156 and 80.9% OR156. Although this sensitivity is relatively lower than those previously reported in other NGS studies (Görzer et al., 2010a; Tsibris et al., 2009), it indicates that strong biases were not introduced in the amplification processes towards a specific genotype. Moreover, it reveals that the experimental and computational protocols employed here could detect minority variants that were present in the population as low as 3.9%. The relative difference in the ratio of input RNA to that of the output result may have been a result of experimental error, such as dilution effect during the generation of the standard curve for RT-qPCR or during the formation of the input ratio pool.

The genetic diversity and complexity of mutation spectra within each virus population were characterised by considering the corrected per-nucleotide

variation of each sample, and the percentage of variant sequences that differed from the sample-specific consensus sequence. Altogether, the result of this assessment showed that genetic diversity and mutation spectra were significantly higher in G1a samples than those of G1b, which were in turn higher than G2 samples. Samples belonging to G1a contained a significantly higher number of sequences that differed from each of the sample-specific consensus sequence (p=0.021), and also demonstrated as higher per-nucleotide variation compared to G1b and G2 samples (p=0.001). In contrast, G2 samples exhibited a significantly lower complexity than all G1 samples (p=0.029). Only one G2 isolate (K59532) displayed a higher per-nucleotide variance than baseline error, yet it was substantially lower than that of G1 samples (Table 4.2). The other two G2 samples (K59536 and K68196) had a lower per-nucleotide variance than the baseline error (plasmid control), indicating little or no evidence of a highly diverse viral populations. Similar to this finding, Jerzak et al. (2005) found no evidence of a complex mutation spectrum in two WNV samples taken from mosquitoes and American crows.

It is worth noting that initially lower numbers of raw reads were obtained for G2 samples (Table 4.2). While the coverage of K59532 sample was comparable to the G1 samples, K59536 and K68196 had substantially lower numbers of reads than any other sample in the study. This may have affected the sampling and detection of minority sequences and low level of genetic variation. Therefore, the level of nucleotide variation for K59536 and K68196 may in fact be higher. Significantly, the lower level of genetic diversity in G2 viruses correlates with them being the minority circulating genotype that is restricted in the Kimberley region of WA. Proposition can be made that the inherent lower level of intra-population genetic diversity in G2 viruses is an underlying factor for the restricted geographical spread and low frequency of these viruses in nature. Overall, the level of genetic diversity and the complexity of mutation spectra in G1 MVEV populations tested in this study are substantially higher than that reported for homogenates of WNV-infected mosquitoes and bird specimens, with 19.5% of sequences and 0.016% of nucleotides differing from consensus sequence (Jerzak et al., 2005). However, in the

latter study, the authors employed conventional cloning and sequencing to determine the depth of genetic diversity in the WNV populations analysed.

SNP analysis revealed that nucleotide polymorphism is higher in G1a samples, compared to G1b, which is in turn higher than G2 viruses. Analysis of SNP supported the observation of lower levels of genetic diversity in G2 viruses as characterised above. Of significance is the observation of SNP at amino acid positions 275 and 277 in the flexible hinge region of samples belonging to G1a and G1b ($S^{275}P$ and $S^{277}N$ substitutions in G1a, and $P^{275}S$ and $S^{277}N$ in G1b minority variants). Changes in the flexible hinge region have been proposed to lead to decreased flexibility of the hinge and lowered fusion efficiency of the viral envelope to the plasma membrane during infection. Mutations at position 277 resulted in loss of MVEV virulence in mice (Hurrelbrink and McMinn, 2001; Prow et al., 2011). Although these substitutions are not reflected in the consensus sequence of any G1a or G1b samples tested here, they may affect their phenotypic characteristics *in vitro* and/or *in vivo.* The phenotypic features of these viruses will be examined in Chapter 5.

The selection pressure analyses on each sequence dataset revealed the dominance of purifying and neutral selection acting on MVEV populations in mosquitoes (Table 4.5). Similar findings have been reported previously for WNV (Jerzak et al., 2005; Jerzak et al., 2008). Minor evidence of positive selection was also observed between sequence pairs of four G1 and one G2 samples, also consistent with previous reports for several other flaviviruses (Baillie et al., 2008; Beasley et al., 2003; Bennett et al., 2006; Bertolotti et al., 2007; Brault et al., 2007; Carney et al., 2012; Twiddy et al., 2002a; Twiddy et al., 2002b). In flaviviruses, as per any other virus, selection operates at the population level and not on individual sequences within the population (Biebricher and Eigen, 2006; Eigen, 1971; Vignuzzi et al., 2006). Therefore, the minor number of sequences showing evidence of positive selection in a large MVEV population may not have a considerable effect on the phenotype of these viruses.

Nucleotide mutations that disrupted the ORF and led to the introduction of premature stop codons were observed in all G1 and one G2 sequence datasets

(Table 4.2). These stop codons were frequently repeated at amino acid positions 217, 225, 233, 250 and 264, all of which reside on domain II of MVEV (Fig. 2.4). Stop codons were the result of a frameshift mutations rather than nucleotide deletions. The presence of a stop codon can result in the production of defective non-viable genomes in a flavivirus population that are highly likely to be removed by natural selection. In previous studies, sequences encoding a stop codon in DENV and WNV were also reported (Aaskov et al., 2007; Aaskov et al., 2006; Jerzak et al., 2005). The prevalence of stop codons in MVEV datasets, detailed in this chapter, were much lower (0.06 to 0.90%) than that found in WNV population (2.25%) reported by Jerzak et al. (2005) and DENV-1 population, in which up to 55% of sequence clones contained defective genomes (Aaskov et al., 2006). The DENV-1 study revealed that viral populations containing stop codons were found to circulate with a high prevalence between mosquitoes and humans for one and half years (Aaskov et al., 2006). The stop codon variant of DENV-1 represented a distinct phylogenetic lineage. The authors proposed that transmission of viruses in the stop codon lineage was most likely due to a complementation process, in which defective genomes used the functional proteins of wild-type virus to complete the replication cycle (Aaskov et al., 2006). A similar phenomenon may be occurring in MVEV populations, whereby defective genomes continue to circulate with wild-type genomes.

Despite using sample homogenates of G1 MVEV with comparable copy numbers, substantially different numbers of raw reads were obtained following NGS. It was not possible to determine the reason for this difference. However, the presence of DI particles in the homogenates might have contributed to this discrepancy. Previously, it has been demonstrated that DI particles of flaviviruses lack most of the structural genes as well as 5’ end of NS1 gene (Lancaster et al., 1998; Tsai et al., 2007; Yoon et al., 2006). To date, the observation of DI particles lacking the 3’UTR (the target of MVEV-specific RT-qPCR assay used to determine the viral copy number in this study; Chapter 3) has not been reported. Therefore, it may be argued that DI particles have been amplified by the RT-qPCR and contributed to the calculation of the viral copy number, but due to the lack of structural genes, they

were not amplified by the RT-PCR and were not sequenced by NGS, hence decreasing the number of raw reads in some samples. This discrepancy may have also been a result of experimental errors during amplification, library preparation and NGS.

An interesting finding in this study was the observation of substantially lower viral genome copy numbers for G2 MVEV compared to G1 (Table 4.2). This may indicate that G2 viruses replicate less efficiently in mosquitoes than G1 viruses. This observation is consistent with the attenuated phenotype of G2 viruses in mice (Chapter 5 and 6). These differences may be due to the unique genetic composition of G2 viruses compared to G1. Previous studies on WNV revealed that a single amino acid substitution in the E protein resulted in higher levels of infection efficiency and virus production in mosquitoes (Beasley et al., 2003; Brault et al., 2007; Moudy et al., 2007). Conversely, G2 strains of MVEV may contain molecular determinants that restrict their replication in mosquitoes than G1 strains.

This is the first report of NGS technology having been used to characterise the genetic diversity and complexity of quasispecies in MVEV populations. It demonstrated that G1 MVEV perpetuates in nature as a highly genetically diverse mix of variants, whereas G2 exists as a more homogeneous population. Results reported here highlight NGS as a powerful technology that can provide a detailed insight into the genetic structure and make up of viral populations.

CHAPTER 5

PHENOTYPIC CHARACTERISATION OF GENOTYPE 1 AND 2 MURRAY VALLEY ENCEPHALITIS VIRUSES: COMPARISON OF GROWTH KINETICS IN CELL CULTURE AND VIRULENCE IN MICE

5.1. Introduction

In Chapter two, the genetic variation of MVEV isolates was characterised. It was reported that G2 MVEV still circulates in the Kimberley region of WA and G1 has two sublineages (G1a and G1b). In Chapter three it was reported that G1 MVEV perpetuates in nature as a complex of genetically heterogeneous variable genomes, whereas the genome of G2 is more homogeneous. There has been little research done to further understand the phenotypic characteristics of the G2 strains, despite circulating in northern Australia for many decades. So far, only two studies have compared the phenotypic features of the two co-circulating Australian genotypes of MVEV, G1 and G2 (Coelen, 1988; Lawson, 1988). These studies revealed that OR156 (the G2 prototype strain) is an attenuated strain of MVEV, whereas G1 strains are virulent. Since 1988, the majority of MVEV studies have focused on assessing the genetic variation and phylogenetic analyses of all MVEV strains (Johansen et al., 2007; Mann et al., 2013; Poindinger et al., 1996; Williams et al., 2013), while others have concentrated on investigating the virulence characteristics of G1 prototype strain, MVE-1-51 (Clark et al., 2007; Hurrelbrink and McMinn, 2001; Hurrelbrink et al., 1999; Kroeger and McMinn, 2002; Lee and Lobigs, 2000, 2002; Lobigs et al., 2009; Lobigs et al., 1990; McMinn et al., 1995; McMinn et al., 1996; Prow et al., 2011) or other G1 isolates (Guirakhoo et al., 1992; Hall et al., 1996; Poidinger et al., 1991; Wallace et al., 2003). Therefore, there is limited knowledge about differences in virulence phenotypes of the current circulating MVEV isolates from various genetic types and subtypes.

The recent detection of four new G2 isolates in the Kimberley region of WA (Chapter 2) provided a unique opportunity to investigate and compare the phenotypic features of recent isolates of G1 and G2 and compare them with their prototype strains.

In Chapter 2, unique amino acids in the full-length prM and E proteins of G2 isolates of MVEV were identified (Table 2.6). It is evident that a small number of mutations (Chapter 1, Section 1.3.5) can have a significant impact on the virulence phenotype of flaviviruses both *in vitro* (Ciota et al., 2007b; Hurrelbrink and

McMinn, 2001; Moudy et al., 2007; Shirato et al., 2004) and *in vivo* (Beasley et al., 2005; Cecilia and Gould, 1991; Chambers et al., 2008; Davis et al., 2003a; Halevy et al., 1994; Hurrelbrink and McMinn, 2001; Lobigs et al., 1990; McMinn et al., 1995; Prow et al., 2011; Shirato et al., 2004). For example, single amino acid substitutions in the genomes of WNV (Liu et al., 2006b; Wicker et al., 2006), JEV (Kim et al., 2008; Ye et al., 2012), DENV-2 (Sanchez and Ruiz, 1996) and TBEV (Holzmann et al., 1990) has been shown to result in attenuation of virulence in mice. Conversely, a single amino acid substitution in WNV (Brault et al., 2007; Moudy et al., 2007) was shown to result in greater vector infection and an explosive southwesterly spread of WNV in the United States in 2002. Moreover, it is also evident that genetically diverse populations of flaviviruses have a significantly higher viral fitness, adaptability, virulence and pathogenesis than less diverse populations (Ciota et al., 2012; Ciota et al., 2007c; Jerzak et al., 2007).

In this chapter, the *in vitro* and *in vivo* phenotype of representative strains of G1a, G1b and G2 isolates, recently isolated from WA, are described. This study aims to shed light on the question of whether differences in the replication and virulence of MVEV are associated with different genotypes or subgenotypes.

5.2. Materials and Methods

5.2.1 Cells

Chicken fibroblast cell line (DF1 cells) were grown in DMEM (Life Technologies, Australia) supplemented with 1% L-glutamine (Life Technologies, Australia) and 5% FBS (Life Technologies, Australia) at 37°C in the presence of 5% CO_2. C6/36 and PSEK cells were grown as described in Chapter 2 (Section 2.2.1). Infected cells were cultured in 2% FBS with 1% L-glutamine (maintenance media).

5.2.2 Viruses

Six isolates of MVEV were tested: MVE-1-51, OR156, and representative recent isolates from G1a (K68838), G1b (K60555), and G2 (K59532 and K62017). MVE-1-51 and OR156 were derived from tissue culture supernatants with multiple (unknown)

passage histories. K68838, K60555, K59532 and K62017 viruses were isolated from tissue culture supernatants infected with the homogenates of mosquito pools, as described in Chapter 2 (Section 2.2.1 and Fig. 2.1). The latter isolates were kindly provided by the Arbovirus Surveillance and Research Laboratory, The University of Western Australia. All virus stocks were titrated in PSEK cells as described in Chapter 3 (Section 3.2.2)

5.2.3 Growth Kinetics in Cell Culture

Viral replication kinetics of each of virus isolate tested was assessed by single-step growth curve analysis in C6/36 and DF1 cells. These two cell lines were selected to represent the mosquito vector and avian host of MVEV, respectively. Briefly, sub-confluent (80%) monolayers of DF1 and C6/36 cells were inoculated with a virus suspension at an MOI of 0.01 in six-well plates. Each virus strain was tested in triplicate. The monolayers were incubated for one hour at 28°C for C6/36 cells and 37°C for DF1 cells to allow the adsorption of virus particles to cells. The inoculum was removed and the monolayer in each well was rinsed twice with 2ml of PBS. Four millilitres of maintenance media was added to each well. Aliquots of 400µl were removed from each well at 0, 12, 24, 36, 48, 72 and 96 hpi and replaced with an equal volume of fresh maintenance media. The titre of virus in each triplicate was determined by titration in PSEK cells. Single-step growth curves were generated using the mean and standard error of the titre at each time-point.

5.2.4 Mouse Virulence Experiments

Groups of six 18-day old Swiss ARC mice (Animal Resources Centre, Murdoch, Western Australia, Australia) were injected i.p. with 50µl of 10-fold dilutions of virus (10^0-10^2 $TCID_{50}$ for G1 strains and 10^2-10^4 for G2 strains). A group of six control mice were inoculated i.p. with 50µl of sterile PBS. Mice were housed in clean individually ventilated cages and were provided with water and food *ad libitum*. Mice were weighed every morning. Infected mice were monitored twice daily until the first signs of the disease were observed, after which, they were examined every two hours. To reduce distress to experimental animals, the 50% humane end point dose (HD_{50}) was employed (Wright and Phillpotts, 1998). In this method, when animals

exhibited severe signs of disease or lost ≥15% body weight, they were euthanised. HD_{50} method does not significantly differ from LD_{50} (Wright and Phillpotts, 1998). The HD_{50} was calculated for each virus using the method of Reed and Muench (1938). The average survival time (AST) was also determined for each infection group by calculating the mean time to death (in days) of the number of mice that succumbed to infection.

5.2.5 Virus Detection and Quantification

The brains of euthanised mice were collected, snap frozen in liquid nitrogen and stored at -80°C. Frozen tissues were thawed and 10% w/v homogenates were prepared in M199 medium (Life Technologies, Australia) using a PYREX® Tenbroeck homogeniser, Corning® (Corning Life Sciences, Australia). Homogenates were centrifuged at 4000*xg* for 10 min at 4°C. Supernatants were removed and re-centrifuged under the same conditions. The clarified supernatants were stored in 200µl aliquots at -80°C. The virus titre in each brain homogenate was determined using the $TCID_{50}$ method. Furthermore, MVEV-specific RT-qPCR assay was performed to determine the viral RNA copy number in each homogenate. Viral RNA was extracted from 140µl of brain homogenates using QIAamp® Viral RNA mini kit (QIAGEN, Australia), according to the manufacturer's protocol. Purified RNA was tested in duplicate using the MVEV-specific RT-qPCR assay, described in Chapter 3 (Section 3.2.5). For each homogenate, the viral RNA copy number was calculated from the standard curve that was generated using *in vitro*- transcribed MVE-1-51 RNA. The concentration of viral RNA present in each homogenate was expressed as the $TCID_{50}$/g as well as the copy number/g of infected mouse brain.

5.2.6 Serological Confirmation of Infection in Surviving Mice

At 21 dpi, surviving mice were euthanised with an i.p. injection of an overdose (150mg/kg body weight) of pentobarbitone. To confirm infection in surviving mice, blood samples were collected by cardiac puncture. Blood was allowed to clot at room temperature for 30 minutes. The samples were then incubated at 4°C for 2 hours and subsequently centrifuged at 1500*xg* for 10 min and serum recovered. An immunofluorescence assay (IFA) was performed in 96-well micro-titre plates

(Greiner Bio-one, Australia) to detect the presence of anti-MVEV antibodies in mice sera. Briefly, monolayers of PSEK cells were inoculated with 50µl of MVE-1-51 at an MOI of 1.0 and incubated for one hour. After removing the inoculum, the cells in each well were washed twice with 100µl of PBS and then 100µl of fresh maintenance media was added to each well. The plates were then incubated at 37°C for 48 hours. The media was removed and cells were fixed using 100µl of a 1:1 acetone-methanol fixative solution at room temperature for 10 minutes. The fixative solution was removed and the plates were rinsed three times using 100µl of 0.5% BSA-PBS blocking solution. Fifty microlitre of each mouse serum was added to individual wells in duplicate as primary antibody. Monoclonal antibodies (mAbs) against MVEV E protein (3H6) and MVEV NS1 protein [10C6; (Hall et al., 1995)] were used as positive controls, at 1:5 dilution, and were tested in duplicate. MAbs 3H6 and 10C6 were kindly provided as cell culture supernatants by Professor Roy Hall of the University of Queensland, Australia. Sterile PBS and sera of control mice were tested in duplicate as negative controls. The monolayers were incubated with sera and controls at room temperature for one hour. Cells were then washed three times with 100µl of PBS prior to adding 50µl of a 1:200 dilution of FITC-conjugated goat anti-mouse antibody (SIGMA Aldrich, Australia). Monolayers were incubated at 37°C for 30 minutes and washed three times with 100µl of PBS. The presence of anti-MVEV antibodies, indicating seroconversion, was confirmed by the observation of antibody staining under UV light (absorption Wavelength: 495nm and emission Wavelength: 528nm) using an Olympus IX51 microscope.

5.2.7 Statistical Analysis

Repeated measures analysis of variance (rmANOVA) was employed to determine whether observed differences were significant. Two-tailed paired Student's *t*-test was performed to determine whether there were any significant differences between the mice mortalities of different test viruses. Statistical significance of the AST values was determined using a two-tailed *t*-test. Animal experiments were approved by the Curtin University Animal Ethics Committee (AEC; approval number: AEC_2011_64). A two-tailed *t*-test was performed to see if the observed differences in the viral copy number and titres were significant. Statistical analyses were

performed using StatistiXL 1.10 software (Roberts and Withers, 2007). A *p*-value of <0.05 was considered to be statistically significant.

5.3. Results

5.3.1 Comparison of Viral Replication in Avian and Mosquito cells

The replication kinetics of recent G1 and G2 isolates were determined using a single-step growth curve analysis in DF1 and C6/36 cells, and compared to the prototypic strain of G1 (MVE-1-51) and G2 (OR156). Statistical significance of any differences in replication kinetics was evaluated using the rmANOVA, which compares the height and shape of the growth curve, taking into consideration both the maximum titre and the rate of viral replication (Roberts and Withers, 2007). The maximum titre and the rate of replication were used in this study as measures of virus replicative fitness (Ebel and Kramer, 2012; Orr, 2009).

5.3.1.1. Viral Fitness in DFI Cells

All viruses, except K62017 (G2), demonstrated comparable replication kinetics in DF1 cells from 0 hpi to 96 hpi. These viruses demonstrated a sharp rise in titre between 0 hpi to 12 hpi, after which the titre increased at a slower rate until it reached its peak (7.25 ± 0.16 Log_{10} $TCID_{50}$) at 48 hpi (Fig. 5.1). The titre of these viruses declined by up to one log after 48 hpi, dropping to 6.26 ± 0.23 Log_{10} $TCID_{50}$ at 96 hpi. For K62017, the titre of the virus was higher at 96 hpi than any other virus in this study (Fig. 5.1), indicating a greater level of stability of this virus culture. However, the statistical analyses of the combined single-step growth curve data revealed no significant differences between the replicative ability of the G1 strains (MVE-1-51, K68838, and K60555) and the G2 strains (OR156, K59532 and K62017; Table 5.1).

5.3.1.2. Viral Fitness in C6/36 Cells

The replicative ability of the G1 and G2 isolates in this study was also assessed in a single-step growth curve assay in C6/36 cells. The replicative ability of the G1

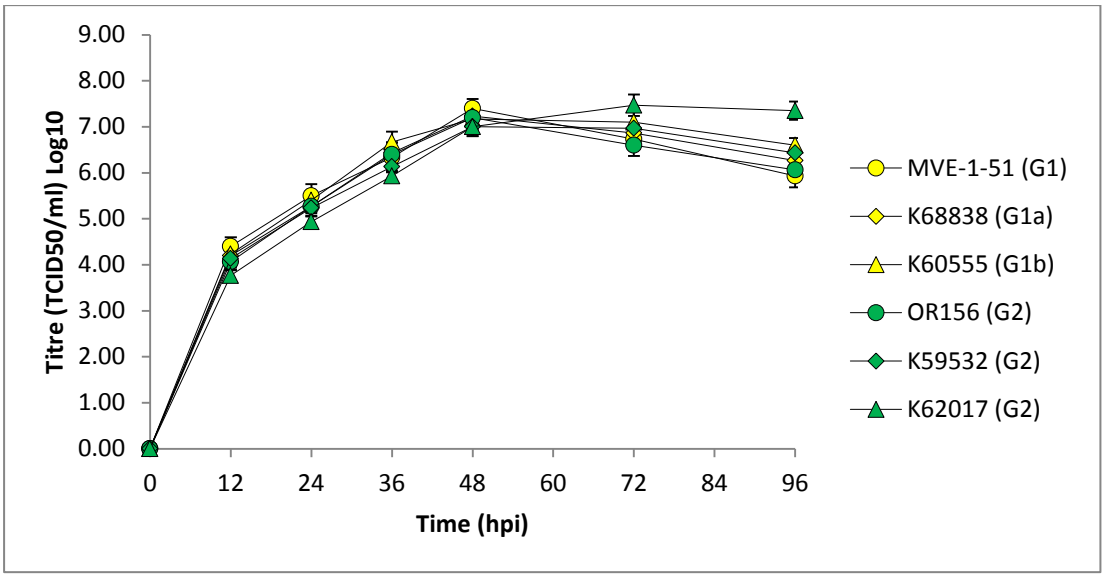


Fig. 5.1. *Growth kinetics of MVE-1-51 and OR156 with recent genotype 1 (K68838, K6055) and genotype 2 (K59532 and K62017) isolates in DF1 cells.*

Error bars represent the mean ± standard deviation of the triplicates at each time point.

Table 5.1. *Statistical analyses of the single-step growth curve data of the MVEV in DF1 cells (rmANOVA).*

Reference	Viruses	Mean Difference	Standard error	q statistic*	*p*-value†
MVE-1-51 (G1)	K68838	0.033	0.658	0.051	0.97
	K60555	-0.767	0.658	1.166	0.42
	OR156	0.700	0.658	1.064	0.94
	K59532	0.400	0.658	0.608	0.90
	K62017	-1.000	0.658	1.521	0.54
K68838 (G1a)	K60555	-0.800	0.658	1.216	0.67
	OR156	0.667	0.658	1.014	0.89
	K59532	0.367	0.658	0.558	0.70
	K62017	-1.033	0.658	1.571	0.69
K60555 (G1b)	OR156	1.467	0.658	2.230	0.62
	K59532	1.167	0.658	1.774	0.60
	K62017	-0.233	0.658	0.355	0.81
OR156 (G2)	K59532	-0.300	0.658	0.456	0.94
	K62017	-1.700	0.658	2.585	0.55
K59532 (G2)	K62017	-1.400	0.658	2.129	0.57

* The q statistic (studentised range statistic) is used for multiple significance testings across a number of means. It is the calculation of the differences in mean divided by the standard error.

† A *p*-value of <0.05 is considered significant.

prototype strain (MVE-1-51) was significantly greater than G2 prototype strain (OR156; Fig. 5.2 and Table 5.2). However, recent G1 strains, K68838 (G1a) and K60555 (G1b), demonstrated a significantly lower replicative ability than MVE-1-51 and the G2 strains up to 48 hpi (Fig. 5.2 and Table 5.2). Although at 96 hpi, their titres were similar to that of MVE-1-51, the shape and the height of the growth curve demonstrates a slower increase in the titre of K68838 and K60555 (Fig. 5.2). A substantial difference (0.9 Log_{10}) could be observed as early as 24 hpi, with these two viruses exhibiting a lower titre compared to MVE-1-51, between 24-72 hpi. The replicative ability of K68838 (G1a) was not significantly different to that of K60555 (Table 5.2).

In the case of the G2 isolates, there was no significant difference in the replicative ability of OR156 and K59532. However, the replicative ability of K62017 was significantly higher than both OR156 and K59532 (Fig. 5.2 and Table 5.2). Significant differences were observed as early as 24 hpi, with K62017 showing an approximately ten-fold higher titre than OR156 between 24-72 hpi (Fig. 5.2).

Overall, there was a significant difference between the replicative ability of strains belonging to G1 (MVE-1-51, K68838 and K60555) to those belonging to G2 (OR156, K59532 and K62017; Fig. 5.2 and Table 5.2). While the prototype strain of G1 (MVE-1-51) demonstrated a higher replicative ability in C6/36 cells than the G2 prototype strain (OR156), the recent G1 isolates (K68838 and K60555) exhibited a lower replicative ability compared to recent G2 isolates (K59532 and K62017; Fig. 5.2 and Table 5.2). This was apparent as a slower replication rate between 12-72 hpi, however equivalent titres were observed at 96 hpi.

5.3.2. Virulence in Mice

To further characterise the phenotypic features of MVEV strains from different genotypes, the neuroinvasiveness of the virus isolates was investigated using a mouse model of pathogenesis (Beasley et al., 2005; Hurrelbrink and McMinn, 2001; Hurrelbrink et al., 1999; May et al., 2006; Prow et al., 2011). To minimize the use of animals, recent G1a (K68838) and G1b (K60555) isolates with only one G2 isolate (K59532) were tested and compared to the prototype viruses.

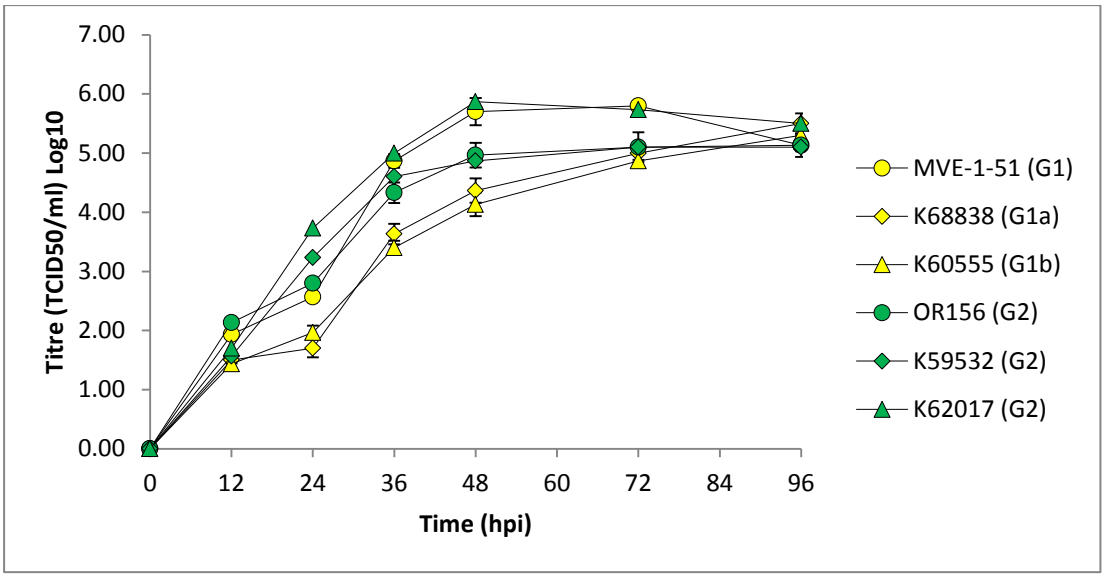


Fig. 5.2. *Growth kinetics of MVE-1-51 and OR156 with recent genotype 1 (K68838, K6055) and genotype 2 (K59532 and K62017) isolates in C6/36 cells.*
Error bars represent the mean ± standard deviation of the triplicates at each time point.

Table 5.2. *Statistical analyses of the single-step growth curve data of the MVEV in C6/36 cells.*

Reference	Viruses	Mean Difference	Standard error	q statistic	*p*-value†
MVE-1-51 (G1)	K68838	4.233	0.210	20.112	**0.00**
	K60555	4.867	0.210	23.121	**0.00**
	OR156	1.367	0.210	6.493	**0.00**
	K59532	1.467	0.210	6.968	**0.00**
	K62017	-1.600	0.210	7.601	**0.00**
K68838 (G1a)	K60555	0.633	0.210	3.009	0.05
	OR156	-2.867	0.210	13.619	**0.00**
	K59532	-2.767	0.210	13.144	**0.00**
	K62017	-5.833	0.210	27.713	**0.00**
K60555 (G1b)	OR156	-3.500	0.210	16.628	**0.00**
	K59532	-3.400	0.210	16.153	**0.00**
	K62017	-6.467	0.210	30.722	**0.00**
OR156 (G2)	K59532	0.100	0.210	0.475	0.74
	K62017	-2.967	0.210	14.094	**0.00**
K59532 (G2)	K62017	-3.067	0.210	14.569	0.00

* The q statistic (studentised range statistic) is used for multiple significance testing across a number of means. It is the calculation of the differences in mean divided by the standard error.
† A *p*-value of <0.05 is considered significant.

The neuroinvasive phenotypes of G1 isolates were determined by i.p. injection of groups of six 18-day old Swiss outbred mice with 10^0-10^2 $TCID_{50}$ of the virus (Table 5.3). This range had previously been found to generate HD_{50} for this genotype of MVEV (Hurrelbrink and McMinn, 2001; Prow et al., 2011). Isolates belonging to G1 were virulent and had HD_{50} values between 0.88 to 2.00 $\log_{10}$ $TCID_{50}$ with an AST of between 5.2 to 7.8 days (Table 5.3). A dose-dependent mortality rate was observed whereby higher numbers of deaths were associated with the greater dose of the virus (Table 5.3, Appendix 5.1). Although fewer mice died after inoculation with K68838 (G1a) and K60555 (G1b), the differences in HD_{50} between all G1 strains tested were not statistically significant (Table 5.3 and Appendix 5.1). Mice remained healthy for 4-6 days, after which they demonstrated signs of disease, such as ruffled fur, hunching, eye closure, anorexia and a reduced weight gain followed by a marked decrease in weight within 24 hours. Severe signs of neurological involvement such as tremor, twitching, lethargy, loss of balance, immobility and hind leg paralysis were observed. The progression of disease was rapid with death occurring within 24 hours of onset of symptoms. Full paralysis in one or both hind legs was observed in 32.5% of mice with lethal infections, whereas 35% displayed partial paralysis, in either one or both hind legs. The remaining animals (32.5%) did not show signs of paralysis but exhibited other severe signs of neurological involvement. All mice that succumbed to the infection lost at least 15% body weight.

In contrast to G1 isolates, higher doses (10^2-10^4 $TCID_{50}$) of G2 isolates were injected into mice to determine the HD_{50}, as it was hypothesised that the G2 isolates of MVEV are attenuated strains (Coelen, 1988; Lawson, 1988). No higher doses were available to continue the experiment for the G2 isolates beyond 10^4 $TCID_{50}$, because higher titres in culture could not be achieved. Interestingly, most mice that received different doses of G2 isolates exhibited no clinical signs of disease or death. Low numbers of mortalities were observed for high doses of G2 virus isolates such that HD_{50} values could not be determined (Table 5.3 and Appendix 5.2). The AST for mice that succumbed to the G2 lethal infection ranged between 7 to 12 days (Table 5.3 and Appendix 5.2), showing significantly higher AST than G1 strains (p=0.002).

Table 5.3. *Average survival time (AST) and HD_{50} values for MVEV isolates following intraperitoneal inoculation of 18-day old Swiss ARC mice.*

Virus	Dose (Log_{10} $TCID_{50}$)	Mortality (%)	AST (days)	HD_{50} (Log_{10} $TCID_{50}$)	*p*-value‡
Genotype 1 isolates					
MVE-1-51 (G1)	0	0/6 (0%)	N/A		
	1	4/6 (66.7%)	6.3		
	2	5/6 (83.3%)	5.2	0.88	
K68838 (G1a)	0	0/6 (0%)	N/A		
	1	0/6 (0%)	N/A		
	2	3/6 (50%)	7.3	2.00	0.225 †
K60555 (G1b)	0	0/6 (0%)	N/A		
	1	0/6 (0%)	N/A		0.423 †
	2	5/6 (83.3%)	7.8	1.60	0.423 ‡
Genotype 2 Isolates					
OR156 (G2)	2	1/6 (16.7%)	12.0 *		
	3	0/6 (0%)	N/A		
	4	0/6 (0%)	N/A	>4.00	
K59532 (G2)	2	2/6 (33.3%)	10.0		
	3	1/6 (16.7%)	10.0 *		
	4	0/6 (0%)	N/A	>4.00	0.184 †

***** The value represents the survival time of the only mice that died in these group.

† *p*-values are based on statistical comparison mice mortalities of each virus with that of the prototype virus of the associated genotype.

‡ *P-value* is based on statistical comparison of mice mortalities of K68838 and K60555. A *p*-value of <0.05 is considered to be significant.

The course of disease for mice that demonstrated lethal infection when inoculated with G2 isolates was similar to those inoculated with G1 isolates, except they had a prolonged time to death. Interestingly, inoculation of mice with lower doses of K59532 (recent G2) caused higher mortality compared to those inoculated with the higher doses (Table 5.3, and Appendix 5.2), but this was not statistically significant. This phenomenon is called a prozone effect (Monath et al., 1980).

In each of the mice that succumbed to infection, the presence and concentration of MVEV in brain homogenates were determined by the RT-qPCR assay described in Chapter 3, and by titration in PSEK cells. As shown in Appendix 5.3, the viral RNA ranged from 11.51 to 13.33 Log_{10} copies per gram of mouse brain. There was no significant difference in the viral copy number in the brains of mice that were inoculated with MVE-1-51 and K68838. However the viral copy number in the brains of mice that inoculated with MVE-1-51 was significantly higher than those inoculated with K60555 (G1b) and the G2 isolates (Appendix 5.4). There was no significant difference in viral copy number between recent G1 isolates (K68838 and K60555) and the two G2 isolates (OR156 and K59532). In addition, virus titres ranged between $10^{7.0}$ to $10^{9.6}$ $TCID_{50}$ per gram of mouse brain (Appendix 5.3). Although significantly higher titres were obtained from homogenates of mice that were administered with MVE-1-51 than any other isolate tested, no significant differences were observed when the titres of recent G1 (K68838 and K60555) and G2 (OR156 and K59532) isolates were compared (Appendix 5.5).

Sera from mice injected with any dose of the prototype strain of G1 (MVE-1-51) contained anti-MVEV antibodies in IFA. However, mice administered with a low dose of recent isolates of G1a (K68838) and G1b (K60555) did not always have detectable antibodies to MVEV (Appendix 5.6). The sera of all mice surviving the infection with G2 MVEV contained anti-MVEV antibodies (titres not determined).

5.4. Discussion

In this chapter, the in vitro replication and in vivo virulence of representative MVEV isolates of recent G1 and G2 were investigated and compared to prototype isolates.

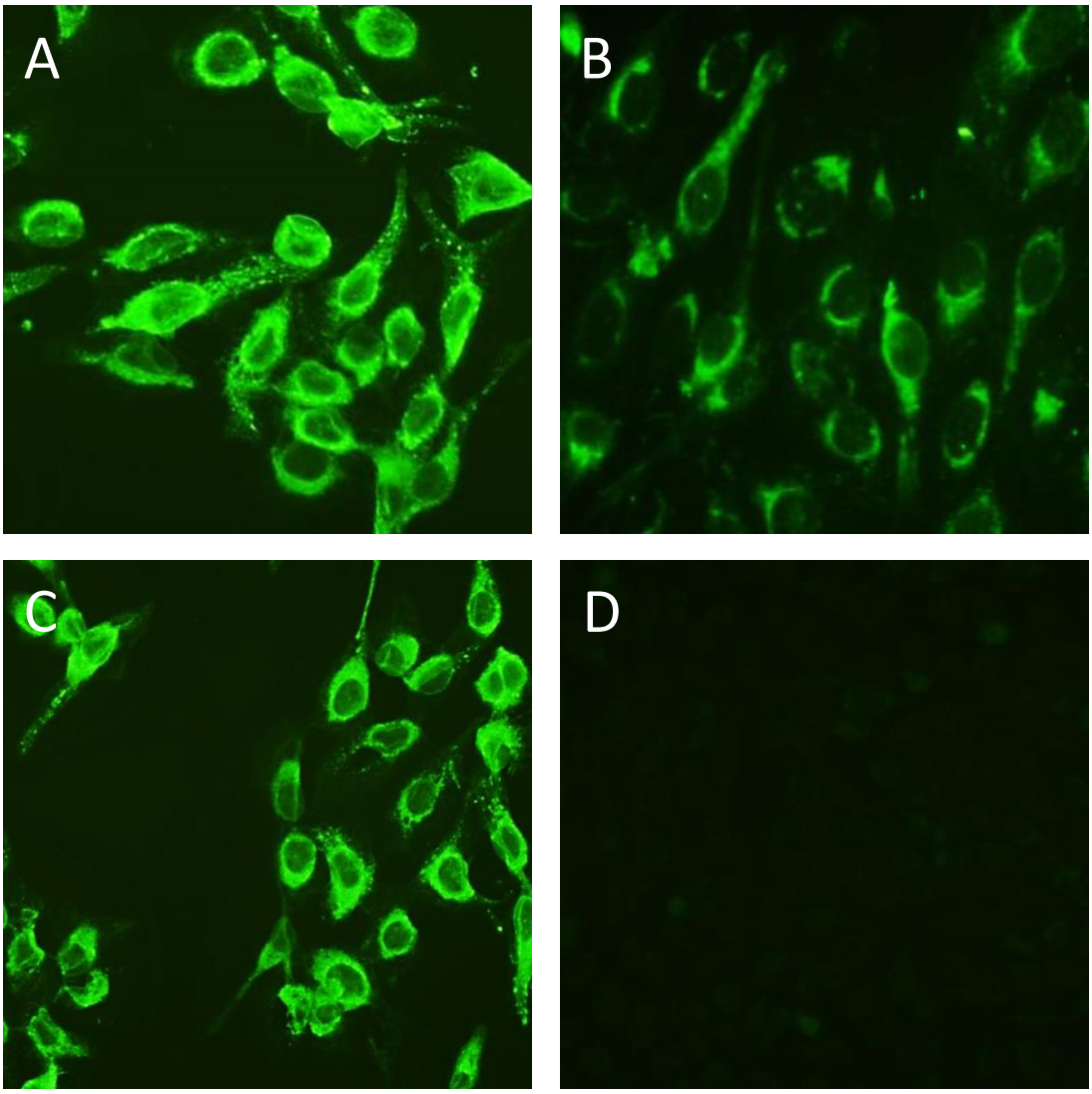


Fig. 5.3. *Immunofluorescence assay for the detection of antibodies to MVEV in the sera of mice following inoculation with MVEV.*

A. 10C6 (positive control), **B.** 3H6 (positive control), **C.** positive mouse serum, **D** negative (uninfected) mouse serum,

The fitness of each isolate was determined by examining its replicative capacity in avian DF1 and mosquito C6/36 cells in single-step growth curve analyses. No significant difference was observed in the replicative ability of recent MVEV isolates compared to their prototype strains in avian cells, suggesting that the ability of MVEV isolates to infect avian cells may have been unchanged over time. In this regard, it is notable that these strains were isolated approximately 60 years apart. A fundamental question that requires further investigation is whether the ability of MVEV to infect its vertebrate hosts in nature has also been unaffected over time.

In C6/36 cells, the significantly lower replication kinetics observed in recent G1 isolates (K68838 and K60555) and the higher replicative ability of one of the recent G2 isolates (K62017) compared to the prototype strains is of interest and may be due to genetic differences between these strains. Variation in the replication of flaviviruses in mosquito and vertebrate cells has been previously demonstrated. The molecular determinants underlying this phenotypic variation are located in the C protein (Groat-Carmona et al., 2012), E protein (Ciota et al., 2007b), NS4B protein (Hanley et al., 2003) and the 3'UTR (Alvarez et al., 2005; Villordo et al., 2010; Villordo and Gamarnik, 2013; Zeng et al., 1998). For example, Hanley et al. (2003) demonstrated that a point mutation in the NS4B protein ($P^{101}L$) resulted in decreased DENV replication in C6/36 cells, while this mutation increases the replication kinetics of the virus in mammalian cells. The recent G1 isolates K68838 and K60555 may contain a mutation(s) of similar effect that decreases their replicative ability in C6/36 cells relative to DF1 cells. Comparison of the E genes of these isolates to that of MVE-1-51 reveals that K68838 (G1a) encodes only one substitution ($M^{180}L$) located in domain I of the E protein (Fig. 2.4), while K60555 (G1b) exhibits two, $T^{230}S$ and $I^{237}V$, both in domain II. Furthermore, comparison of the E gene of K62017 to that of OR156 reveals three unique mutations ($S^{7}G$, $V^{68}I$ and $A^{367}V$, located in domain I and II, respectively (Fig. 2.4). These locations on the E gene have not been previously associated with any molecular determinants of fitness and virulence. Further genetic and phenotypic studies may reveal the biological importance of these substitutions as well as substitutions in other parts of the genome that are associated with a lower replicative ability in mosquito cells.

To extend the *in vitro* characterisation, *in vivo* neuroinvasion was examined by determining the HD_{50} and AST values after i.p. injection of groups of 18-day old mice with different doses of each virus. Consistent with the studies of Coelen (1988) and Lawson (1988), this study also revealed that significant variation in the neuroinvasive potential of MVEV isolates correlates to their genotypes, with G1 viruses being highly neuroinvasive and G2 viruses showing a low neuroinvasive phenotype. The mortality rate and mean time to death of mice that were infected with the G1 isolates were dependent on the doses administered, a feature of highly virulent strains (Davis et al., 2003a). The HD_{50} and AST values were reliably obtained for G1 isolates and were consistent with the values reported in previous MVEV studies (Coelen, 1988; Hurrelbrink and McMinn, 2001; Lawson, 1988; Prow et al., 2011; Shueb, 2008). In contrast, groups of mice inoculated with G2 isolates demonstrated sporadic deaths across most doses, so that HD_{50} and AST could not reliably be determined.

Of interest was a significantly delayed time to death for G2 isolates (10.50 ± 2.65 days) compared to that of the G1 isolates (6.13 ± 0.92 days; p=0.002) a distinguishing feature of an attenuated strain of flaviviruses (Beasley et al., 2005; Hurrelbrink and McMinn, 2001; Lawson, 1988; Prow et al., 2011). Delayed time to death was reported for OR156 [up to 13 days; (Lawson, 1988)] and attenuated mutants of MVE-1-51 [10.2 dpi, (Hurrelbrink and McMinn, 2001)]. Similarly, a mean time to death of 11.4-13.3 dpi were reported for three WNV attenuated strains (Beasley et al., 2002; Beasley et al., 2005). It has been hypothesised that the inability of the host to entirely clear the virus, the persistence of the virus in peripheral tissues and some as-yet-unidentified immunopathological reactions might be involved in this late disease state (Lawson, 1988).

Also of interest was the observation of mice that received higher doses of the G2 isolates which displayed lower level of mortality rates than those that received lower doses. This phenomenon is called prozone effect, and has been observed for many flaviviruses, such as MVEV (Hurrelbrink and McMinn, 2001; Lobigs et al., 1988; Lobigs et al., 1990; Prow et al., 2011), WNV (Beasley et al., 2002; Beasley et al., 2005; Shirato et al., 2004) and SLEV (Monath et al., 1980). The

immunopathologic factors governing the prozone effect for G2 isolates of MVEV is not yet known.

The titres of viruses in the brains of infected mice obtained in this study are comparable with those reported previously (Beasley et al., 2005; Chambers et al., 2008). The observation of a three to four log differences between viral copy numbers and titres of MVEV isolates in the brains of individually infected mice (Appendix 5.3) indicates a high ratio of non-infectious to infectious virus particles. This may be due to the much higher sensitivity of the MVEV RT-qPCR assay (discussed in Chapter 3), compared to cell culture. Similar differences between the viral copy numbers and titres were reported by (Jerzak et al., 2005), where the concentration of WNV-infected samples taken from different bird and mosquito species were tested. The presence of DI particles in the infected mouse brains and subsequent cultures may also have contributed to this difference. DI particles can reduce the cytopathic effect of MVEV infection in cell culture (Poidinger et al., 1991), but the genomes of these particles can be amplified by the RT-qPCR. Although the genomes of DI particles of flaviviruses lack most of the structural genes and the 5' end of NS1 gene (Lancaster et al., 1998; Yoon et al., 2006), there are no reports of deletions in the 3'UTR. Therefore, the presence of DI particles (if any) could have been detected by the RT-qPCR assay employed in this study, which targets the 3'UTR, thereby leading to an apparently high ratio of genome copies to infectious particles.

The variation in the neuroinvasivness of MVEV strains from different genotypes observed in this study is comparable with results of similar studies investigating flavivirus virulence phenotype (Beasley et al., 2002; Coelen, 1988; Lawson, 1988; Monath et al., 1980). In these studies researchers correlated the variation in the virulence of flaviviruses such as MVEV (Coelen, 1988; Lawson, 1988) WNV (Beasley et al., 2002), and SLEV (Monath et al., 1980) to different genotypes. This suggests that certain molecular determinants of pathogenesis specific for each genotype are responsible for the virulence properties of these viruses. In this regard, the variability in neuroinvasiveness of flaviviruses has been associated with microevolution. While some amino acid substitutions result in an increase in

virulence (Brault et al., 2004; Brault et al., 2007; Kilpatrick et al., 2008; Moudy et al., 2007), others significantly reduce virulence of flaviviruses (Beasley et al., 2005; Gualano et al., 1998; Guirakhoo et al., 2004; Holbrook et al., 2001; Holzmann et al., 1990; Hurrelbrink and McMinn, 2001; McMinn et al., 1995; Monath et al., 1980; Ye et al., 2012). Comparison of full-length prM and E proteins of G2 to that of G1 revealed several amino acid substitutions, already discussed in Chapter 2 (see also Section 2.3.2 and Table 2.7). The biological significance of many of these substitutions is as yet not known. However G2 exhibits a conservative $S^{275}T$ and a non-conservative $S^{276}G$ substitution in the flexible hinge region between domain I and domain II. Previous research revealed that mutation in this region can greatly influence the fusogenic activity and neuroinvasiveness of MVEV in mice (Hurrelbrink and McMinn, 2001; Prow et al., 2011). In addition, as mentioned in Chapter 6 (Section 6.3.6) the G2 isolates of MVEV exhibit several other amino acid differences compared to G1 in the structural and non-structural proteins as well as 5' and 3'UTRs (Chapter 6). These genetic changes may also contribute to or account for their attenuated phenotype.

The results in Chapter 4 indicated that G2 viruses exist as less genetically diverse populations than G1 viruses. This is significant because previous research has revealed that more diverse populations of RNA viruses have a significantly higher replication, pathogenesis and virulence than less diverse populations (Ciota et al., 2007a; Domingo et al., 2006; Lancaster and Pfeiffer, 2012; Lauring and Andino, 2010; Perales et al., 2010; Vignuzzi et al., 2006). It can be hypothesised that less complex mutant spectra in G2 viruses, coupled with the unique mutations observed in viruses within this genotype, have significantly attenuated its virulence phenotype. However, it should be noted that in this chapter, phenotypic characterisation was performed on virus isolates, whereas in Chapter 4, homogenates of mosquito pools were examined.

The data presented in this chapter supports the hypothesis that G2 isolates of MVEV are attenuated. Unfortunately, due to an overwhelming number of mutations scattered in structural and non-structural proteins of the G2 (Chapter 6, Section 6.3.6), a specific mutation/s cannot be readily associated with this

attenuation. Further genetic and molecular studies are required to elucidate the mechanism of virulence or attenuation of these two very closely related genotypes of MVEV. The availability of an infectious clone for MVEV [pMVE-1-51; (Hurrelbrink et al., 1999)] offers a unique opportunity for reverse genetic studies. Introduction of point mutations or a complete gene of G2 isolates into the MVEV infectious clone, similar to those reported by previous researchers (Chambers et al., 2005; Hurrelbrink and McMinn, 2001; Matusan et al., 2001; Prow et al., 2011; Pryor et al., 1998) may provide valuable information on revealing the molecular determinants of attenuation in the G2 isolates.

CHAPTER 6

GENETIC AND PHENOTYPIC CHARACTERISATION OF MURRAY VALLEY ENCEPHALITIS VIRUS FOLLOWING ADAPTATION IN MOSQUITO AND AVIAN CELLS

6.1 Introduction

MVEV, as for any other arbovirus, circulates in nature by alternating replication (cycling) between arthropod vectors and vertebrate hosts. This places substantial evolutionary constraints, which may have limited the evolution of this virus throughout its history (Ciota and Kramer, 2010; Weaver, 2006). As addressed in Chapter 2 (Sections 2.3.2.1 and 2.3.2.2), MVEV has shown a high level of genetic conservation over time.

Experimental evolution studies that have employed cell culture systems (Chen et al., 2003; Coffey and Vignuzzi, 2010; Moutailler et al., 2011; Vasilakis et al., 2009) and animal models (Ciota et al., 2009; Coffey et al., 2008; Lin et al., 2004) have provided conflicting conclusions with regards to the effect of host cycling on the genetic stability of arboviruses. A review of these studies is provided in Chapter 1 (Section 1.3.1). In short, some studies have indicated that host cycling results in genetic stability (Coffey et al., 2008; Coffey and Vignuzzi, 2010; Moutailler et al., 2011), whereas, others conclude that evolutionary conservation of arboviruses is the result of adaptation in a single host cell type (Chen et al., 2003; Ciota et al., 2009; Lin et al., 2004; Vasilakis et al., 2009). To date, no experimental evolution studies have been carried out to explain the reason behind the evolutionary stasis of MVEV.

This chapter describes an *in vitro* experimental evolution study carried out to examine the evolutionary constraints imposed by sequential passaging or cycling of MVEV in mosquito and/or avian cell lines. This study aims to investigate the molecular basis of such adaptation phenotypes. Three hypotheses were simultaneously tested:

1. Sequential passaging of MVEV in single host cells remove the selective pressures imposed by cycling and will facilitate the accumulation of mutations;

2. A significant fitness trade-off exists in viruses derived from avian and mosquito cell cycling, whereas higher levels of fitness and adaptation may be observed in

single host cell-specialised viruses, in which increased fitness in one host cell line will be accompanied by decreased fitness in the other; and

3. A minor number of mutations can have a significant impact on the phenotype of MVEV both *in vitro* and *in vivo.*

Results obtained in this study may provide an insight into the mechanism(s) of MVEV evolution and indicate whether a significant fitness trade-off exists in the MVEV population as a result of the requirements to replicate in mosquito and vertebrate hosts.

6.2 Materials and Methods

6.2.1 Cells

DF1, C6/36 and PSEK cells were grown as described in Chapter 5 (Section 5.2.1).

6.2.2 Clone-Derived Viruses

Clone-derived virus representing G1 of MVEV was generated from full-length *in vitro*-transcribed RNA prepared from the pMVE-1-51 infectious clone (Hurrelbrink et al., 1999), as described in Chapter 3 (Section 3.2.4). In order to generate infectious viral RNA, 5mM cap analogue [m7G(5')ppp(5')G; Life Technologies, Australia] was added to the T7 RNA synthesis reaction. The *in vitro*-transcribed MVE-1-51 RNA was then purified using a QIAGEN RNeasy Kit (QIAGEN, Australia), according to the manufacturer's protocol. Subsequently, a sub-confluent monolayer of PSEK cells was transfected with the capped *in vitro*-transcribed MVE-1-51 RNA using Lipofectamine 2000^{TM} reagent in a 25cm^2 tissue culture flask (Greiner Bio-one, Australia). Briefly, 1.5µg of purified RNA was added to 500µl of Opti-MEM media (Life Technologies, Australia) in a 1.5ml eppendorf tube and incubated at room temperature for 20 minutes. The sub-confluent (80%) monolayer of cells was washed twice with 5ml of PBS and once with 5ml of Opti-MEM. Cells were transfected with transfection mixture (500µl of Opti-MEM containing *in vitro*-transcribed RNA) at 37°C for 3 hours. The media was removed and cells were washed twice with 5ml of PBS, prior to adding 3ml of maintenance

M199 media (Life Technologies, Australia). The cells were incubated under standard cell culture conditions for five days or until approximately 75% CPE was observed. Tissue culture supernatants were removed and clarified by centrifugation at 1500*xg* for 5 minutes. The rescued viruses were further cultured in a 25cm^2 tissue culture flask (Greiner Bio-one, Australia) of C6/36 cells under standard conditions for 5 days. Supernatants were removed and clarified as above before they were used as working stock for parental virus of G1 (G1-P0) in serial passage experiments.

A full-length infectious clone representing G2 of MVEV has not been reported and was not available. Therefore, biological clones of G2 MVEV were prepared using a cloning by limiting dilution method. Initially, 5-fold serial dilutions of OR156 were prepared and used to infect monolayers of PSEK cells in a 96-well micro-titre plate (Greiner Bio-one, Australia). The monolayers were incubated at 37°C for 3-4 days or until CPE was observed in the highest dilution of virus. The tissue culture supernatant of the well exhibiting CPE in the end-point dilution was harvested and clarified as above. A further set of 2-fold serial dilutions was prepared from the resultant supernatant and were used to infect PSEK cells in a 96-well micro-titre plate (Greiner Bio-one, Australia). The supernatants of the wells with the highest dilution that demonstrated CPE were further expanded individually by infecting C6/36 cells in a 25cm^2 tissue culture flask (Greiner Bio-one, Australia) for 5 days. Supernatants were harvested and clarified as described above and used as working stock for parental virus of G2 (G2-P0) in cell passage experiments.

6.2.3 Experimental Cell Passages

The cell passage experiment reported in this chapter was performed essentially as described elsewhere (Chen et al., 2003; Ciota et al., 2007b; Coffey et al., 2008; Coffey and Vignuzzi, 2010; Jerzak et al., 2008; Moutailler et al., 2011). Briefly, G1-P0 and G2-P0 were subjected to ten sequential passages in C6/36 cells (yielding viruses G1-C10 and G2-C10, respectively) or DF1 cells (G1-D10 and G2-D10, respectively; Fig. 6.1). As a control, G1-P0 and G2-P0 were also passaged sixteen times alternately between C6/36 and DF1 cells (G1-A16 and G2-A16, respectively; Fig. 6.1).

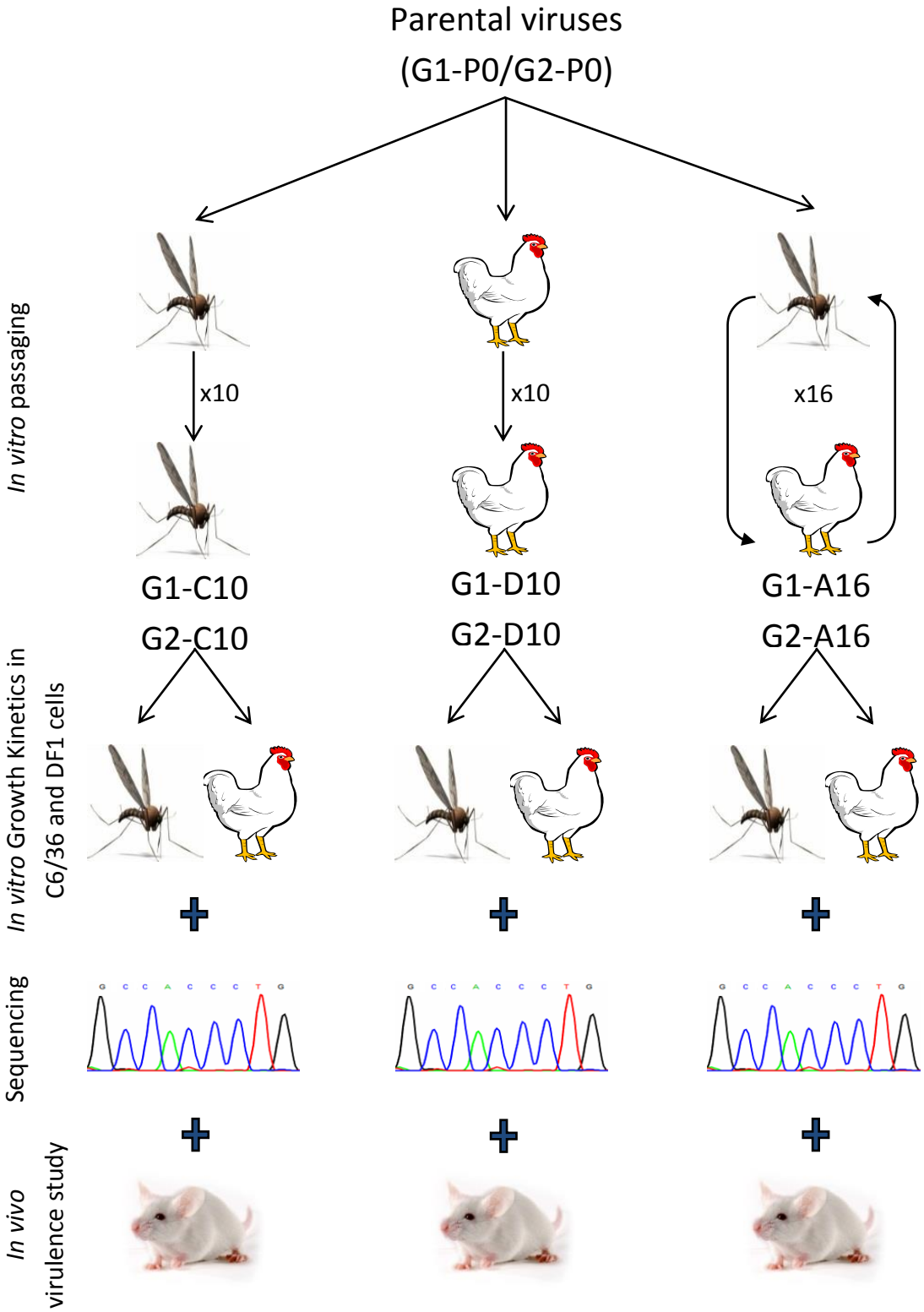


Fig. 6.1. *Schematic representation of the methodology used in this chapter.*

G1-C10 and G1-D10 indicate G1 viruses passaged ten times in C6/36 cells or DF1 cells, respectively. G1-A16 indicates G1 virus passaged 16 times alternately between C6/36 cells and DF1 cells. G2 passaged virus nomenclature is similarly shown.

For each passage, sub-confluent monolayers of DF1 and C6/36 cells ($1x10^6$ cells) were infected at an MOI of 0.01 in a $25cm^2$ tissue culture flask (Greiner Bio-one, Australia) and incubated for one hour at appropriate growth temperature. Inoculum was then removed and cell monolayers were washed twice with 5ml of PBS. Next, 5ml of fresh maintenance media was added to each flask and cells were incubated for three days. The cell culture supernatants were centrifuged at 3000*xg* for 5 min and stored at -80°C. The titre of the supernatants was determined in PSEK cells after each passage, as described in Chapter 3 (Section 3.2.2).

6.2.4 Growth Kinetics in Cell Culture

The replication kinetics of the passaged viruses was determined in DF1 and C6/36 cells and compared to their parental viruses, as per the method described in Chapter 5 (Section 5.2.3). Additional samples were taken at 6 hpi for both DF1 and C6/36 cells. Since the growth kinetics of all viruses in DF1 cells indicated a reduction in titre after 48 hpi, the 96 hpi time-point was omitted from DF1 infection.

6.2.5 Mouse Neuroinvasive Experiments

Animal studies were performed as per methodology detailed in Chapter 5 (Section 5.2.4). The animal study conducted in this chapter received approval from the Curtin University's AEC (AEC approval number: AEC_2011_64).

6.2.6 Virus Detection and Quantification

The brains of mice that succumbed to the lethal infection were homogenised as detailed in Chapter 5 (Section 5.2.7). The concentration of MVEV in the brain homogenates was determined by titration in PSEK cells and using the MVEV-specific RT-qPCR assay as detailed in Chapter 5 (Section 5.2.5).

6.2.7 Serological Confirmation of Infection in Surviving Mice

At 21 dpi, all surviving mice were euthanised and their sera were collected as described in Chapter 5 (Section 5.2.6). IFA was performed on the sera of all surviving mice as detailed in Chapter 5 (Section 5.2.6).

6.2.8 RNA Extraction and RT-PCR of the Complete Genomes

RNA was extracted from 140µl of each sample (tissue culture supernatants or brain homogenates) using the QIAamp® Viral RNA mini kit (QIAGEN, Australia), as per the manufacturer's protocol. The cDNA was synthesised using 10µl of purified RNA, reverse primers [VD8 (Pierre et al., 1994) or MVEV16Rb; Appendix 6.1] and SuperScript® III Reverse Transcriptase kit (Life Technologies, Australia), as described in Chapter 2 (Section 2.2.2). The complete genome of G1 viruses were amplified using 19 overlapping sets of primers (Appendix 6.1) and PCR SuperMix (Life Technologies, Australia) as described in Chapter 2 (Section 2.2.2). Similarly, the complete genomes of G2 viruses were amplified using seventeen overlapping sets of primers (Appendix 6.2). All PCR reactions were performed as described in Chapter 2 (Section 2.2.2), except that some G2 amplicons underwent an elongation step at 72°C for 90 seconds (indicated by asterisks in Appendix 6.2). The PCR products were purified using QIAquick® PCR purification kit (QIAGEN, Australia), according to the manufacturer's protocol.

6.2.9 Sequencing and Sequence Analyses

Sequencing were carried out at AGRF, Perth, as described in Chapter 2 (Section 2.2.3). The raw sequences were compiled, edited and assembled in BioEdit (Hall, 1999) and MEGA 5.2.1 (Tamura et al., 2011) software. Multiple alignment of the nucleotide sequences and the deduced amino acid chains were carried out using ClustalW in MEGA 5.2.1. The authenticity of all observed mutations was confirmed by resequencing the associated region using freshly extracted RNA from the respective viral stock. The eight complete genome sequences obtained in this study were deposited in the GenBank database (KC852189 - KC852196; Table 6.1).

6.2.10 Statistical Analyses

Statistical analyses were performed as per methodology described in Chapter 5 (Section 5.2.7).

Table 6.1. *Details of all viruses used in this chapter.*

Virus	GenBank ID	Experimental Passages	Accession No
G1-P0 *	MVE-1-51-P0	N/A	KC852189
G1-C10	MVE-1-51-C10	C6/36 (10x)	KC852190
G1-D10	MVE-1-51-D10	DF1 cells (10x)	KC852191
G1-A16	MVE-1-51-CD8	C6/36 ↔ DF1 (16x)	KC852192
G2-P0 †	OR156-P0	N/A	KC852193
G2-C10	OR156-C10	C6/36 (10x)	KC852194
G2-D10	OR156-D10	DF1 cells (10x)	KC852195
G2-A16	OR156-CD8	C6/36 ↔ DF1 (16x)	KC852196

***** Derived from a molecular clone of MVE-1-51 (CDV-1-51), which was rescued in C6/36.

† Derived from a biological clone of OR156 (CDV OR156), and passed once in C6/36.

6.3 Results

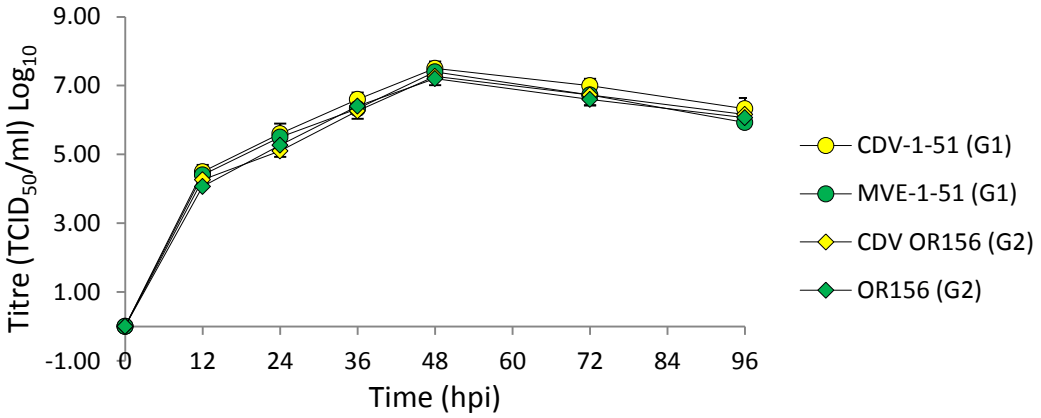
6.3.1 Comparison of the Clone-Derived and Wild-Type Viruses: *in Vitro* Replication in Cells and *in Vivo* Pathogenesis in Mice

In order that genetically homogeneous virus populations were used as parental viruses in the serial cell passage experiment, clone-derived viruses of G1 and G2 were generated. To ensure that clone-derived viruses (CDV-1-51 and CDV OR156) were not phenotypically different to those of un-cloned laboratory strains (MVE-1-51 and MVE OR156), the phenotypes of these viruses were compared *in vitro* in a single-step growth curve assay and *in vivo* in a mouse model of pathogenesis. No significant differences were seen in the replication kinetics of the clone-derived viruses to those of un-cloned laboratory strains both in C6/36 and DF1 cells (Fig. 6.2). Similarly, following infection in mice, no significant differences in virulence of clone-derived viruses were observed, when compared to those of un-cloned laboratory strains (Table 6.2). Similar to the results reported for G1 MVEV in Chapter 5 (Section 5.3.2), CDV-1-51 caused dose-dependent mortality in mice, characteristic of highly virulent strains. In contrast, for G2 viruses, a sporadic pattern of deaths across the virus doses tested (10^2-10^4), characteristic of attenuated flaviviruses, was observed. These results indicate that no significant phenotypic differences between the clone-derived viruses and un-cloned laboratory strains were apparent. Therefore, CDV-1-51 and CDV OR156 were used as parental viruses of G1 (G1-P0) and G2 (G2-P0), respectively, in the cell passage experiment described below.

6.3.2 Experimental Passage

G1-P0 and G2-P0 were sequentially passaged ten times in either mosquito (C6/36) or avian (DF1) cells and as a control, they were also passaged for a total of sixteen times alternately between the two cell lines (eight passages in each; Fig. 6.1). This resulted in the generation of virus populations designated G1-C10, G1-D10, and G1-A16 for G1 MVEV and G2-C10, G2-D10, and G2-A16 for G2 MVEV (Fig. 6.1). Fig. 6.3 shows the infectious titres of all viruses at each passage. Although the titres of

A



Reference	Viruses	Mean Difference	Standard error	q statistic *	*p*-value †
CDV-1-51 (G1)	MVE-1-51	1.167	0.658	1.774	0.60
CDV OR156 (G2)	OR156	0.200	0.658	0.304	0.83

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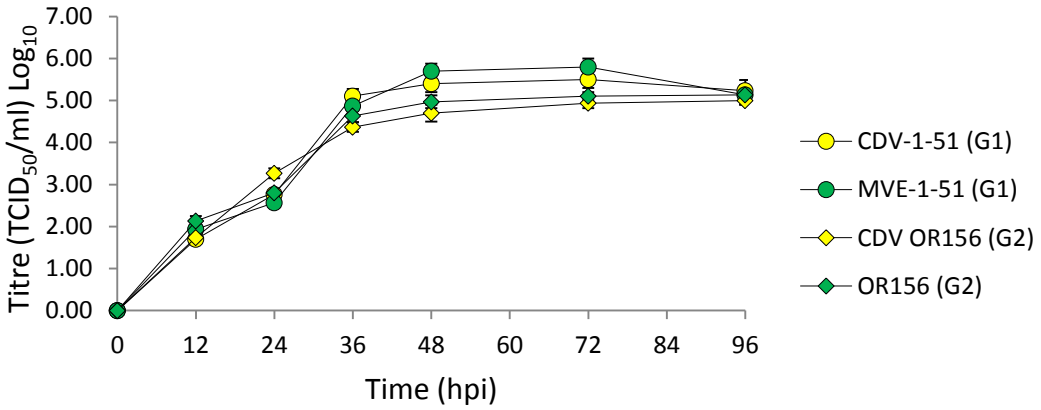

Reference	Viruses	Mean Difference	Standard error	q statistic *	*p*-value †
CDV-1-51 (G1)	MVE-1-51	-0.300	0.210	1.425	0.33
CDV OR156 (G2)	OR156	-0.433	0.210	2.059	0.34

Fig. 6.2. *Growth kinetics of clone-derived vs. un-cloned laboratory strains of genotype 1 (CDV-1-51, MVE-1-51) and genotype 2 (CDV OR156 and OR156) MVEV in **(A)** DF1 cells and **(B)** C6/36 cells.* Statistical analyses for assays in each cell line are shown below each graph.

* **The q statistic** (studentised range statistic) is used for multiple significance testing across a number of means. It is the calculation of the differences in means divided by the standard error.
† A p-values of <0.05 is considered significant.

Mean differences is the difference between the sum of means of triplicates of two given samples.
Standard error in the table refers to the standard error of the differences between means.

Table 6.2. *Average survival time (AST) and HD_{50} values of clone-derived vs. un-cloned laboratory strains of genotype 1 (CDV-1-51, MVE-1-51) and genotype 2 (CDV OR156 and OR156) MVEV in a mouse model of pathogenesis.*

Virus	Dose (Log_{10} $TCID_{50}$)	Mortality (%)	AST (days)	HD_{50} (Log_{10} $TCID_{50}$)	*p-value* *
Genotype 1					
	0	0/6 (0%)	N/A		
CDV-1-51 (G1)	1	1/6 (16.7)	7		
	2	4/6 (66.7)	7	1.64	
	0	0/6 (0%)	N/A		
MVE-1-51 (G1)	1	4/6 (66.7%)	6.3		
	2	5/6 (83.3%)	5.2	0.88	0.27
Genotype 2					
	2	2/6 (33.3%)	7.5		
CDV OR156 (G2)	3	1/6 (16.7%)	7		
	4	2/6 (33.3%)	5	>4.00	
	2	1/6 (16.7%)	12		
OR156 (G2)	3	0/6 (0%)	N/A		
	4	0/6 (0%)	N/A	>4.00	0.06

* *p*-value of > 0.05 is considered not significant.

N/A: Not applicable

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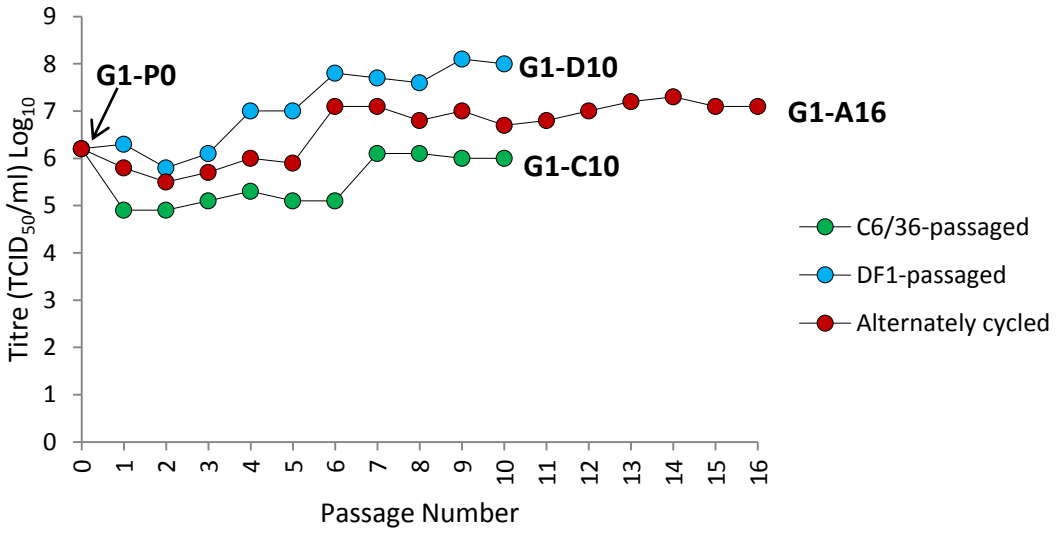

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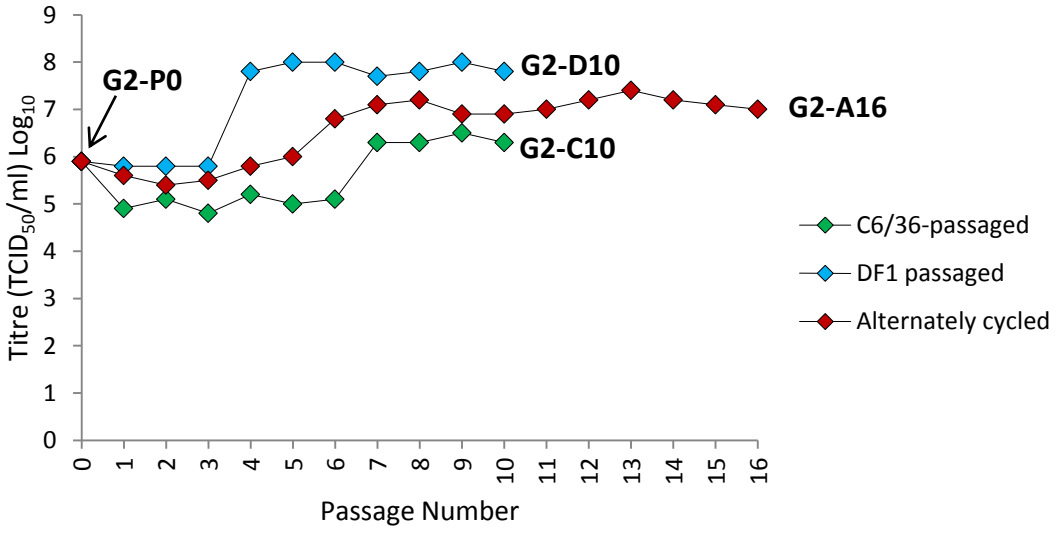

Fig. 6.3. *The titre of genotype 1* ***(A)*** *and genotype 2* ***(B)*** *MVE viruses during sequential passage in mosquito C6/36 cells and avian DF1 cells, and alternately cycled between the two.*

Titrations were performed in PSEK cells.

C6/36-cell-passaged viruses were approximately 1.0 $\log_{10}$ lower than the unpassaged parental viruses for the first six passages, the titres of G1-C10 and G2-C10 were similar to that of their respective parental viruses, suggesting that the viruses adapted to C6/36 cells after six passages. In contrast, the titre of DF1-cell-passaged viruses were similar to their parental viruses for the first three passages, but increased from passage 4 onwards, such that the titres of G1-D10 and G2-D10 were approximately 2.0 $\log_{10}$ higher than G1-P0 and G2-P0, respectively. This indicates selection of viruses with a higher replicative ability and fitness in DF1 cells. In comparison, the titres of cycled-viruses (G1-A16 and G2-A16) were not substantially different to those of the respective parental viruses for the first five passages, after which an approximately 1.0 $\log_{10}$ increase in titre was observed. The titres of the G1-A16 and G2-A16 were comparable between P6 and P16 (Fig. 6.3).

6.3.3 Comparison of Viral Fitness

The viral replicative ability of the passaged viruses of each genotype were assessed by single-step growth curve analyses and compared to that of the associated parental virus in both DF1 and C6/36 cells.

6.3.3.1 G1 Viruses

In C6/36 cells, no significant differences were observed in the replicative ability of G1-C10 compared to G1-P0 (p=0.31; Fig. 6.4A and Appendix 6.3A). The titre of these two viruses increased rapidly during the first 6 hpi, then increased steadily until 48 hpi, after which it plateaued to 96 hpi (end of experiment). In contrast, G1-D10 demonstrated a small, but statistically significant decrease in replicative ability compared to G1-P0 (p=2.26E-04) and G1-C10 (p=1.94E-04; Fig. 6.4A and Appendix 6.3A). Moreover, the replicative fitness of G1-A16 was substantially and significantly lower than G1-P0 (p=1.93E-07), G1-D10 (p=1.93E-07) and G1-C10 (p=2.14E-07; Fig. 6.4A and Appendix 6.3A), indicating that G1-A16 has a lower level of replicative fitness in C6/36 cells than any other G1 isolates included in this study (Fig 6.4A). In DF1 cells, no significant difference in the replicative ability of G1-C10 compared to G1-P0 was observed over the course of the experiment (p =0.36; Fig. 6.4B and Appendix 6.3B). However, G1-D10 displayed a higher replicative ability than G1-P0

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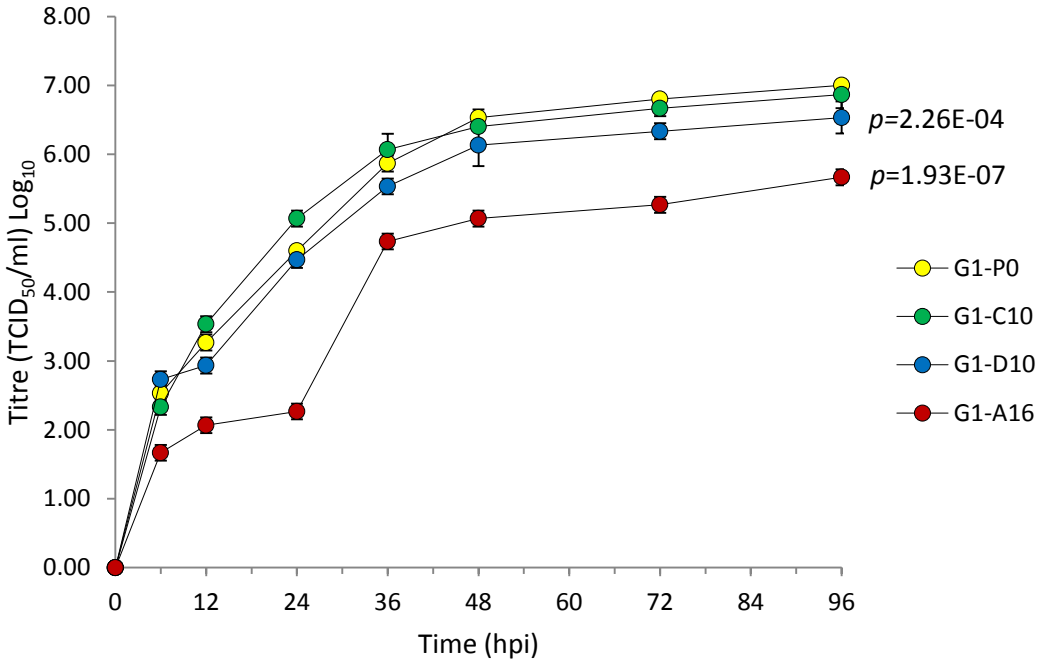

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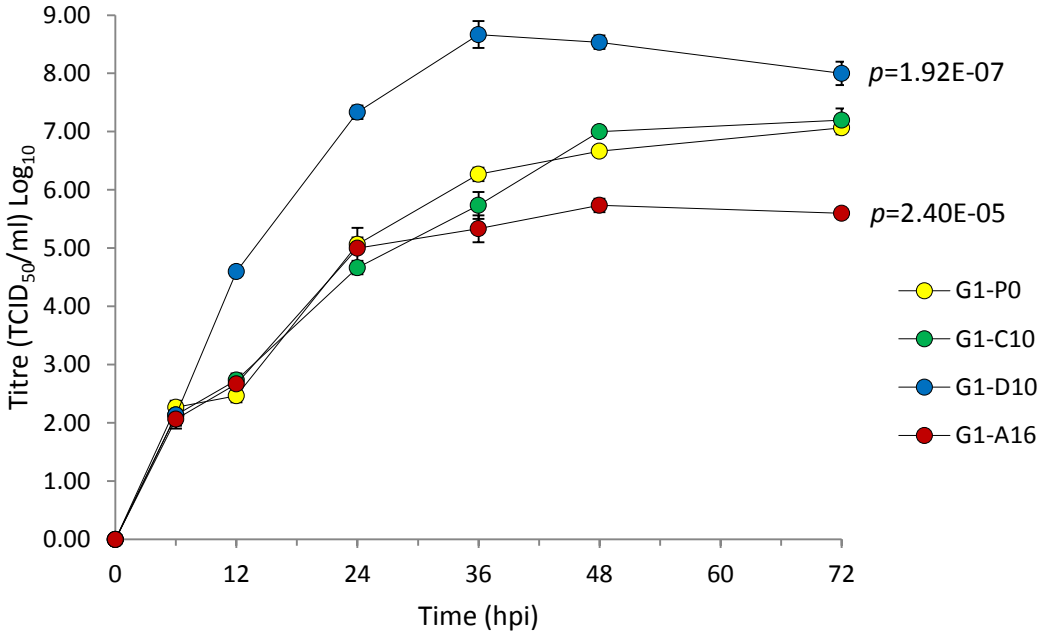

Fig 6.4. *Comparison of growth kinetics of MVEV G1-D10, G1-C10 and G1-A16 with their parental virus (G1-P0) in C6/36* ***(A)*** *and DF1 cells* ***(B)****.*

Titres are presented as the mean and standard error from three individual replicates obtained at each time-point. **G1-D10:** DF1-cells-passaged virus; **G1-C10:** C6/36-cells-passaged virus; **G1-A16:** C6/36-DF1-passaged virus; **G1-P0:** Parental unpassaged virus.

(p=1.92E-07) and G1-C10 (p=1.95E-07; Fig. 6.4B and Appendix 6.3B). This indicates that avian DF1-cell-derived G1-D10 has a higher level of replicative fitness in these cells, compared to the other serially-passaged G1 strains and that adaptation to this cell line has taken place over the ten sequential passages. In contrast, the replicative ability of G1-A16 in DF1 cells, as in C6/36 cells, was significantly lower than G1-P0 (p=2.40E-05), G1-C10 (p=1.97E-05) and G1-D10 (p=2.13E-07; Fig. 6.4B and Appendix 6.3B) over the duration of the time course of infection.

Taken together, sequential passages of G1-P0 in mosquito C6/36 cells did not alter the growth kinetics of the resulting virus (G1-C10) in both C6/36 and avian DF1 cells. However, virus derived from sequential passage in avian DF1 cells (G1-D10) demonstrated a lower replicative ability in C6/36 cells, but increased replicative fitness in DF1 cells, indicating adaptation to these cells. Alternately-cycled virus G1-A16 showed lower replication kinetics than the parental virus in both C6/36 and DF1 cells, suggestive of a lower replicative fitness for this virus in both host cell lines.

6.3.3.2 G2 Viruses

In C6/36 cells, no difference in replication kinetics was observed between G2-P0 and G2-C10 (p=0.32; Fig. 6.5A and Appendix 6.4A). These viruses exhibited comparable titres across all time-points tested. In contrast, G2-D10 and G2-A16 demonstrated marginally, but statistically significant, lower titres in mosquito cells than G2-P0 (p=5.63E-05 and p=9.12E-05, respectively; Appendix 6.4A) and G2-C10 (p=4.32E-05 and p=8.85E-05, respectively; Fig. 6.5A and Appendix 6.4A). There were no differences between the replicative ability of G2-D10 and G2-A16 (p=0.14; Appendix 6.4A), indicating they have a similar growth phenotype in C6/36 cells. As observed in C6/36 cells, there was no difference between the replicative ability of G2-P0 and G2-C10 in the avian DF1 cells (p=0.28; Fig. 6.5B and Appendix 6.4B). In contrast, the replication curves of G2-D10 and G2-A16 were significantly higher than G2-P0 (p=1.92E-07 and p=2.91E-07, respectively; Appendix 6.4B) and G2-C10 (p=2.13E-07 and p=3.55E-07, respectively; Appendix 6.4B). A significant difference in replication kinetics between G2-D10 and G2-A16 was also found (p=3.85E-07).

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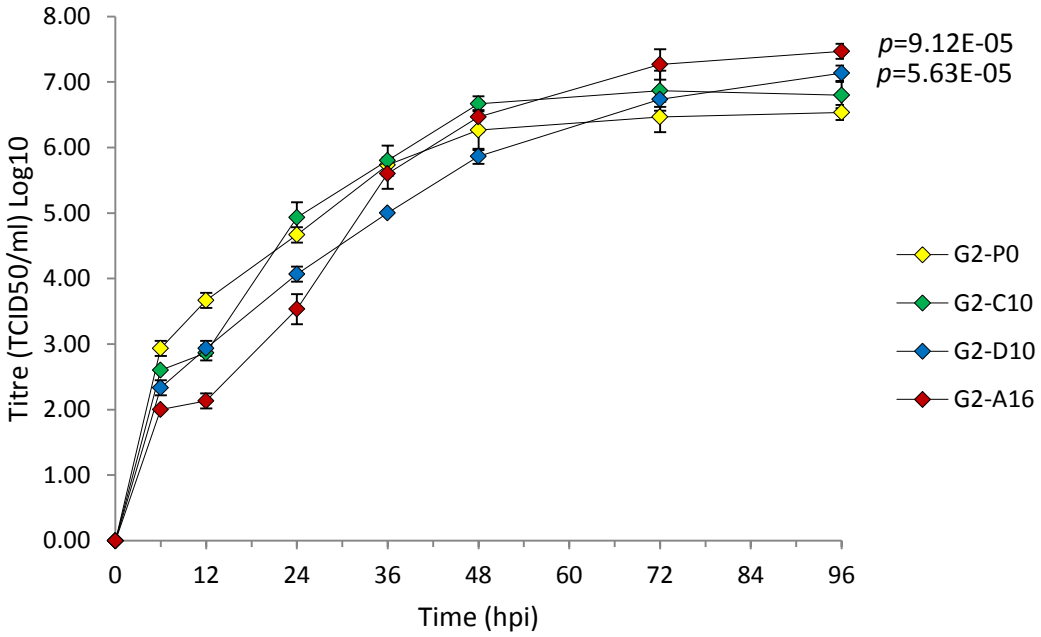

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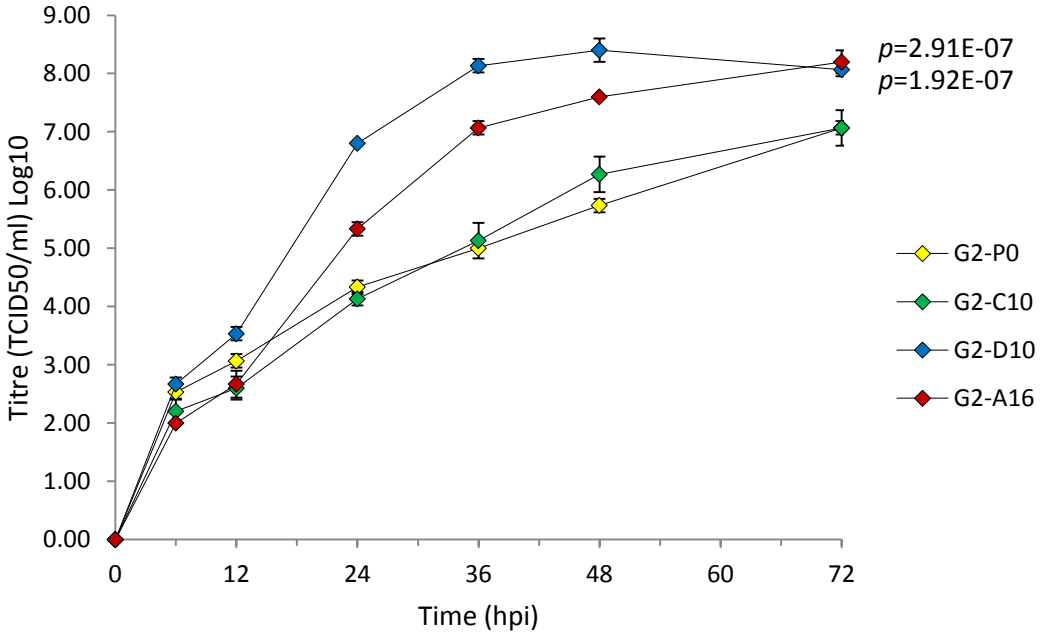


Fig 6.5. *Comparison of growth kinetics of G2-D10, G2-C10 and G2-A16 with their parental virus (G2-P0) in C6/36* ***(A)*** *and DF1 cells* ***(B)****.*

Titres are presented as the mean and standard error from three individual replicates obtained at each time-point. **G2-D10:** DF1-cells-passaged virus; **G2-C10:** C6/36-cells-passaged virus; **G2-A16:** C6/36-DF1-passaged virus; **G2-P0:** Parental unpassaged virus

Titres of G2-were approximately 1.0 log_{10} higher titre than G2-A16 from 6 hpi to 48 hpi (Fig. 6.5B).

As for G1 virus, sequential passage of G2 parental virus (G2-P0) in C6/36 cells did not significantly affect the growth kinetics of the virus in either C6/36 or DF1 cells. In contrast, sequential passages in avian DF1 cells (yielding G2-D10) and alternate cycling between C6/36 and DF1 cells (G2-A16) resulted in slightly lower replicative replication kinetics in C6/36 cells, but a higher replicative fitness in DF1 cells. This suggests that differences in the replication kinetics of these viruses are due to passage in DF1 cells, either sequentially or alternately.

6.3.4 Mouse Virulence Study

Neuroinvasiveness of each passaged virus was determined by i.p. inoculation of groups of 18-day old Swiss ARC mice with a range of virus doses. For each virus, the HD_{50} and AST values were determined (when possible) and compared to their parental viruses. Virus concentrations (genome copy numbers and infectious titres) in the brain of euthanised mice were also determined and compared.

6.3.4.1 G1 Viruses

Initially, mice were inoculated with 10^0-10^2 $TCID_{50}$ of G1 isolates. Mice were healthy for 4-6 dpi, after which they exhibited early signs of disease, similar to those described in Chapter 5 (Section 5.3.2). The HD_{50} and AST values of G1-C10 were lower, but not significantly different, to those of G1-P0 (Table 6.3 and Appendix 6.5) and mice injected with these viruses demonstrated a dose-dependent mortality rate (Table 6.3 and Appendix 6.5). Virus concentrations in the brain of euthanised mice were not significantly different as determined by genome copy number (p=0.648) or $TCID_{50}$ (p=0.639; Appendix 6.7). Similar to the findings described in Chapter 5 (Section 5.3.2), approximately 3.0 to 4.5 log_{10} differences were observed between the RNA copy number (12.39 to 13.51 log_{10} copies/g) and the titre (9.00 to 9.8 log_{10} $TCID_{50}$/g) of these viruses in the brains of individually infected mice. Remarkably, HD_{50} and AST values could not be obtained for G1-D10 and G1-A16 viruses when mice were inoculated with the same dose range. Therefore, additional

Table 6.3. *HD_{50} and average survival time (AST) values of the passaged variants compared to the parental viruses.*

Virus	Dose (Log_{10} $TCID_{50}$)	Mortality (%)	AST (days)	HD_{50} (Log_{10} CID_{50})	*p-value**
Genotype 1 isolates					
G1-P0	0	0/6 (0%)	N/A		
G1-P0	1	1/6 (16.7)	7		
G1-P0	2	4/6 (66.7)	7	1.64	
G1-C10	0	0/6 (0%)	N/A		
G1-C10	1	5/6 (83.3%)	6.6		
G1-C10	2	6/6 (100%)	5.35	0.60	0.225
G1-D10	0	0/6 (0%)	N/A		
G1-D10	1	0/6 (0%)	N/A		
G1-D10	2	0/6 (0%)	N/A		
G1-D10	3	0/6 (0%)	N/A		
G1-D10	4	0/6 (0%)	N/A		
G1-D10	5	0/6 (0%)	N/A		
G1-D10	6	0/6 (0%)	N/A	>6.0	**0.018**
G1-A16	0	0/6 (0%)	N/A		
G1-A16	1	1/6 (16.7)	N/A		
G1-A16	2	0/6 (0%)	N/A		
G1-A16	3	0/6 (0%)	N/A	>4.0	**0.028**
G1-A16	4	0/6 (0%)	N/A		
Genotype 2 Isolates					
G2-P0	2	2/6 (33.3%)	7.5		
G2-P0	3	1/6 (16.7%)	7		
G2-P0	4	2/6 (33.3%)	5	>4.0	
G2-C10	2	2/6 (33.3%)	10		
G2-C10	3	2/6 (33.3%)	8		
G2-C10	4	1/6 (16.7%)	6	>4.0	0.423
G2-D10	3	0/6 (0%)	N/A		
G2-D10	4	0/6 (0%)	N/A		
G2-D10	5	0/6 (0%)	N/A		
G2-D10	6	0/6 (0%)	N/A	>6.0	**0.001**
G2-A16	3	0/6 (0%)	N/A		
G2-A16	4	0/6 (0%)	N/A		
G2-A16	5	0/6 (0%)	N/A		
G2-A16	6	0/6 (0%)	N/A	>6.0	**0.001**

* *p*-values are based on statistical comparison of mice mortalities for the same doses of each virus with that of the associated parental virus. A *p*-value of <0.05 is considered to be significant. Numbers in bold indicate statistically significant *p*-values.

groups of mice were inoculated with up to 10^6 $TCID_{50}$ of G1-D10 and 10^4 $TCID_{50}$ of G1-A16 viruses (a higher titre was not available for G1-A16). These higher doses also did not cause lethal infections, except for one mouse that displayed paralysis in both hind legs 7 dpi with 10^1 $TCID_{50}$ of G1-A16 (Table 6.3 and Appendix 6.5). Thus, although G1 virus passaged in C6/36 cells (G1-C10) showed an unchanged (virulence) phenotype compared to that of parental virus (G1-P0), passages in DF1 cells either sequentially (G1-D10) or alternately (G1-A16) resulted in an attenuated phenotype.

Surviving mice were euthanised 21 dpi and their sera were tested for the presence of anti-MVEV antibodies. Almost all mice demonstrated strong seroconversion. Only one mouse inoculated with 100 TCID50 (lowest concentration) of G1-A16 did not have detectable antibody to MVEV (Appendix 6.8).

6.3.4.2 G2 Viruses

Similar to the experiment conducted in Chapter 5 (Section 5.3.2), mice were initially inoculated with 10^2-10^4 $TCID_{50}$ of G2-P0 and G2-C10. Inoculation of mice with G2-C10 resulted in scattered deaths across the dose range, similar to that of G2-P0 (Table 6.3 and Appendix 6.6). This pattern of mortality is also consistent with that observed for the un-cloned G2 laboratory strain OR156 and recent G2 isolate K59532, reported in Chapter 5 (Section 5.2.3). Since titres higher than 10^4 $TCID_{50}$ could not be achieved in culture, no higher doses were available for G2-P0 and G2-C10 viruses to inoculate into mice. Mice that succumbed to infection by G2-P0 and G2-C10 displayed comparable viral copy numbers (11.19 to 12.58 log_{10}/g; p=1.00) and titres (7.20 to 8.60 log10 $TCID_{50}$/g; p=0.877) in harvested brains (Appendix 6.7). Similar to the results reported in Chapter 5 (Section 5.3.2), no HD_{50} was achieved for G2-P0 and G2-C10. Since higher titres were available for G2-D10 and G2-A16, 10^3-10^6 $TCID_{50}$ doses were attempted. Surprisingly, no deaths occurred when mice were inoculated with these doses of G2-D10 and G2-A16. This contrasts with the sporadic pattern of deaths observed for G2-P0 and G2-C10, indicating that G2-D10 and G2-A16 have a more pronounced attenuation phenotype in mice than G2-P0

and G2-C10. As a result, HD_{50} and AST values could also not be obtained for these viruses (Table 6.3).

Of interest were the clinical signs of disease (loss of weight, and manifestation of slightly ruffled fur) at 11 to 13 dpi in 50% of mice inoculated with G2-D10 and G2-A16 (data not shown). However, these signs of infection only persisted for 24-48 hours, after which all mice showed a dramatic recovery and survived until the end of the experiment. All surviving mice (except two mice that were inoculated with the lowest concentration of G2-D10 and G2-A16) demonstrated seroconversions (Appendix 6.8).

6.3.5 Complete Genome Sequencing and Consensus Changes

In order to examine whether sequential passages of MVE G1 and G2 viruses in a single host cell or alternate cycling resulted in nucleotide and amino acid changes, the genomes of all viruses were sequenced and compared to their associated parental virus.

6.3.5.1 G1 Viruses

Table 6.4 summarises the sequencing results of all four G1 viruses. Consistent with their phenotypic characterisation, no genetic changes were found in the consensus sequence of the complete genome of G1-C10 compared to G1-P0. However, nucleotide mutations were observed in the prM, E, NS3 and NS4B genes of G1-D10, and the E and NS3 genes of G1-A16. The nucleotide mutations in the prM and NS4B genes of G1-D10 were synonymous and did not cause any changes in the encoded amino acid sequence. However, two amino acid substitutions in the E protein ($D^{390}N$, and $H^{398}Y$) and one amino acid substitution in the NS3 protein ($A^{27}T$) of G1-D10 were observed. For G1-A16, two nucleotide mutations in the E gene were synonymous, whereas one amino acid substitution in the E protein ($E^{308}K$) and one amino acid substitution in the NS3 protein ($G^{477}C$) were observed. All amino acid substitutions were non-conservative. This sequence analysis indicated that passaging G1 parental virus in mosquito C6/36 cells preserves the genetic stability of this virus. In contrast, sequential passage in avian DF1 cells or alternate cycling

Table 6.4. *Genetic changes in the passaged viruses of genotype 1 (G1-C10, G1-D10 and G1-A16) compared to their parental virus (G1-P0).*

	MVE viruses *						
Nt position †	**G1-P0** ‡	**G1-C10** ‡	**G1-D10** ‡	**G1-A16** ‡	**Aa change**	**Location** ◊	**Note** ○
737	C	-	T	-		prM	
1259	G	-	-	T		E	
1655	T	-	-	C		E	
1890	G	-	-	A	E to K	E-308	Non-conservative
2139	G	-	A	-	D to N	E-390	Non-conservative
2163	C	-	T	-	H to Y	E-398	Non-conservative
4683	G	-	A	-	A to T	NS3-27	Non-conservative
6033	G	-	-	T	G to C	NS3-477	Non-conservative
7646	T	-	C	-		NS4B	-

* Dashes denote no changes in the full-length genome sequence

† Nucleotides are numbered according to the complete genome of MVE-1-51 (NC000943).

‡ **G1-P0:** unpassaged; **G1-C10:** 10 passages in mosquito cells; **G1-D10:** 10 passages in avian cells; **G1-A16:** 16 passages alternately between mosquito and avian cells.

◊ Amino acids are numbered according to the beginning of each individual protein.

○ Amino acids are categorised as conservative and non-conservative as mentioned in Chapter 2 (Section 2.3.3, Fig. 2.4)

Nt: nucleotide; **Aa:** amino acid.

between mosquito and avian cells led to accumulation of genetic changes in the complete genome of G1-P0. The results also suggest that the involvement of replication in DF1 cells may have led to the acquisition of mutation in G1 mutants.

6.3.5.2 G2 Viruses

Table 6.5 summarises the sequencing results of the G2 viruses. Only one nucleotide mutation was found in the complete genome of G2-C10, resulting in a non-conservative amino acid substitution in the NS4B protein ($E^{253}K$). In contrast, three identical nucleotide mutations were observed in the E, NS2A and NS4B genes of G2-D10 and G2-A16 when compared to the complete genome of G2-P0. The nucleotide mutation in the NS2A gene was synonymous and did not affect the amino acid sequence; however, nonsynonymous nucleotide mutations resulted in amino acid substitutions in the E protein ($E^{50}K$) and the NS4B protein ($T^{21}I$), both of which are non-conservative.

To confirm that the identical mutations in G2-D10 and G2-A16 were authentic and not the result of cross contamination, sequencing was repeated using RT-PCR amplicons derived from re-extracted viral RNA. In addition, the stage in the sequential passages at which mutations occurred was investigated by sequence analyses of viruses recovered from selected passages (Table 6.6). For DF1-passaged G2 virus (G2-D10), mutations occurred at different stages: $G^{1118}A$ at passage five, $C^{3760}T$ at passage three, and $C^{6984}T$ at passage seven. In alternately-passaged G2 virus (G2-A16), however, all mutations occurred at passage six. Therefore, mutations in the two viruses were acquired at different stages of the experiment, providing support that the observed mutations were authentic and not result of contamination.

6.3.6 Comparison of Full-Length Genomes of MVE-1-51 and OR156

Appendix 6.9 shows the observed substitutions in the complete ORF of CDV OR156 when compared to CDV-1-51. One hundred and nineteen amino acid substitutions were observed within all structural (except prM/M) and non-structural proteins. These are discussed below (section 6.4). It is notable that substitutions observed in

Table 6.5. *Genetic changes in the genotype 2 passaged viruses (G2-C10, G2-D10, and G2-A16) compared to their parental virus (G2-P0).*

Nt position †	MVE viruses *				Aa change	Location ◊	Note ○
	G2-P0‡	G2-C10‡	G2-D10‡	G2-A16‡			
1118	G	-	A	A	E to K	E-50	Non-conservative
3760	C	-	T	T		NS2A	
6984	C	-	T	T	T to I	NS4B-21	Non-conservative
7664	G	A	-	-	E to K	NS4B-253	Non-conservative

For legend please refer to page 22.

‡ **G2-P0:** unpassaged; **G2-C10:** 10 passages in mosquito cells; **G2-D10:** 10 passages in avian cells; **G2-A16:** 16 passages alternately between mosquito and avian cells.

Table 6.6. *Occurrence of mutations during sequential passages of G2-P0 in avian DF1 cell (G2-D) or alternate passage between avian DF1 and mosquito C6/36 cells (G2-A).*

Passage Number	G2-A			G2-D		
	Nucleotide positions *			Nucleotide positions *		
	1118	3760	6984	1118	3760	6984
0	**G**	**C**	**C**	**G**	**C**	**C**
1					C	
2					C	
3				G	T	
4	G	C	C	G		
5	G	C	C	A	T	C
6	A	T	T			C
7						T
8	A	T	T			
9				A	T	T
10				A	T	T
11						
12						
13						
14	A	T	T			
15						
16	A	T	T			

* Nucleotide position is according to according to the complete genome of MVE-1-51 (NC000943).

the genomes of G1-D10 and G1-A16, associated with an attenuated virulence phenotype, were not found in the genomes of OR156 or any of the G2 viruses analysed in this study.

6.4 Discussion

The aim of this study was to investigate the basis of evolutionary constraints that are hypothesised to exist for MVEV by using an experimental approach involving sequential or alternate passaging of the virus in mosquito C6/36 cells and avian DF1 cells, representing vector and reservoir host of MVEV, respectively. In addition, for passaged viruses, changes in replicative fitness, adaptation to host cells, mouse neuroinvasiveness as well as molecular microevolution underlying observed phenotypic changes of MVEV were assessed. This study revealed that sequential passage of the parental viruses of both G1 and G2 in mosquito C6/36 cells preserved their genetic and phenotypic characteristics. On the other hand, passaging parental viruses through avian DF1 cells and alternately between C6/36 and DF1 cells led to accumulation of mutations which were associated with significant differences in their growth kinetics *in vitro* and neuroinvasiveness *in vivo*. These changes are discussed at genotype level below.

6.4.1 G1 viruses

Fig. 6.6 summarises the results of the experimental evolution of G1 of MVEV, reported in this chapter. The sequential passage of G1-P0 through mosquito C6/36 cells did not result in a consensus nucleotide change in the complete genome of the virus, nor was its growth kinetics altered in both DF1 and C6/36 cells. This indicates that no cell-specific adaption occurred in the virus after ten sequential passages in mosquito cells. Furthermore, both viruses exhibited a high virulence phenotype in mice with a dose-dependent mortality rate, characteristic of highly virulent strains of flaviviruses (Davis et al., 2003b). A lack of genetic and phenotypic changes in G1-C10 indicates that mosquito C6/36 cells may impose an evolutionary constraint on G1 MVEV. This is in accord with DENV studies which revealed that mosquito cells preserve the genetic stability of flaviviruses (Chen et al., 2003; Lin et al., 2004; Vasilakis et al., 2009). Results also support the findings of Ciota et al. (2009) on

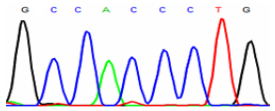
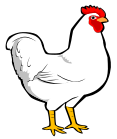
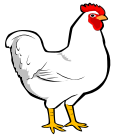
In vitro passaging	Viruses	Genetic Changes 	Growth Kinetics (*in vitro*)  C6/36 cells  DF1 cells	*In vivo* Neuroinvasiveness 
 10x → 	G1-C10	N/C	N/C	N/C
 10x → 	G1-D10	E, NS3	↓	↑	↓
 16x ↔ 	G1-A16	E, NS3	↓	↓	↓

Fig. 6.6. *Schematic representation of Murray Valley encephalitis virus genotype 1 experimental evolution.*

N/C: no change; **↓:** decrease; **↑:** increase

SLEV, which demonstrated that the evolutionary conservation of flaviviruses is not a result of cycling between invertebrate vector and vertebrate host, but rather it is the result of sequential passages separately in either hosts.

In contrast, the sequential passage of the parental virus in avian DF1 cells (G1-D10), and alternate passages between both host cells (G1-A16) resulted in the accumulation of mutations in their nucleotide and amino acid sequences, along with significantly altered growth kinetics *in vitro* and virulence *in vivo*. The biological role of many of the mutations observed in these viruses is yet to be characterised. For both viruses, mutations were found in the E protein, that are likely to be responsible for their attenuation in the mouse model of pathogenesis. G1-D10 acquired a $D^{390}N$ mutation in the E protein, which is located in the receptor binding RGD motif, involved in cell attachment (Lobigs et al., 1990; Rey et al., 1995). Consistent with the virulence phenotype of G1-D10, Lobigs et al. (1990) demonstrated that when CDV-1-51 was sequentially passaged ten times in the human adrenal cortex carcinoma-derived cell line (SW13 cells), mutations in the RGD motif were selected. This mutation caused a reduction in negative charge and diminished the acidic property of this motif. In the study reported here, it is also likely that adaptation to avian DF1 cell resulted in the acquisition of $D^{390}N$ in the RGD motif. Previous studies have demonstrated that this mutation increases the binding affinity for glycosaminoglycans (GAGs) on the surface of host cells (Lee and Lobigs, 2000, 2002). It was proposed that host tissues containing a high amount of GAGs rapidly remove the infectious virus from the blood before it can infect CNS, thereby diminishing neuroinvasiveness of the virus (Lee and Lobigs, 2000, 2002).

The other mutation in the E protein of G1-D10 ($H^{398}Y$) altered the conserved histidine residue that connects domain I and domain III of the E protein (Nybakken et al., 2006). Conserved histidine residues are essential for the conformational changes of the fusion peptide of the E protein (Roussel et al., 2006). It was hypothesised that histidine at position 398 contributes to the formation of functionally important salt bridges within the E protein of MVEV (Hurrelbrink and McMinn, 2001). Therefore, substitution of the histidine at this position may disrupt formation of salt bridges and/or changes the fusion efficiency of the virus with cell

membrane. Moreover, the $A^{27}T$ substitution of the G1-D10 NS3 protein is located in the serine protease domain, crucial for the cleavage and release of flavivirus non-structural proteins into the cytoplasm for viral genome replication and translation (except NS1; Fig. 1.1A). It is not yet clear that mutation at position 27 of NS3 enhances or diminishes the proteolytic activity of the NS3 protein. However, in this study, $A^{27}T$ substitution may have contributed to the more efficient replication kinetics of G1-D10 observed in DF1 cells. Further investigation is required to assess the functional role of this and other mutations found for G1-D10.

The significantly lower replicative ability of G1-D10 in C6/36 cells, combined with its higher fitness in DF1 cells compared to G1-P0, suggests that this virus had adapted to this cell line during the sequential passaging (Fig. 6.3A and Fig 6.4B). Given the observed mutations in the RGD motif of the E protein, a change in receptor usage may be a key factor of this adaptation. The adaptation of G1-D10 in DF1 cells is in agreement with the theory of cell-specific adaptation, which indicates that sequential passages of a virus in a single host increases its fitness in that host, while it reduces or does not substantially change the virus fitness in the other host (Ciota et al., 2007b).

The significantly lower replicative ability and titre of G1-A16 compared to G1-P0 in both C6/36 and DF1 cells (Fig 6.4) is consistent with the fitness trade-off hypothesis which postulates that arboviruses display a lower level of fitness in both hosts compared to viruses that specialise to a single host environment (Ciota and Kramer, 2010; Deardorff et al., 2011). As for G1-D10, non-conservative amino acid substitutions in the E ($E^{308}K$) and NS3 ($G^{477}C$) proteins were found for G1-A16 (Table 6.4). The $E^{308}K$ substitution is located on the loop connecting domain I and domain III of the E protein (Fig. 2.4). This substitution considerably changes the charge of the loop (E is negatively charged while K is strongly positive), which may greatly affect the conformation and flexibility of the loop. This may invite speculation that $E^{308}K$ may have contributed in reducing the replication kinetics of G1-A16 in both C6/36 and DF1 cells. In this regard, Jiang et al. (1993) reported that a substitution at position 308 of the E protein of Louping ill virus (*Flavivirus*, *Flaviviridae*) was associated with the loss of virulence in mice. Moreover, the $G^{477}C$ substitution of

the NS3 protein is located on the helicase domain, involved in unwinding dsRNA during replication. This substitution may have affected the function of this domain, changing the efficiency of this enzyme, contributing to the observed decrease in *in vitro* replication kinetics of G1-A16 and attenuated virulence in mice. Further genetic and phenotypic studies may reveal the biological importance of this substitution.

In addition to altered replication kinetics *in vitro*, G1-D10 and G1-A16 displayed a dramatic attenuation when inoculated in 18-day old Swiss ARC mice. This suggests that the mutations acquired in these two viruses dramatically attenuated their neuroinvasiveness. This is not surprising, since the mutations in the E protein of G1-D10 ($D^{390}N$) and G1-A16 ($E^{308}K$) were previously found to be associated with attenuation of the neuroinvasiveness of flaviviruses in mice (Hurrelbrink and McMinn, 2001; Jiang et al., 1993; Lee and Lobigs, 2002; Lobigs et al., 1990). It is highly likely that altered receptor binding properties may have contributed to the attenuated virulence phenotype of G1-D10.

The results reported here for MVEV G1 viruses contrasts to the experimental evolution studies of Jerzak et al. (2007) that involved *in vivo* passaging of WNV in mosquitoes and chickens. Chicken-passed WNV produced similar mortality rates to the parental virus, while mosquito-passed WNV strains were less pathogenic than the parental virus in mice. The conflicting results may be due to different experimental passage conditions (*in vitro* vs. *in vivo*) and/or some unique inherent as yet unidentified characteristics of these two closely-related flaviviruses. Future *in vivo* passage experiments using relevant vectors and hosts of MVEV may reveal whether the experimental strategy used here provides an appropriate model to test MVEV evolution *in vitro*.

6.4.2 G2 viruses

Fig. 6.7 summarises the results of experimental evolution study of G2 viruses carried out in this chapter. Significantly, consistent with results obtained for G1-C10 (described above), and those reported in DENV studies (Chen et al., 2003; Lin et al., 2004; Vasilakis et al., 2009), it appears that sequential passages of G2 parental virus

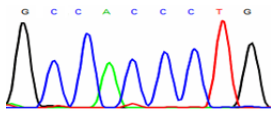
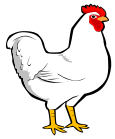
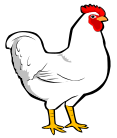
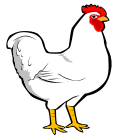
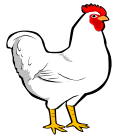
In vitro passaging	Viruses	Genetic Changes 	Growth Kinetics (*in vitro*)  C6/36 cells  DF1 cells	*In vivo* Neuroinvasiveness 
 10x → 	G2-C10	NS4B	N/C	N/C
 10x → 	G2-D10	E, NS4B	↓	↑	↓
 16x ↔ 	G2-A16	E, NS4B	↓	↑	↓

Fig. 6.7. *Schematic representation of Murray Valley encephalitis virus genotype 2 experimental evolution.*

N/C: no change; **↓:** decrease; **↑:** increase

in mosquito C6/36 cells did not significantly alter the genetic and phenotypic characteristics of the virus, supporting the hypothesis that passaging MVEV in mosquito cells preserves both genetic and phenotypic characteristics of the virus. Consistent with the results for G2-P0, as well as the laboratory strain of OR156 and the recent G2 isolate (K59532) reported in Chapter 5 (Section 5.3.2), scattered mortalities occurred when mice were inoculated with different doses of this virus. This pattern of mortalities in the mouse model of pathogenesis is characteristic of low virulent strains and has been found in other flaviviruses (Beasley et al., 2002; Beasley et al., 2005; Hurrelbrink and McMinn, 2001; Lobigs et al., 1988; Lobigs et al., 1990; Monath et al., 1980; Prow et al., 2011; Shirato et al., 2004). Therefore, G2-P0 and G2-C10 were classified as low virulent viruses.

In the growth kinetics analysis, similar to G1-D10, G2-D10 displayed a cell-specific adaptation (lower replicative ability in C6/36 cells, but higher replicative fitness in DF1 cells), suggesting that during the sequential passages in DF1 cells, MVEV strains belonging to both genotypes, adapt to these cells. Considering the titres of each sequential passage (Fig. 6.3), it appears that this adaptation occurred after four passages. The observation of identical nucleotide mutations in G2-D10 and G2-A16, suggests it is passaging in avian DF1 cells, either sequentially or alternately with C6/36 cells, that leads to accumulation of genetic and phenotypic changes in MVEV. In contrast, and unlike G1 viruses, the result of the G2 *in vitro* growth kinetics is not in accord with the fitness trade-off hypothesis. Although the initial stages of replication dynamics of G2-A16 were lower than G2-P0 and G2-C10 in C6/36 cells, at the later stages (>48 hpi), the titre of G2-A16 was comparable or higher than G2-C10. Several other studies also concluded that there is not enough support that cycling imposes a fitness trade-off (Ciota and Kramer, 2010; Vasilakis et al., 2009). According to Ciota and Kramer (2010), arboviruses have different capabilities to adapt to both host cell environments with no or minimal change in the alternating environment due to differences in host utilisation, genome organisation, the size of mutant swarm and quasispecies, and the routes and mechanism of transmission.

Furthermore, the accumulated mutations in G2-D10 and G2-A16 were found to be associated with further attenuation of these viruses in mice. The observed

mutations in the E and NS4B proteins of G2-D10 and G2-A16 have not previously been associated with phenotypic changes, and their potential biological significance is not yet clear. However, the $E^{50}K$ mutation in the E protein is in close proximity to position 52, which resides at the polar interface that links domain I and domain II of the E protein (Hurrelbrink and McMinn, 2001). Mutation at this position of JEV (Hasegawa et al., 1992) and YFV (Schlesinger et al., 1996) has been associated with low neuroinvasiveness in mice. Further investigation is required to assess the functional effect of residue $E^{50}K$ on this interface.

Taken together, the results of the *in vivo* experiments in this study support the findings of other studies on molecular determinants of flavivirus virulence. Many studies have associated a small number of mutations in the flavivirus genome with a loss of virulence in mice (Hurrelbrink and McMinn, 2001; Liu et al., 2006b; May et al., 2006; McMinn et al., 1995; McMinn et al., 1996; Prow et al., 2011; Wicker et al., 2006; Ye et al., 2012). Moreover, this study did not find any apparent correlation between cytopathicity and replication dynamics of G1-D10 and G1-A16 *in vitro* and their virulence *in vivo*. Both these viruses demonstrated relatively efficient growth kinetics in cell culture while in the mouse model of pathogenesis, they demonstrated a dramatic attenuation. A similar pattern was seen in the MVEV studies carried out by Lobigs et al. (1990) and Hurrelbrink and McMinn (2001). In these studies, the authors reported that mutant viruses had adequate replication dynamics in cell culture while they were significantly attenuated in mice.

Alignment of the clone-derived viruses of G1 (CDV-1-51) and G2 (CDV OR156), revealed 119 amino acid differences scattered through the coding region of both viruses. The roles of most of these substitutions have not yet been characterised. However, OR156 encodes a substitution at position 126, which is located on the surface of the E protein on domain II. A study on DENV-2, revealed that a substitution at this position is associated with the loss of neurovirulence in mice (Gualano et al., 1998). OR156 also exhibits two substitutions in the flexible hinge region (S275T and S276G) of the E protein. As mentioned above, substitutions in this region can reduce hinge flexibility, and fusion efficiency with the plasma membrane, leading to the loss of virulence in mice (Hurrelbrink and McMinn, 2001;

May et al., 2006; McMinn et al., 1995; Prow et al., 2011). Moreover, the T157S substitution in the E protein of CDV OR156 is adjacent to the glycosylation site of flaviviruses (154-156). Previous research has revealed that substitution at the glycosylation site of flaviviruses are associated with a loss of neurovirulence (Pletnev et al., 1993) and neuroinvasiveness (Beasley et al., 2005; Chambers et al., 2008; Prow et al., 2011) in mice. Further research is needed to reveal if the substitution at position 157 impacts on the functionality of the glycosylation site too. Similarly, there are two mutations just adjacent to position 368 of the E protein in the CDV OR156 (A367V and A369S). Position 368 of the E protein is also a molecular determinant of flavivirus virulence, with substitutions at this position shown to significantly decrease the neuroinvasiveness of TBEV in mice (Holzmann et al., 1997). Further genetic and phenotypic studies are also needed to determine if substitutions at positions 367 and 369 are also associated with any phenotypic changes. In the non-structural protein, substitutions at position 106 (A106V) and 107 (I107V) of the NS3 protein of OR156 are next to position 105 of this protein. Ni et al. (1995) demonstrated that substitution in the latter position is associated with loss of neurovirulence in wild-type JEV SA14. The roles of other substitutions in the non-structural proteins of CDV OR156 compared to CDV-1-51 have not as yet been characterised. Taken together, it can be speculated that the sum effect of these molecular differences between MVE-151 and OR156 may account for the difference observed in virulence phenotype.

6.5 Conclusion

Altogether, results from this *in vitro* study suggest that genetic stability of MVEV is not the result of host cycling. The evidence of the existence of a significant fitness trade-off in both MVEV genotypes following alternate mosquito-avian cell passage was also not found. Contrary to the hypothesis tested, but consistent with DENV studies (Chen et al., 2003; Vasilakis et al., 2009), passaging parental virus through mosquito cells preserves both genetic and phenotypic characteristics of MVEV, whereas passaging it through avian cells and alternating cycles changed these characteristics. Taken together, the evolution of MVEV seems to be driven by alternating replication in mosquito cells, where purifying selection is strong, and in

avian cells in which purifying selection appears to be relaxed. Several associated mutations affecting MVEV replication in cell culture and virulence in mice were also identified, which may serve as a basis for further mutagenesis study.

In conclusion, arbovirus evolution studies produced conflicting conclusion regarding the effect of cycling on viral genetic stability and fitness. Further studies especially experimental *in vivo* passage studies may shed light and broaden our knowledge on the effect of cycling on evolutionary conservation and the phenotypic characteristics of flaviviruses. With the new advances in reverse genetics and mutagenesis, the biological role of each individual mutation observed in this study can be identified.

CHAPTER 7

GENERAL DISCUSSION

MVEV is the most important encephalitogenic arbovirus in Australia. The virus has shown intermittent increases in activities in recent years. For the first time since 1974, human MVE cases were reported in the southern and south-eastern states of Australia in 2011 (Knox et al., 2012). This outbreak was also associated with widespread seroconversion in sentinel chicken flocks and considerable mortality in humans (Knox et al., 2012) and horses (Mann et al., 2013; Roche et al., 2013). This highlighted the importance of surveillance programs for prediction of MVEV activity. In this regard, the MVEV-specific RT-qPCR assay reported in Chapter 3 may be a useful diagnostic tool for the detection and quantification of MVEV. Application of the MVEV-specific RT-qPCR assay, reported herein, in the research laboratory or in surveillance and clinical settings, has the advantage of comparably high efficiency, sensitivity and specificity for the amplification of viral RNA from all MVEV genotypes (Tables 3.3, and 3.5). Therefore, it is expected that this assay will enable detection of G2 MVEV as well as possible circulation of G3 and G4 in Australasian region.

Prior to this study, only five G2 isolates of MVEV had been identified up to 1995 (Johansen et al., 2007; Liehne et al., 1976), all from the Kununurra district of the Kimberley region in WA. It should be noted that prior to the work presented in Chapter 2 of this thesis, only representative MVEV isolates from each year were sequenced as part of molecular epidemiological studies of MVEV isolates from WA (Johansen et al., 2007; Lobigs et al., 1986; Williams et al., 2013). Thus, an accurate distribution of MVEV genotypes had not been properly elucidated. A primary aim of this thesis was to perform genotyping by sequencing a partial E gene fragment of all MVEV strains isolated between 2005 and 2009, in order to accurately estimate the prevalence and distribution of different MVEV genotypes. The main finding of this study was the detection of four new G2 isolates from the Kimberley region (Fig. 2.2 and Appendix 2.1). Significantly, these isolates were found for the first time outside of the Kununurra district, in Fitzroy Crossing (2006 and 2009) and Broome (2006), and extended the known geographic range of G2 viruses by approximately 600km west of Kununurra.

NGS technology revealed that G1 viruses exist as highly genetically heterogeneous populations in vector mosquitoes, while G2 viruses exhibited a more homogeneous genetic structure (Table 4.2). Higher levels of genetic diversity and complexity of virus populations can directly affect replication, pathogenicity and virulence (Ciota et al., 2012; Ciota et al., 2007c; Jerzak et al., 2007; Lancaster and Pfeiffer, 2012; Perales et al., 2010; Vignuzzi et al., 2006). Therefore, the low level of genetic diversity in G2 viruses may have contributed to its inability to spread and adapt to new environments, outside the Kimberley region. However, the level of genetic diversity is higher in G1a viruses than those of G1b, which is inconsistent with the hypothesis that higher genetic diversity may correlate with superior transmission. Therefore, other as yet un-identified factors such as biodiversity of mosquito vectors and reservoir hosts may contribute to these relative distributions.

Mutagenesis studies have also revealed that altering the natural level of genetic diversity in a viral population (both increasing or decreasing) significantly reduces the viral cytopathicity and virulence in mice (Coffey et al., 2011; Perales et al., 2011; Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006; Vignuzzi et al., 2008). For example, $G^{64}S$ variant of poliovirus encodes a high fidelity polymerase, generating a less genetically diverse viral population (Pfeiffer and Kirkegaard, 2005). Unlike the wild-type viruses, the high-fidelity variant was not able to invade the CNS of mice (Pfeiffer and Kirkegaard, 2005). When the level of genetic diversity was expanded by mutagenesis and reverse genetics, the pathogenicity and virulence of the virus was restored (Borderia et al., 2011). Therefore, intra-population genetic diversity and the complexity of mutation spectra are hidden virulence determinants of replication and pathogenesis in RNA viruses. Unfortunately, due to the availability of very small volumes of mosquito homogenates from the samples analysed by NGS, a direct comparison of the level of genetic diversity, observed in Chapter 4, with the associated *in vitro* and *in vivo* phenotypes could not be performed. Instead, viruses isolated from tissue culture were used.

In addition to virological properties influencing the restricted geographic range, competition for vectors and hosts by G1 may also play a significant role. Since G1 MVEV is predominant [67 of the 71 (94.4%) isolates sequenced in this study] and is

associated with large epizootics (Broom, 2003; Broom et al., 2002a; Broom et al., 2002b; Mann et al., 2013), it is plausible that a substantial number of vertebrate reservoir hosts have protective antibodies against this genotype. Hence, the presence of cross-reacting antibodies against G2 may have a significant impact on reducing the levels of amplification in reservoir hosts and the activity of the G2 viruses. Moreover, as detailed in Chapter 4, a number of G2 viruses were found to contain lower concentrations of viral genome in two mosquito vectors (*Cx. annulirostris* and *Cx. pullus;* Table 4.2), relative to G1 viruses. This may indicate that vector competence of these two *Culex* species is substantially lower for G2 viruses than for G1 viruses. Research on vector competence of *Cx. annulirostris* for MVEV has only been reported for G1 isolates (Kay et al., 1989). Further investigation of this kind is required to investigate the vector competence of *Culex* mosquitoes for G2 viruses. Furthermore, considering the proposition that it is unlikely that all likely hosts of MVEV are fully elucidated (P. Neville 2012; personal communication), it is possible that a distinct vertebrate reservoir host(s) exists for G2 viruses, which may not be as abundant in nature as those of G1, or is not a preferred source for *Culex* vectors. G2 MVEVs may also not replicate as efficiently as G1 in reservoir hosts and do not produce a sufficiently high level of viraemia required for efficient transmission.

Finally, infection of mosquito vectors with G1 viruses may exclude superinfection with G2 viruses. Previously, this phenomenon has been postulated to be responsible for interfering and suppressing secondary infection with the same or closely-related viruses (Folimonova, 2012; Hobson-Peters et al., 2013; Ramírez et al., 2010; Zou et al., 2009). Superinfection exclusion has been previously observed in flaviviruses such as WNV (Zou et al., 2009), SLEV (Randolph and Hardy, 1988), DENV (Pepin et al., 2008) and between PCV and MVEV/KUNV (Hobson-Peters et al., 2013). It has been shown that the primary infecting virus competes for or modifies cellular factors that are responsible for virus entry and RNA replication (Folimonova, 2012; Lee et al., 2005; Ramírez et al., 2010; Zou et al., 2009). Therefore, a primary infection of mosquito vectors by MVEV strains of the most prevalent genotype (G1) may further contribute to reduced levels of activity observed for G2 viruses. *In vitro*

experimental approaches using mosquito cell lines such as the C6/36 clone, or *in vivo* in *Culex* species of mosquitoes will be needed to address this possibility. Similarly, a prior infection of reservoir hosts with G1 viruses may exclude the secondary infection with G2 viruses. Co-infection studies involving infection of animal reservoir hosts by both G1 and G2 will address this question too.

Potential molecular determinants of MVEV virulence and/or attenuation were explored in this study. Significantly, the prM protein of OR156 did not show any amino acid differences to that of MVE-1-51, indicating that this protein is unlikely to be under selection or influence transmission and pathogenesis. In contrast, the E protein of OR156 demonstrated 26 amino acid substitutions (including two substitutions in the flexible hinge region) that are highly likely to be involved in the attenuation of G2. Similarly a high number of mutations were observed in the NS proteins of OR156 that may also contribute to the attenuation phenotype, affecting replication, translation, assembly and production of G2 MVE viruses. However, due to an abundance of mutations throughout the NS proteins, a specific mutation could not be reliably associated with attenuation of G2 MVEV strains. A mutagenesis and reverse genetics approach may be undertaken in future studies using the pMVE-1-51 infectious clone, allowing defined gene swaps, followed by site-directed mutagenesis to define the attenuating determinants of G2. In addition, results reported in Chapter 6 identified other molecular determinants that may have contributed to the attenuation of neuroinvasiveness in mice observed for DF-1-passaged, and alternately-passaged viruses (Table 6.4 and 6.5). The biological importance of these mutations has not yet been investigated. Site-directed mutagenesis and reverse genetic studies employing pMVE-1-51 (Hurrelbrink et al., 1999) could be undertaken to address the effect of the observed mutations in G1-D10 and G1-A16.

Results reported in Chapter 2 of this thesis support the hypothesis that MVEV has undergone restricted genetic diversity over time. A maximum of 5.7% nucleotide differences were observed within consensus sequences of G1 strains isolated between 1951 and 2011, while a maximum of 3.3% nucleotide variation was found within G2 strains isolated from 1973 to 2009 (Table 2.6). An experimental evolution

study, using a cell culture approach, was carried out to examine the evolutionary constraints imposed by sequential passaging or cycling of MVEV in mosquito and/or avian cells. Genetic diversity was characterised by sequencing the consensus sequence of the complete genome of each passaged virus using conventional sequencing. Results indicated that contrary to the general belief for arboviruses, genetic conservation of MVEV is not a result of cycling between invertebrate vectors and vertebrate hosts. Instead, the evolutionary stasis appears to be driven by virus passage in mosquito C6/36 cells (Figs. 6.4, and 6.5 and Tables 6.3, 6.4, and 6.5). These results are in accord with studies on DENV (Chen et al., 2003; Vasilakis et al., 2009) and SLEV by Ciota et al. (2009) reporting that passage of these viruses in mosquito cells preserved their genetic and phenotypic characteristics. Consistent with this, sequential passage of MVEV in avian DF1 cells or alternately between C6/36 and DF1 cells was found to result in accumulation of consensus mutations, associated with significant differences in virus growth kinetics in cell culture and virulence in mice.

Although no changes in the consensus genome sequence were observed for virus derived from passage in mosquito cells (G1-C10), this virus was found to have a more pronounced neuroinvasive phenotype, with approximately ten-fold lower HD_{50}, and a shorter AST compared to parental G1-P0 (Table 6.3; statistically not significant). In this regard, the depth of genetic diversity and complexity of mutation spectra may have increased during the sequential passages in C6/36 cells. Research has shown that when a clonal population of RNA viruses or even a single infectious particle is used to infect an animal or cell culture, it quickly transforms into an ensemble of multiple related variants to generate a quasispecies (Biebricher and Eigen, 2006; Domingo et al., 2001; Lauring and Andino, 2010; Perales et al., 2010). Supporting this hypothesis, Ciota et al. (2007c) revealed that sequential passage of clone-derived WNVs in mosquito C6/36 cells resulted in a linear increase in the intra-population genetic diversity. The authors suggested that increases in fitness, adaptation and replicative ability were not only due to the consensus changes in the genome of the virus, but were directly associated with the size of the mutant swarm. In the case of G1-C10, ten sequential passages in C6/36 cells may have

produced multiple minority yet phenotypically-important variants, which could not be detected by the Sanger sequencing, but may have altered neuroinvasiveness of this virus in mice. NGS technology could help characterise the depth of genetic diversity in the G1-C10 population, but due to constraints in time and resources, this analysis was not carried out as part of the investigations of this thesis.

Taken together, the results and findings of this thesis reveal important insights into the genetic and phenotypic diversity of MVEV and how this virus may evolve. In particular, clear differences were observed in the level of inter- and intra-population genetic diversity as well as virulence phenotype between the dominant genotype (G1) and the minority (G2). G2 MVEV continues to circulate in the Kimberley region of WA, beyond its previously known geographic range of Kununurra, and accounted for approximately 5% of all isolates over the study period. The intra-population genetic diversity and mutation spectra were also found to be significantly lower in G2-infected mosquito samples than those of G1, indicating the existence of a highly homogeneous population for G2 viruses. The major phenotypic difference found between G1 and G2 viruses was neuroinvasiveness in mice, with G1 showing a virulence phenotype and G2 an attenuated one. Although differences were observed in genetic diversity between viruses belonging to G1a and G1b, both had a virulent phenotype in mice. Results obtained from this thesis have led to the proposal that the lower level of genetic diversity in G2 viruses is a contributing factor for their geographic restriction in the Kimberley region of WA. In addition, this thesis identified several molecular determinants of MVEV adaptation, replication and virulence, the roles of some of which have already been characterised. Finally, experimental evolution studies in cell culture indicated that the observed genetic conservation of MVEV may be maintained by alternating cycles between mosquito cells, where purifying selection appears to be strong and avian cells, in which purifying selection is relaxed.

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APPENDICES

Appendix 1.1*: Members of the genus Flavivirus.*

1. TICK-BORNE VIRUSES

Mammalian tick-borne virus group

Gadgets Gully virus (GGYV)

Gadgets Gully virus (GGYV)

Kyasanur Forest disease virus (KFDV)

Kyasanur Forest disease virus (KFDV)

Alkhumra haemorrhagic fever virus (AHFV)

Langat virus (LGTV)

Langat virus (LGTV)

Louping ill virus (LIV)

British subtype (LIV-Brit)

Irish subtype (LIV-Ir)

Spanish subtype (LIV-Spain)

Turkish sheep encephalitis virus subtype (TSEV)

Greek goat encephalitis virus subtype (GGEV)

Omsk hemorrhagic fever virus (OHFV)

Omsk hemorrhagic fever virus (OHFV)

Powassan virus (POWN)

Powassan virus (POWN)

Royal Farm virus (RFV)

Royal Farm virus (RFV)

Tickborne encephalitis virus (TBEV)

European subtype (TBEV-Eu)

Far Eastern subtype (TBEV-FE)

Siberian subtype (TBEV-Sib)

Seabird tick-borne virus group (probably tick-borne)

Meaban virus (MEAV)

Meaban virus (MEAV)

Saumarez Reef virus (SREV)

Saumarez Reef virus (SREV)

Tyuleniy virus (TYUV)

Tyuleniy virus (TYUV)

Kadam virus group

Kadam virus (KADV)

Kadam virus (KADV)

Appendix 1.1. *Continued.*

2. MOSQUITO-BORNE VIRUSES

Aroa virus group

Aroa virus (AROAV)

Aroa virus (AROAV)

Bussuquara virus (BSQV)

Iguape virus (IGUV)

Naranjal virus (NJLV)

Dengue virus group

Dengue virus (DENV)

Dengue virus 1 (DENV-1)

Dengue virus 2 (DENV-2)

Dengue virus 3 (DENV-3)

Dengue virus 4 (DENV-4)

Japanese encephalitis virus group

Cacipacore virus (CPCV)

Cacipacore virus (CPCV)

Japanese encephalitis virus (JEV)

Japanese encephalitis virus (JEV)

Koutango virus (KOUV)

Koutango virus (KOUV)

Murray Valley encephalitis virus (MVEV)

Alfuy virus (ALFV)

Murray Valley encephalitis virus (MVEV)

St. Louis encephalitis virus (SLEV)

St. Louis encephalitis virus (SLEV)

Usutu virus (USUV)

Usutu virus (USUV)

West Nile virus (WNV)

Kunjin virus (KUNV)

West Nile virus (WNV)

Yaounde virus (YAOV)

Yaounde virus (YAOV)

Kokobera virus group

Kokobera virus (KOKV)

Kokobera virus (KOKV)

Stratford virus (STRV)

Appendix 1.1. *Continued.*

Ntaya virus group

Bagaza virus (BAGV)

Bagaza virus (BAGV)

Ilheus virus (ILHV)

Ilheus virus (ILHV)

Rocio virus (ROCV)

Israel turkey meningoencephalitis virus (ITV)

Israel turkey meningoencephalitis virus (ITV)

Ntaya virus (NTAV)

Ntaya virus (NTAV)

Tembusu virus (TMUV)

Tembusu virus (TMUV)

Zika virus (ZIKV)

Zika virus (ZIKV)

Yellow fever virus group

Sepik virus (SEPV)

Sepik virus (SEPV)

Wesselsbron virus (WESSV)

Wesselsbron virus (WESSV)

Yellow fever virus (YFV)

Yellow fever virus (YFV)

PROBABLY MOSQUITO-BORNE

Kedougou virus group

Kedougou virus (KEDV)

Kedougou virus (KEDV)

Edge Hill virus group

Banzi virus (BANV)

Banzi virus (BANV)

Bouboui virus (BOUV)

Bouboui virus (BOUV)

Edge Hill virus (EHV)

Edge Hill virus (EHV)

Jugra virus (JUGV)

Jugra virus (JUGV)

Saboya virus (SABV)

Potiskum virus (POTV)

Saboya virus (SABV)

Uganda S virus (UGSV)

Uganda S virus (UGSV)

Appendix 1.1. *Continued.*

3. VIRUSES WITH NO KNOWN ARTHROPOD VECTOR (NKV)

Entebbe bat virus group

Entebbe bat virus (ENTV)

Entebbe bat virus (ENTV)

Sokoluk virus (SOKV)

Yokose virus (YOKV)

Yokose virus (YOKV)

Modoc virus group

Apoi virus (APOIV)

Apoi virus (APOIV)

Cowbone Ridge virus (CRV)

Cowbone Ridge virus (CRV)

Jutiapa virus (JUTV)

Jutiapa virus (JUTV)

Modoc virus (MODV)

Modoc virus (MODV)

Sal Vieja virus (SVV)

Sal Vieja virus (SVV)

San Perlita virus (SPV)

San Perlita virus (SPV)

Rio Bravo virus group

Bukalasa bat virus (BBV)

Bukalasa bat virus (BBV)

Carey Island virus (CIV)

Carey Island virus (CIV)

Dakar bat virus (DBV)

Dakar bat virus (DBV)

Montana myotis leukoencephalitis virus (MMLV)

Montana myotis leukoencephalitis virus (MMLV)

Phnom Penh bat virus (PPBV)

Batu cave virus (BCV)

Phnom Penh bat virus (PPBV)

Rio Bravo virus (RBV)

Rio Bravo virus (RBV)

TENTATIVE SPECIES IN THE GENUS (not approved)

Mammalian tick-borne viruses

Karshi virus (KSIV)

Mosquito-borne viruses

Spondweni virus (SPOV)

Appendix 1.1. *Continued.*

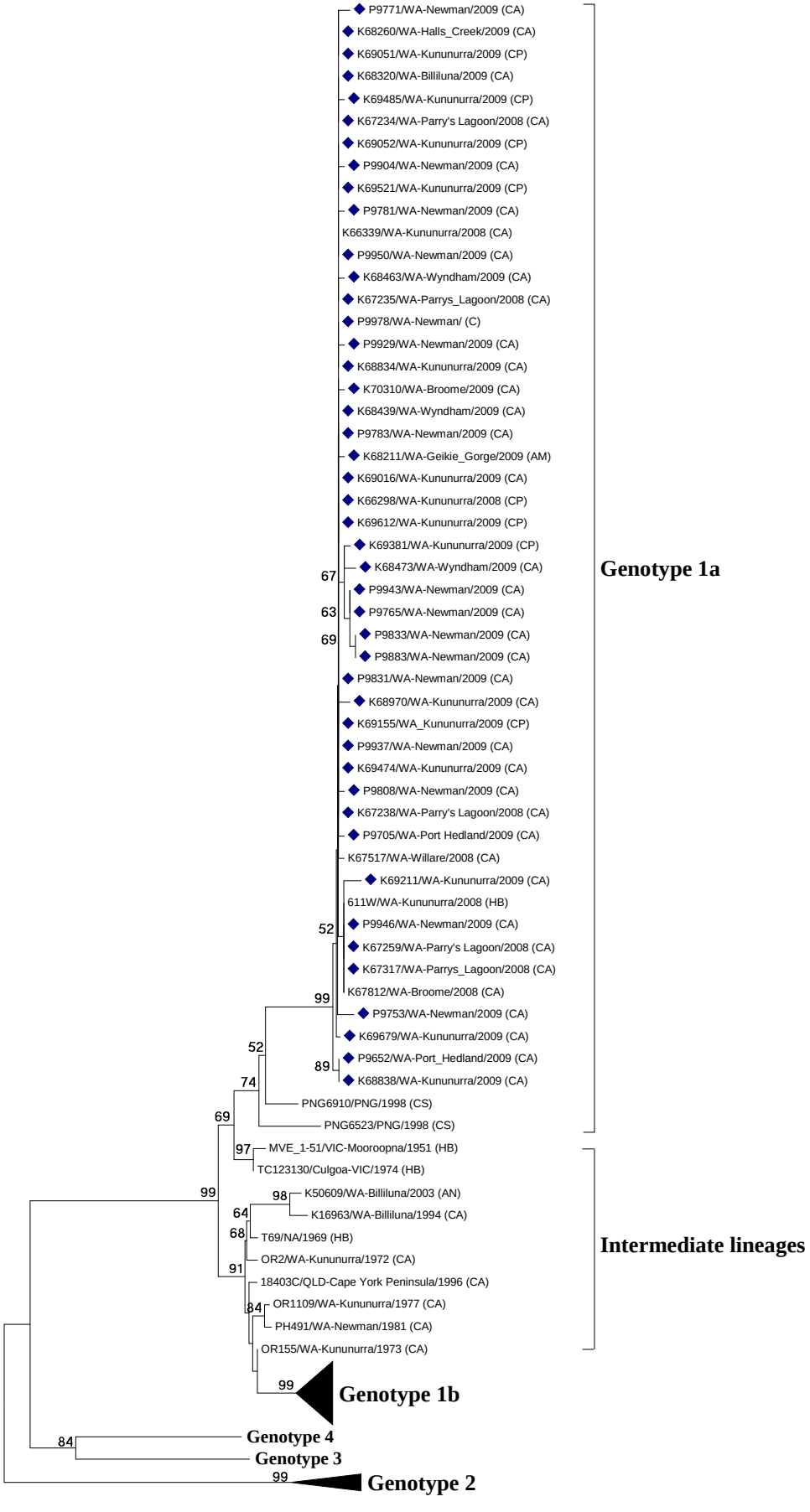
Probably arthropod-borne viruses
Aedes flavivirus (AEFV)
Cell fusing agent virus (CFAV)
Culex flavivirus (CXFV)
Kamiti River virus (KRV)
Nakiwogo virus (NAKV)
Quang Binh virus (QBV)
Viruses with no known arthropod vector
Chaoyang virus (CHAOV)
Lammi virus (LAMV)
Ngoye virus (NGOV)
Nounané virus (NOUV)
Tamana bat virus (TABV)

The main groups are typed in bold UPPERCASE, subgroups are in bold lowercase; species names are in italics; the names of viruses are in normal script.

The main groups are typed in bold capital scripts, subgroups are in bold lower case; species names are in italics; the subtype of each species is in normal script under each species name.

Adapted from (Simmonds et al., 2012).

A.


P9771/WA-Newman/2009 (CA)
K68260/WA-Halls_Creek/2009 (CA)
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K68320/WA-Billiluna/2009 (CA)
K69485/WA-Kununurra/2009 (CP)
K67234/WA-Parry's Lagoon/2008 (CA)
K69052/WA-Kununurra/2009 (CP)
P9904/WA-Newman/2009 (CA)
K69521/WA-Kununurra/2009 (CP)
P9781/WA-Newman/2009 (CA)
K66339/WA-Kununurra/2008 (CA)
P9950/WA-Newman/2009 (CA)
K68463/WA-Wyndham/2009 (CA)
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P9978/WA-Newman/ (C)
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K70310/WA-Broome/2009 (CA)
K68439/WA-Wyndham/2009 (CA)
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K68970/WA-Kununurra/2009 (CA)
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K67517/WA-Willare/2008 (CA)
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611W/WA-Kununurra/2008 (HB)
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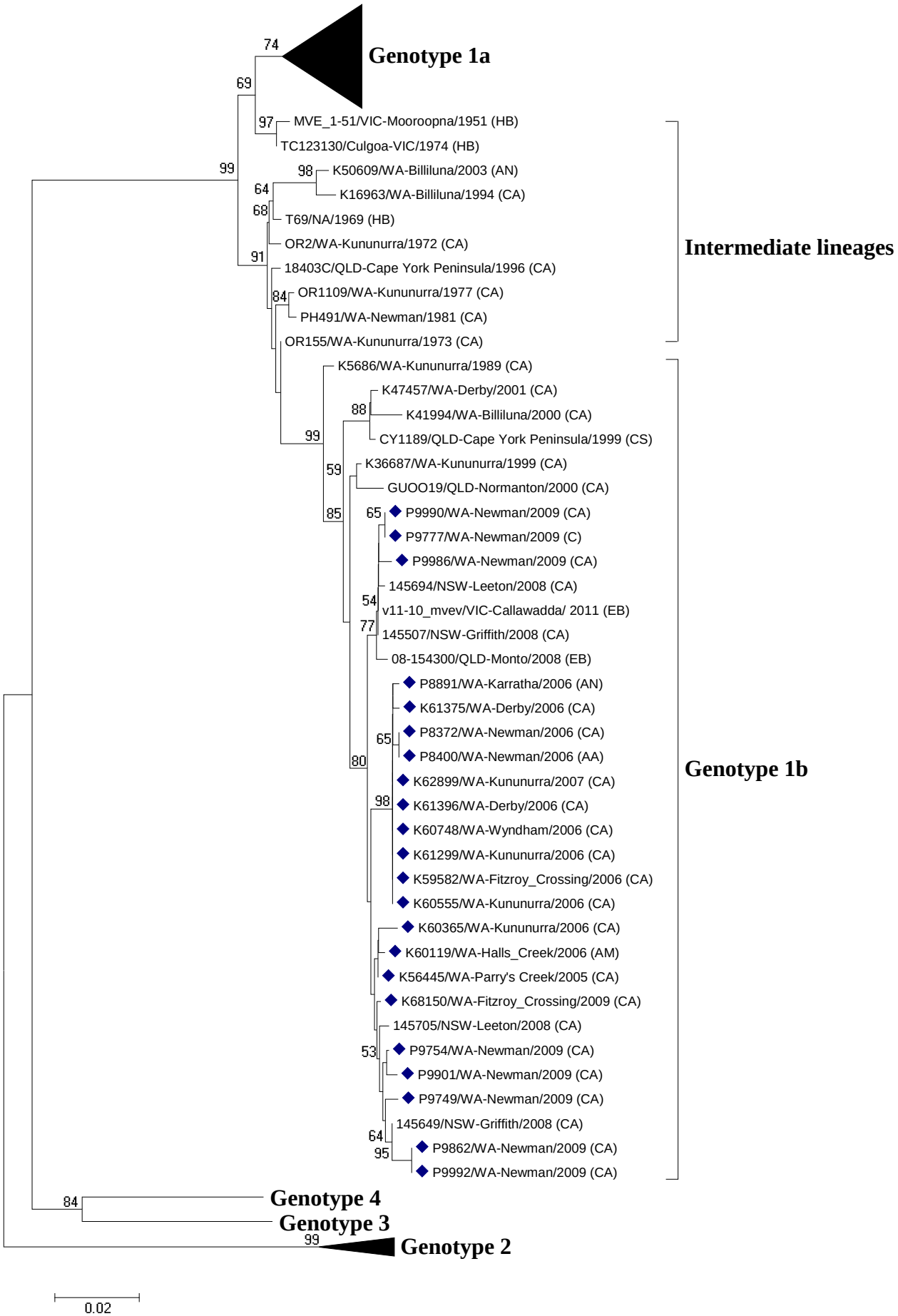
K68838/WA-Kununurra/2009 (CA)
PNG6910/PNG/1998 (CS)
PNG6523/PNG/1998 (CS)
MVE_1-51/VIC-Mooroopna/1951 (HB)
TC123130/Culgoa-VIC/1974 (HB)
K50609/WA-Billiluna/2003 (AN)
K16963/WA-Billiluna/1994 (CA)
T69/NA/1969 (HB)
OR2/WA-Kununurra/1972 (CA)
18403C/QLD-Cape York Peninsula/1996 (CA)
OR1109/WA-Kununurra/1977 (CA)
PH491/WA-Newman/1981 (CA)
OR155/WA-Kununurra/1973 (CA)
Genotype 1a
Intermediate lineages
Genotype 1b
Genotype 4
Genotype 3
Genotype 2
0.02

B.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TC123130/Culgoa-VIC/1974 (HB)
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T69/NA/1969 (HB)
OR2/WA-Kununurra/1972 (CA)
18403C/QLD-Cape York Peninsula/1996 (CA)
OR1109/WA-Kununurra/1977 (CA)
PH491/WA-Newman/1981 (CA)
OR155/WA-Kununurra/1973 (CA)
Intermediate lineages
K5686/WA-Kununurra/1989 (CA)
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K41994/WA-Billiluna/2000 (CA)
CY1189/QLD-Cape York Peninsula/1999 (CS)
K36687/WA-Kununurra/1999 (CA)
GUOO19/QLD-Normanton/2000 (CA)
P9990/WA-Newman/2009 (CA)
P9777/WA-Newman/2009 (C)
P9986/WA-Newman/2009 (CA)
145694/NSW-Leeton/2008 (CA)
v11-10_mvev/VIC-Callawadda/ 2011 (EB)
145507/NSW-Griffith/2008 (CA)
08-154300/QLD-Monto/2008 (EB)
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K59582/WA-Fitzroy_Crossing/2006 (CA)
K60555/WA-Kununurra/2006 (CA)
K60365/WA-Kununurra/2006 (CA)
K60119/WA-Halls_Creek/2006 (AM)
K56445/WA-Parry's Creek/2005 (CA)
K68150/WA-Fitzroy_Crossing/2009 (CA)
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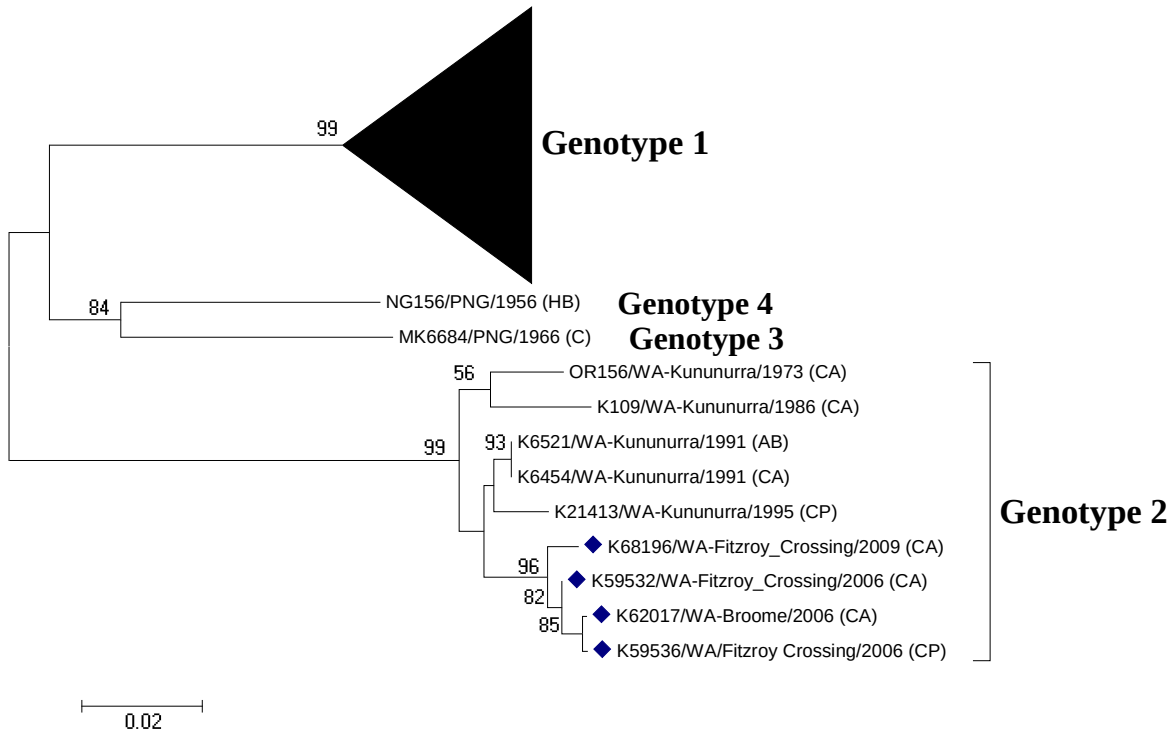
P9992/WA-Newman/2009 (CA)
Genotype 1b
Genotype 4
Genotype 3
Genotype 2
0.02

C.

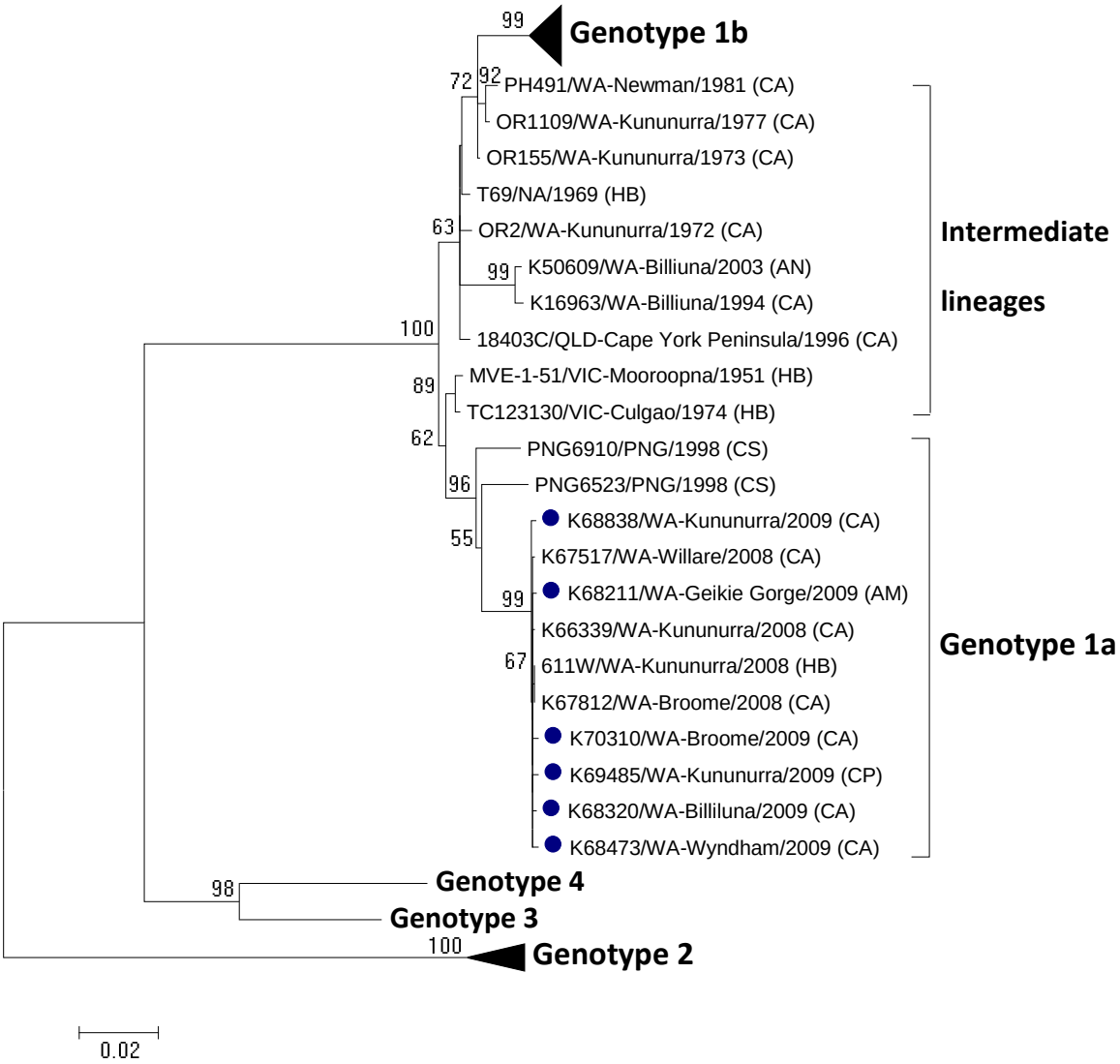


Appendix 2.1. *Neighbour Joining phylogeneitic tree constructed using partial E gene sequences*

Four genotypes are clearly identified (Genotype 1 to Genotype 4). Genotype 1 is further classified as genotype 1a and 1b. Genotypes 1b, 2, 3 and 4 are collapsed in **(A)**. Genotypes 1a, 2, 3 and 4 are collapsed in **(B)**. Genotype 1 is collapsed in **(C)**. Numbers above the internal nodes are the bootstrap support values from 1000 replicates. The scale represents 0.02 substitutions per nucleotide site. **WA:** Western Australia; **NSW:** New South Wales; **VIC:** Victoria; **QLD:** Queensland; **PNG:** Papua New Guinea; **NA:** Northern Australia; **CA:** *Cx. annulirostris*; **CP:** *Cx. pullus*; **CS:** *Cx. sitiens* group; **CX:** Mixed culicine; **C:** Culex species (damaged); **AM:** *Aedes* (Macleaya) species; **AN:** *Aedes normanensis*; **AB:** *Anopheles bancroftii*; **AA:** *Anopheles amictus*; **HB:** Human Brain; **EB:** Equine brain;

◆ indicates the isolates that were sequenced in this study.

A.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92
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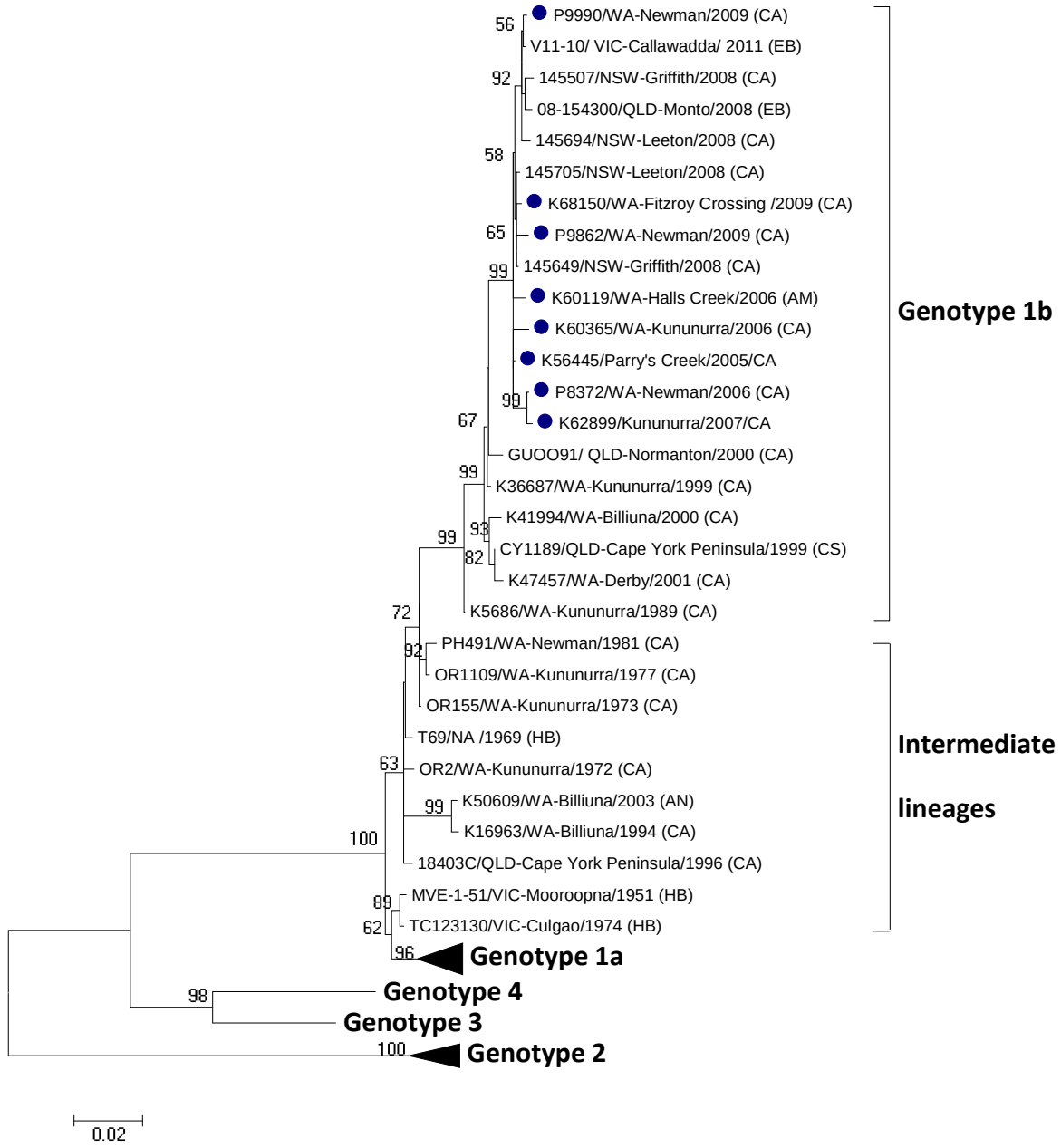
OR155/WA-Kununurra/1973 (CA)
T69/NA/1969 (HB)
63
OR2/WA-Kununurra/1972 (CA)
99
K50609/WA-Billiuna/2003 (AN)
K16963/WA-Billiuna/1994 (CA)
100
18403C/QLD-Cape York Peninsula/1996 (CA)
89
MVE-1-51/VIC-Mooroopna/1951 (HB)
TC123130/VIC-Culgao/1974 (HB)
62
PNG6910/PNG/1998 (CS)
96
PNG6523/PNG/1998 (CS)
K68838/WA-Kununurra/2009 (CA)
55
K67517/WA-Willare/2008 (CA)
99
K68211/WA-Geikie Gorge/2009 (AM)
K66339/WA-Kununurra/2008 (CA)
67
611W/WA-Kununurra/2008 (HB)
K67812/WA-Broome/2008 (CA)
K70310/WA-Broome/2009 (CA)
K69485/WA-Kununurra/2009 (CP)
K68320/WA-Billiluna/2009 (CA)
K68473/WA-Wyndham/2009 (CA)
Intermediate
lineages
Genotype 1a
98
Genotype 4
Genotype 3
100
Genotype 2
0.02

B.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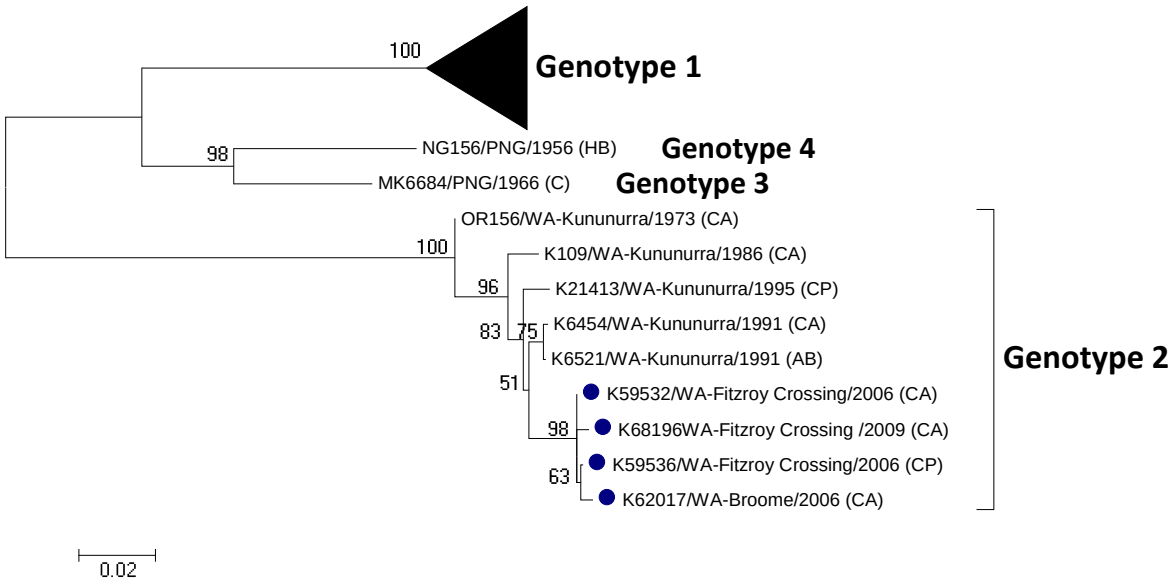
V11-10/ VIC-Callawadda/ 2011 (EB)
145507/NSW-Griffith/2008 (CA)
08-154300/QLD-Monto/2008 (EB)
145694/NSW-Leeton/2008 (CA)
145705/NSW-Leeton/2008 (CA)
K68150/WA-Fitzroy Crossing /2009 (CA)
P9862/WA-Newman/2009 (CA)
145649/NSW-Griffith/2008 (CA)
K60119/WA-Halls Creek/2006 (AM)
K60365/WA-Kununurra/2006 (CA)
K56445/Parry's Creek/2005/CA
P8372/WA-Newman/2006 (CA)
K62899/Kununurra/2007/CA
GUOO91/ QLD-Normanton/2000 (CA)
K36687/WA-Kununurra/1999 (CA)
K41994/WA-Billiuna/2000 (CA)
CY1189/QLD-Cape York Peninsula/1999 (CS)
K47457/WA-Derby/2001 (CA)
K5686/WA-Kununurra/1989 (CA)
PH491/WA-Newman/1981 (CA)
OR1109/WA-Kununurra/1977 (CA)
OR155/WA-Kununurra/1973 (CA)
T69/NA /1969 (HB)
OR2/WA-Kununurra/1972 (CA)
K50609/WA-Billiuna/2003 (AN)
K16963/WA-Billiuna/1994 (CA)
18403C/QLD-Cape York Peninsula/1996 (CA)
MVE-1-51/VIC-Mooroopna/1951 (HB)
TC123130/VIC-Culgao/1974 (HB)
Genotype 1a
Genotype 4
Genotype 3
Genotype 2
Genotype 1b
Intermediate lineages
0.02

C.

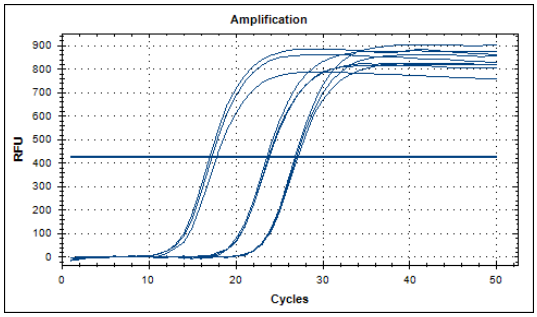


Appendix. 2.2. *Maximum Likelihood tree demonstrating relationships between different strains of MVEV when complete prM-E genes were analysed.*

For legend, please refer to page 76.

Appendix 2.3. *Nucleotide and amino acid identities within and between complete prM-E sequences of genotypes of Murray Valley encephalitis virus.*

Genotypes	**Nucleotide identity %**	**Amino acid identity %**
Within G1a	97.5-100	99.7-100
Within G1b	98.0-99.8	99.1-100
Within G1	94.3-96.0	98.9-99.4
Within G2	96.7-99.8	98.9-100
G1 vs G2	85.5-87.5	95.8-96.7
G1 vs G3 and G4	88.8-90.6	97.7-98.9
G2 vs G3 and G4	86.5-88.0	95.9-96.8
Within all MVEV strains	85.5-100	95.6-100



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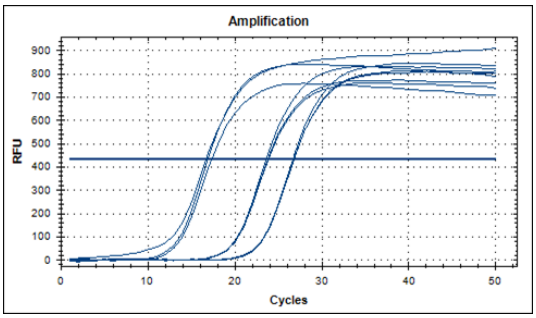
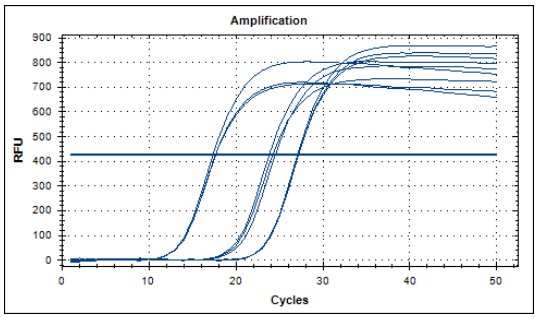


B



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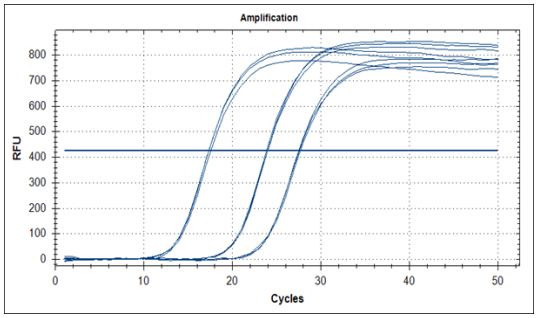

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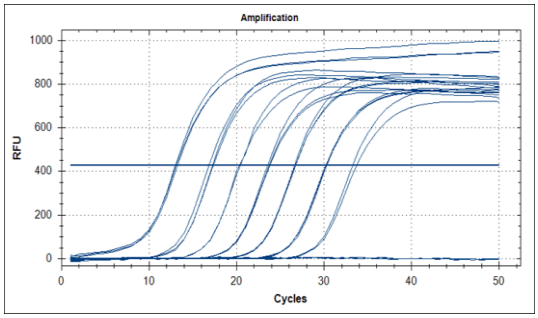


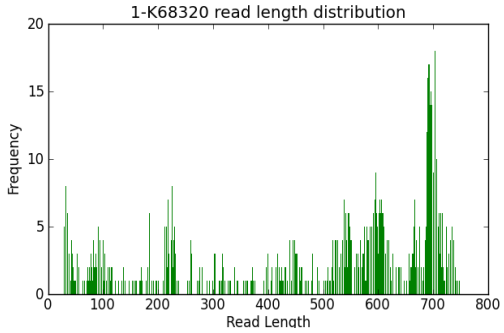
E

Appendix 3.1. *Amplification plots demonstrating the inter- and intra-assay precisions of MVEV RT-qPCR assay.*

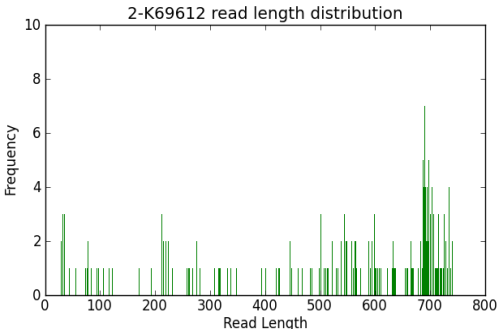
The intra-assay precision was tested using seven ten-fold dilution of MVE-1-51 in vitro RNA transcripts in triplicate in a single run (A). Inter-assay precision was tested by running three dilutions (10^6, 10^4, and 10^3) of MVE-1-51 in vitro RNA transcripts in triplicates over four independent tests run in four different days (B, C, D and E). Mean, S.D. and CV were calculated for each dilution, and tabulated in table 3. Straight line in each graph represents the threshold.

Appendix 4.1. *The number of raw reads obtained from each sample with their maximum, average and median lengths.*

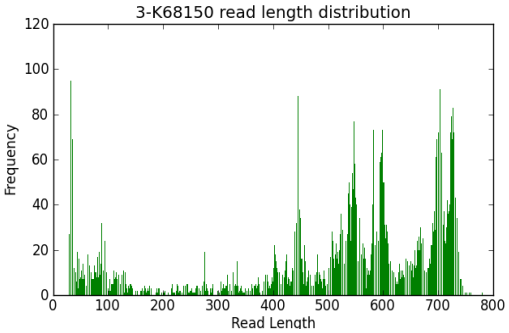
Samples	Genotype	Number of reads	Maximum Length	Average Length	Median Length
K68320	G1a	1,439	744	492	580
K69612	G1a	354	744	514	587
K68150	G1b	10,250	779	514	561
K60555	G1b	3,141	770	535	598
P8891	G1b	21,393	762	521	588
K68196	G2	71	738	562	631
K59532	G2	1,707	744	518	594
K59536	G2	272	737	501	598
pMVE-1-51		8,696	779	533	594
Ratio control		11,127	764	500	588



A



B



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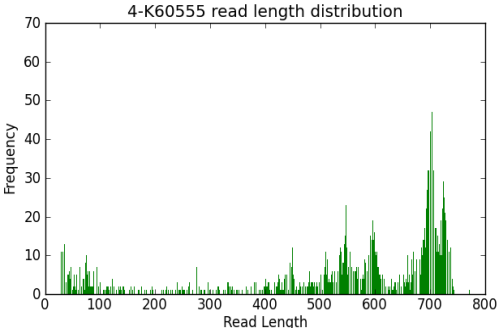
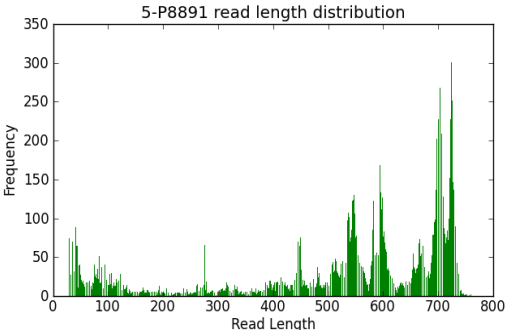
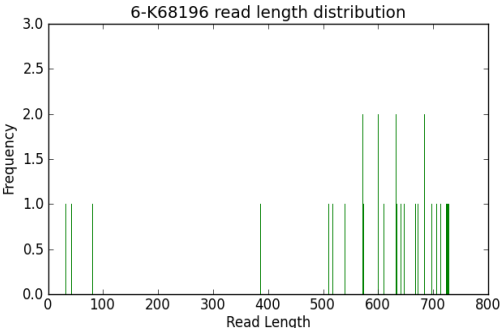


D

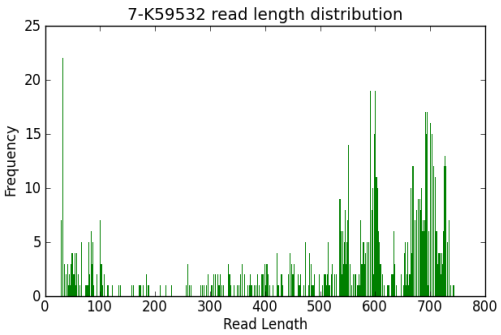


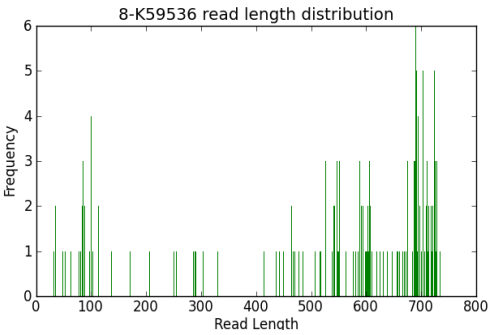
E



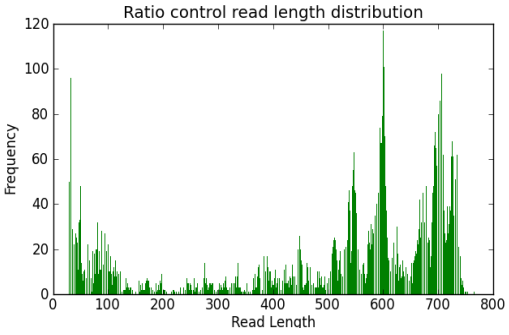
F

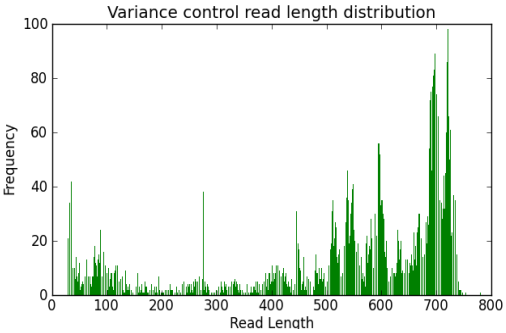
Appendix 4.2. *The read length distribution over the partial envelope gene sequences for the samples and controls used in this study.*





G **H**





I **J**

Appendix 4.2. *Continued*.

A: K68320; **B:** K69612; **C:** H68150; **D:** K60555; **E:** P8891; **F:** K68196; **G:** K59532; **H:** K59536; **I:** Ratio control; **J:** MVE-1-51 control.

Appendix 4.3. *Two-tailed t-tests analysis comparing the number of clones that differed from the sample-specific consensus sequence obtained at the completion of filtering step.*

Group 1	Group2	Ho. Diff	Mean Diff.	SE Diff.	*t*-value	DF	*p*-value*
G1a	G1b	0.000	27.655	6.179	4.476	3.000	**0.021**
	G2	0.000	39.808	4.238	9.393	3.000	**0.003**
G1b	G2	0.000	12.153	4.912	2.474	4.000	**0.006**

Ho. Diff: Hypothesised difference

Mean diff: Mean difference

SE diff: The standard error of the difference in the means

DF: Estimated degrees of freedom

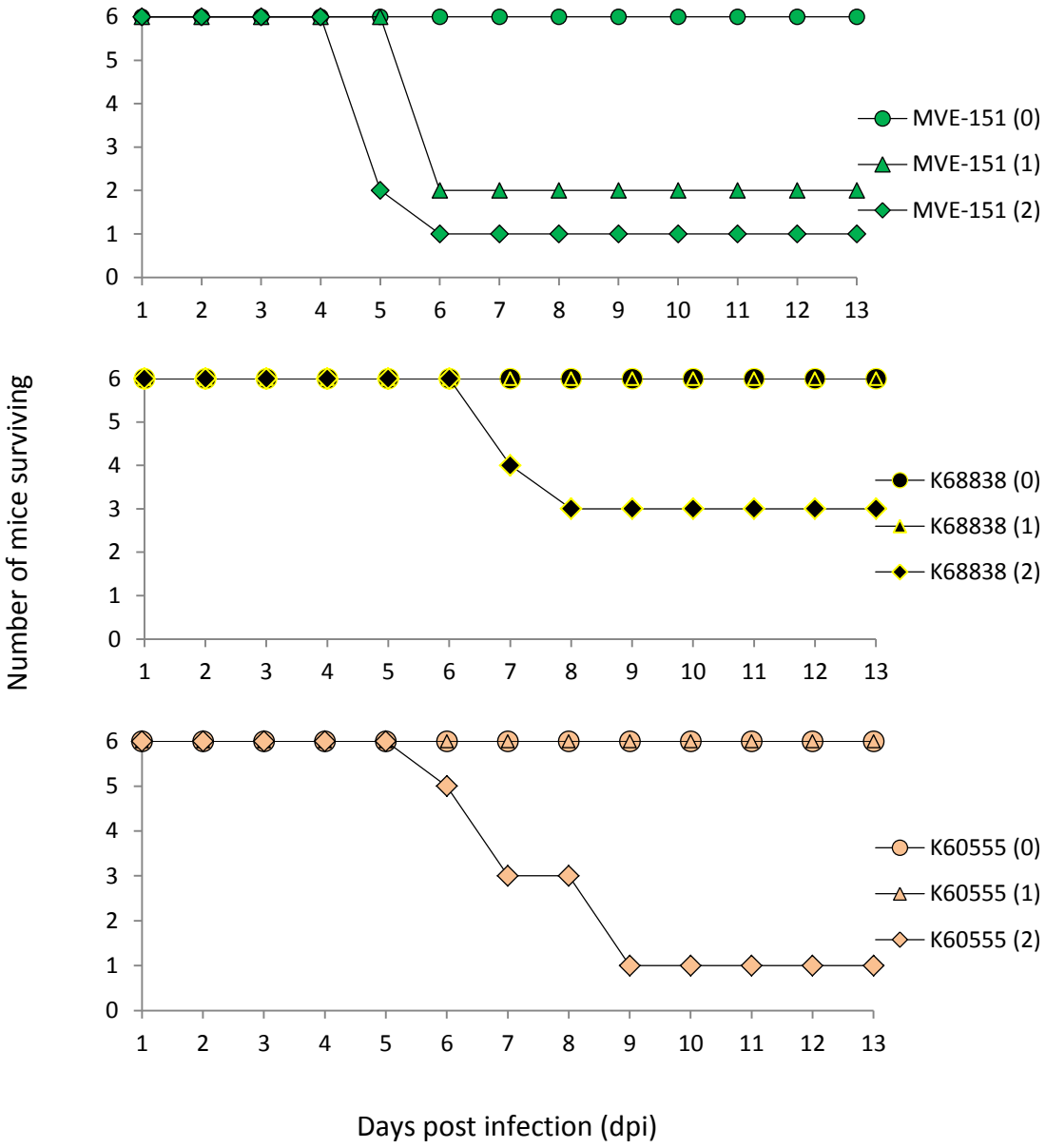
t-value is calculated as (the mean difference of the samples minus hypothesised difference) divided by standard error of the difference in the means.

*A *p*-value of <0.05 is considered significant.

Appendix 4.4. *Two-tailed t-tests analysis comparing the per-nucleotide variance within the partial envelope gene sequences analysed at the completion of data filtering for each genotype and subgenotypes.*

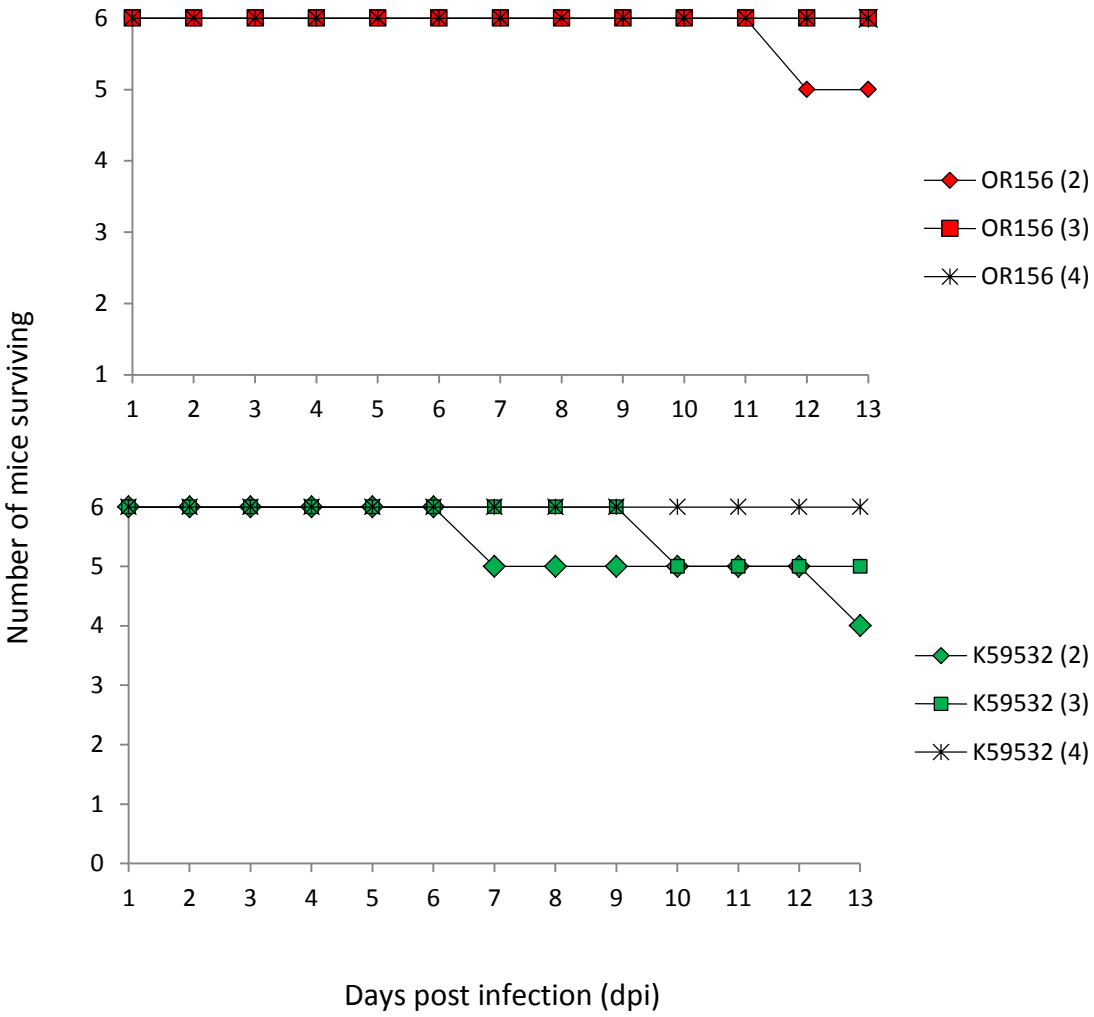
Group 1	Group2	Ho. Diff	Mean Diff.	SE Diff.	*t*-value	DF	*p*-value*
G1a	G1b	0.000	0.023	0.002	14.315	3.000	**0.001**
	G2	0.000	0.028	0.002	13.390	3.000	**0.001**
G1b	G2	0.000	0.005	0.001	3.342	4.000	**0.029**

Legends as above.



Appendix 5.1. *Mortality profile in mice infected with different strains of G1.*

Numbers in the parentheses indicate the Log_{10} $TCID_{50}$ dose of each virus. Days 14-21 post infection are not included in the graphs.



Appendix 5.2. *Mortality profile in mice infected with different strains of G2.*

Numbers in the parentheses indicate the Log_{10} $TCID_{50}$ dose of each virus. Days 14-21 post infection are not included in the graphs.

Appendix 5.3. *The copy number and titre of MVEV in the homogenates of mice brain.*

Viruses	Dose (Log_{10} $TCID_{50}$)	Copy Number/g mouse brain (log_{10})	Titration/g mouse brain (log_{10})
Genotype 1 isolates			
MVE-1-51 (G1)	1	12.44	8.20
	1	12.78	8.60
	1	13.21	9.00
	1	12.56	8.20
	2	13.17	9.00
	2	12.63	9.00
	2	13.33	9.60
	2	12.91	8.60
	2	11.61	7.80
K68838 (G1a)	2	12.50	8.00
	2	12.26	8.20
	2	11.78	7.00
K60555 (G1b)	2	11.51	7.60
	2	12.23	8.00
	2	12.73	8.40
	2	11.67	7.40
	2	11.90	7.80
Genotype 2 isolates			
OR156 (G2)	2	12.32	7.40
K59532 (G2)	2	12.08	8.40
	2	11.51	7.60
	3	12.34	8.20

Appendix 5.4. *Statistical analysis of the viral copy number in the homogenates of mice brains inoculated with different isolates of MVEV.*

Group 1	Group2	Ho. Diff	Mean Diff.	SE Diff.	*t*-value	DF	*p*-value
MVE-1-51	K68838	0	0.558	0.331	1.685	10.00	0.123
	K60555	0	0.730	0.285	2.558	12.00	**0.025**
	G2 isolates	0	0.675	0.295	2.292	11.00	**0.043**
K68838	K60555	0	0.172	0.328	0.524	6.000	0.619
	G2 isolates	0	0.118	0.289	0.406	5.000	0.701
K60555	G2 isolates	0	-0.055	0.299	-0.182	7.000	0.861

Appendix 5.5. *Statistical analysis of the titres of the virus in the homogenates of mice brains inoculated with different isolates of MVEV.*

Group 1	Group2	Ho. Diff	Mean Diff.	SE Diff.	*t*-value	DF	*p*-value
MVE-1-51	K68838	0	0.933	0.379	2.465	10.00	**0.033**
	K60555	0	0.827	0.279	2.968	12.00	**0.012**
	G2 isolates	0	0.767	0.318	2.411	11.00	**0.035**
K68838	K60555	0	-0.107	0.355	-0.300	6.00	0.774
	G2 isolates	0	-0.167	0.419	-0.398	5.00	0.707
K60555	G2 isolates	0	-0.060	0.286	-0.210	7.00	0.840

Ho. Diff: Hypothesised difference

Mean diff: Mean difference

SE diff: The standard error of the difference in the means

DF: Estimated degrees of freedom

A *p*-value of <0.05 is considered significant.

Appendix 5.6. *The result of Immunofluorescence Assays for antibodies to G1 and G2 isolates in mice following intraperitoneal inoculation.*

Viruses	Dose (Log_{10} $TCID_{50}$)	IFA Results* Positive
Genotype 1 isolates		
MVE-1-51 (G1)	0	6/6 (100%)
	1	2/2 (100%)
	2	1/1 (100%)
K68838 (G1a)	0	5/6 (83.33%)
	1	6/6 (100%)
	2	3/3 (100%)
K60555 (G1b)	0	4/6 (66.67%)
	1	5/6 (83.33%)
	2	1/1 (100%)
Genotype 2 isolates		
OR156 (G2)	2	5/5 (100%)
	3	6/6 (100%)
	4	6/6 (100%)
K59532 (G2)	2	4/4 (100%)
	3	5/5 (100%)
	4	6/6 (100%)

* Denominator is the number of mice that survived the infection.

Appendix 6.1. *Details of oligonucleotide primers used for the amplification of G1 viruses in this study.*

Set No.	Primers	Region	Position	size	Sequence	Product length
1	MVEV1F	5'UTR	1	20	AGACGTTCATCTGCGTGAGC	720
	MVEV1R	prM	720	20	CACGTGTGCACCTTCCATAG	
2	prM-E-368F	C	368	24	GATTGATGTGGTGAACAAAAGGGG	720
	prM-E-1086R	E	1086	21	GTTTRTCAGCRGCCATGATGG	
3	prM-E-991F	E	991	22	GYAGCCGTGAYTTYATTGAAGG	805
	prM-E-1975R	E	1795	25	GHRAACTCGACTGGTAYGGCTCCAG	
4	prM-E-1657F	E	1657	24	CAARYACGGAMTGGAGGAAYAGAG	905
	prM-E-2562R	NS1	2562	25	CAAYCCARGCYTCAACATCATTGTG	
5	MVEV5F	NS1	2520	20	GCAGCGGAATATTCATACAC	681
	MKM5R	NS1	3200	20	CACTGGGATTATCAACTCCG	
6	MVEV6F	NS1	3100	19	CATGGAAACTTGAGAGAGC	613
	MVEV6R	NS2A	3710	19	CCAATATGAGATACCGCAC	
7	MVEV7F	NS2A	3615	20	CTTGAGGAAGAGGTGGACGG	330
	MVEV7R	NS2A	3945	20	GGGCATGGCTATTGCTGAGG	
8	MVEV8F	NS2A	3820	21	GTAGATGGACTAATCAAGAG	720
	MVEV8R	NS2B	4540	20	GGCCCTTGGAGTGATGGCAG	
9	MVEV9F	NS2B	4495	21	AATGACCCTGGAGTTCCATG	690
	MVEV9R	NS3	5185	22	AGGTCCAGCACGGTTAGTTG	
10	MVEV10F	NS3	5110	20	GTCAGTGCTATTGTTCAAGG	680
	MVEV10R	NS3	5790	19	GTATTCCGTATCATAGGAC	
11	MVEV11F	NS3	5650	20	GAGTGGATAACCGACTATGC	775
	MVEV11R	NS3	6425	21	GACTGGTGGTCTGAATACAC	
12	MVEV12F	NS3	6360	21	CGAAGTGGAAATCATAACAAG	370
	MVEV12F	NS4A	6730	20	CTACCAAGACGAGTGCTCCG	
13	MKM17F	NS4A	6620	20	CCATAACACTCATTGCAGCG	630
	MKM17R	NS4B	7250	20	ATCAAGGTGGTGAGCGTCAC	
14	MKM18F	NS4B	7110	20	GTTACCACCTCATTGGCATC	655
	MKM18R	NS5	8040	21	CCATAGCTCTGCATCAACATG	
15	MVEV13F	NS5	7975	22	AGCAACCATGAAGAACGTCC	755
	MVEV13R	NS5	8630	21	GATGGCTTCACCTCATAACTC	
16	MVEV14F	NS5	8545	20	AACTGAAGGAGGAGTATGC	625
	MVEV14R	NS5	9300	20	CAACCAGCTGTGTCATCGGC	
17	MVEV15F	NS5	9200	22	GGAGGAGGAGTTGAAGGAG	520
	MVEV15R	NS5	9825	20	CTCTGCAGGGCACCACCAAG	
18	MKM19F	NS5	9800	20	CTGGTATGACTGGCAACAAG	700
	MKM19R	NS5	10320	20	CTATGACTGACCTCACTTGG	
19	MVEV16F	NS5	10250	21	CTGGTGCGGAAGTCTCATAG	761
	MVEV16Rb	3'UTR	11010	17	AGATCCTGTGGTCTTCTC	

Appendix 6.2. *Details of oligonucleotide primers used for the amplification of genotype 2 viruses in this study.*

Set No.	Primers	Region	Position	size	Sequence	Product length
1	MVEV1F	5'UTR	1	20	AGACGTTCATCTGCGTGAGC	385
	MKM1R-2	C	385	20	GTTCACCACATCAATCAGTG	
	prM-E-368F	C	368	24	GATTGATGTGGTGAACAAAAGGGG	720
2	prM-E-1086R	E	1086	21	GTTTRTCAGCRGCCATGATGG	
	prM-E-991F	E	991	22	GYAGCCGTGAYTTYATTGAAGG	805
3	prM-E-1975R	E	1795	25	GHRAACTCGACTGGTAYGGCTCCAG	
	prM-E-1657F	E	1657	24	CAARYACGGAMTGGAGGAAYAGAG	905
4	prM-E-2562R	NS1	2562	25	CAAYCCARGCYTCAACATCATTGTG	
5	OR156_2040F	E	2040	23	CCATATGTTGCCTCATCAACTGC	901
	OR156_2941R	NS1	2914	22	CAGACCCTAGTGGACGTTATTC	
	OR156_2620F	NS1	2620	20	CATAAGAGCGGTATATGTGG	882
6	OR156_3502R	NS1	3502	21	GGACCCTCGATTTCACTAAAG	
	OR156_3178F	NS1	3178	23	CTGATCATTCCAGTGACTCTCGC	701
7	OR156_3879R	NS2A	3879	23	GACAGTTCCAGATCTGAAGCTGC	
	OR156F_3769F	NS2A	3769	24	CATCGCTGTGTTCAAAGTGCAGCC	1262
8*	OR156_5030R	NS3	5030	27	CATAAAGACCGACTATCTCTCCATTGC	
	MKMV7F_2	NS3	4888	18	GGAATGGAGTGGATGACG	772
9	MKMV7R_2	NS3	5660	20	GTTATCCACTCAAATCCAC	
10*	OR156_5030F	NS3	5030	27	GCAATGGAGAGATAGTCGGTCTTTATG	1082
	OR156_6112R	NS3	6112	23	CTCTTTCAGGACCATACAGCTGG	
	MVE_6039F	NS3	6039	20	GAACCAGTGAGGATGACACC	788
11	OR156_6827R	NS4A	6827	19	GCTCAGGGATTAGCACGAC	
	OR156_6747F	NS4A	6747	25	GGATGTCAGATGTTTCTGGAACTGG	737
12	OR156_7484R	NS4B	7484	24	CACACATAATGATGCTACGCTGGC	
	OR156_7406F	NS4B	7406	21	GAAAGAACGACTCCACAGATG	996
13	OR156_8401R	NS5	8401	21	CACCTGACTAGTCATGTTCAC	
	OR156_8360F	NS5	8360	23	CGGGAAATATCGTGCATGCTGTG	817
14	OR156_9177R	NS5	9177	24	GGCTTCAAATTCCAAGAACCTGGC	
15	OR156_8945F	NS5	8945	23	GAATGCACGAGAAGCCGTAGAAG	679
	OR156_9624R	NS5	9624	24	CAACGCTCTCGATGTCATCTGGTC	
	OR156_9489F	NS5	9489	22	GAGGAAGTGGACAAGTGGTAAC	901
16	OR156_10390R	NS5	10390	19	GTCTTCACTCACATGGGCC	
	MVEV16F	NS5	10250	21	CTGGTGCGGAAGTCTCATAG	761
17	MVEV16Rb	3'UTR	11010	17	AGATCCTGTGGTCTTCTC	

* Elongation step at 72°C for 90 seconds.

Appendix 6.3. *Statistical analyses of the single growth curve data of the genotype 1 isolates of Murray Valley encephalitis virus.*

Repeated measures Analysis of Variance for Variable = Titre
Post Hoc Test For Factor = Viruses

A. In C6/36 cells

Reference	Viruses	Mean Difference	Standard error	q statistic*	*p-value†*
G1-P0	G1-C10	-0.33	0.22	1.54	0.31
	G1-D10	1.93	0.22	8.95	**2.26E-04**
	G1-A16	9.87	0.22	45.67	**1.93E-07**
G1-C10	G1-D10	2.27	0.22	10.49	**1.94E-04**
	G1-A16	10.20	0.22	47.22	**2.14E-07**
G1-D10	G1-A16	7.93	0.22	36.72	**1.93E-07**

B. In DF1 cells

Reference	Viruses	Mean Difference	Standard error	q statistic*	*p-value†*
G1-P0	G1-C10	0.33	0.24	1.37	0.36
	G1-D10	-9.47	0.24	39.01	**1.92E-07**
	G1-A16	3.40	0.24	14.01	**2.40E-05**
G1-C10	G1-D10	-9.80	0.24	40.38	**1.95E-07**
	G1-A16	3.07	0.24	12.64	**1.97E-05**
G1-D10	G1-A16	12.87	0.24	53.02	**2.13E-07**

* The q statistic (studentised range statistic) is used for multiple significance testing across a number of means. It is the calculation of the differences in mean divided by the standard error.
† A *p*-value of <0.05 is considered significant. Significant values are emboldened.

Appendix 6.4. *Statistical analyses of the single growth curve data of the genotype 2 isolates of Murray Valley encephalitis virus.*

Repeated measures Analysis of Variance for Variable = Titre
Post Hoc Test For Factor = Viruses

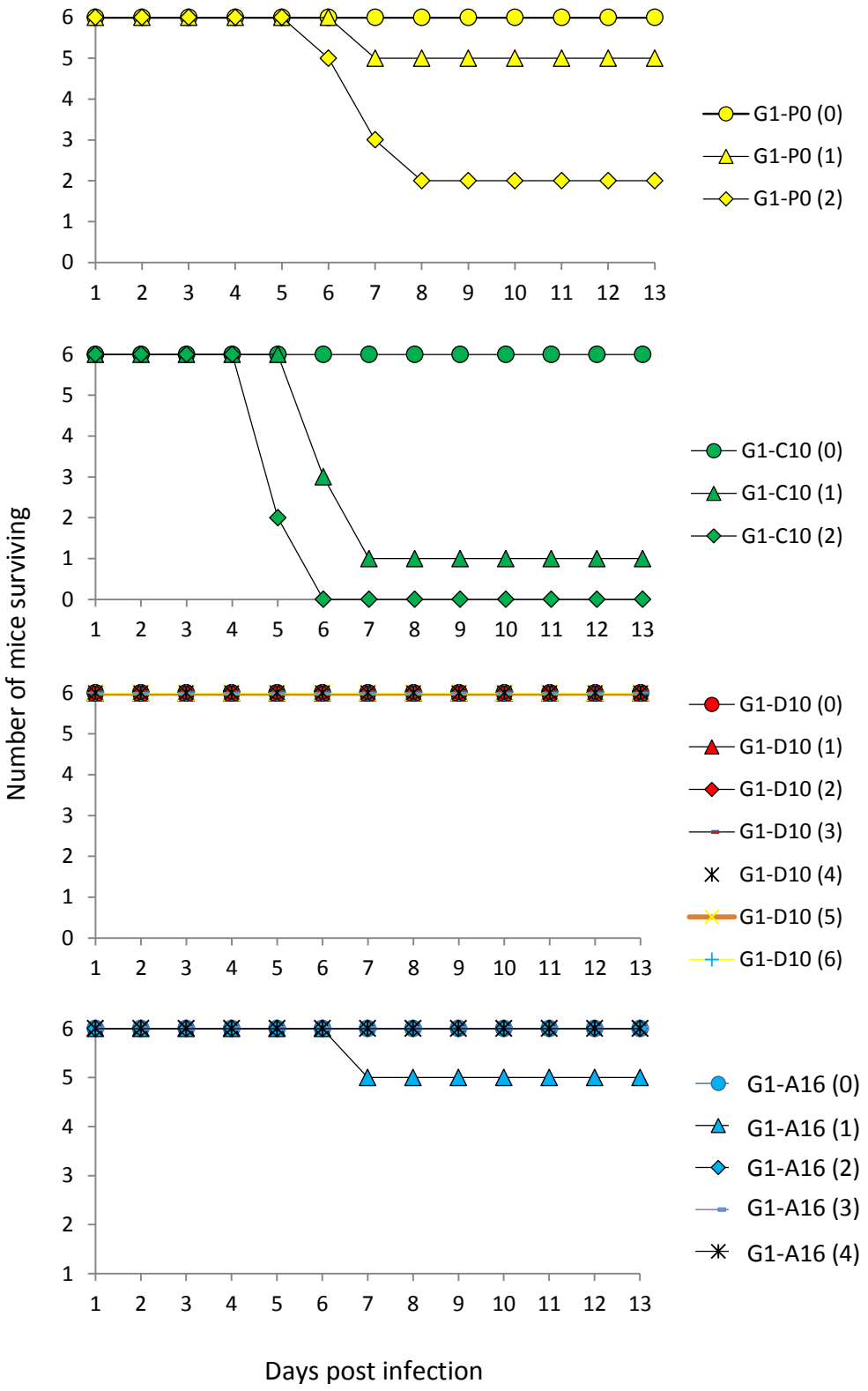
A. In C6/36 cells

Reference	Viruses	Mean Difference	Standard error	q statistic*	*p-value†*
G2-P0	G2-C10	-0.27	0.18	1.51	0.32
	G2-D10	2.20	0.18	12.47	**5.63E-05**
	G2-A16	1.80	0.18	10.21	**9.12E-05**
G2-C10	G2-D10	2.47	0.18	13.98	**4.32E-05**
	G2-A16	2.07	0.18	11.72	**8.85E-05**
G2-D10	G2-A16	-0.40	0.18	2.27	0.15

B. In DF1 cells

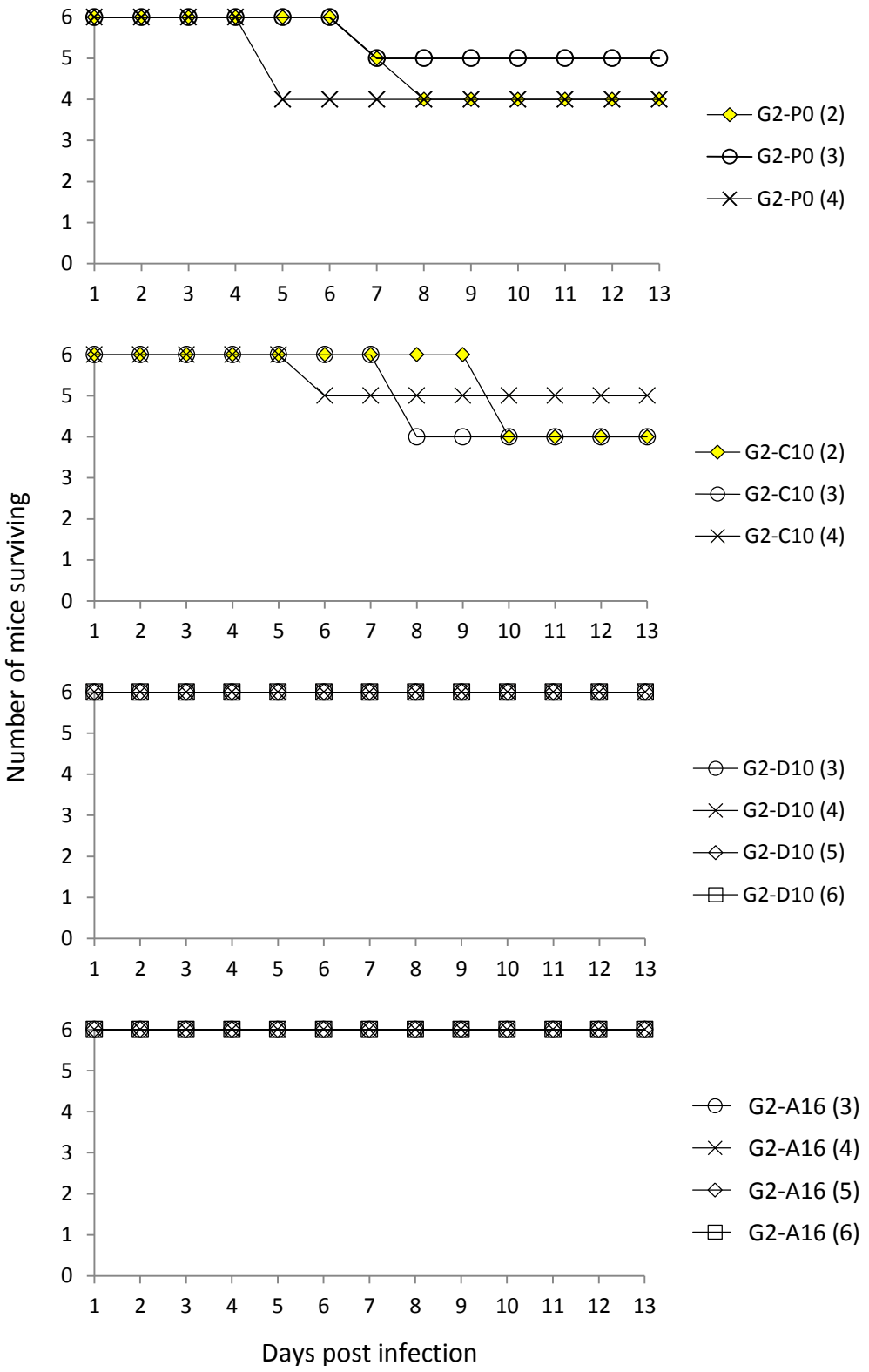
Reference	Viruses	Mean Difference	Standard error	q statistic*	*p-value†*
G2-P0	G2-C10	0.33	0.21	1.62	0.28
	G2-D10	-9.87	0.21	48.02	**1.92E-07**
	G2-A16	-5.13	0.21	24.98	**2.91E-07**
G2-C10	G2-D10	-10.20	0.21	49.64	**2.13E-07**
	G2-A16	-5.47	0.21	26.60	**3.55E-07**
G2-D10	G2-A16	4.73	0.21	23.04	**3.85E-07**

* The q statistic (studentised range statistic) is used for multiple significance testing across a number of means. It is the calculation of the differences in mean divided by the standard error.
† A *p*-value of <0.05 is considered significant. Bolded values indicate the significant values.



Appendix 6.5. *Mortality profiles of the genotype 1 MVEV isolates after i.p. injection of groups of 18-day old mice with different doses of each virus.*

Numbers in the parenthesis indicate the $\log_{10}$ $TCID_{50}$ concentration of the virus administered in each group of mice. Days 14-21 post infection are not included in the graphs.



Appendix 6.6. *Mortality profiles of the genotype 2 isolates after i.p. injection of groups of 18-day old mice with different doses of each virus.*

Numbers in the parenthesis indicate the $\log_{10}$ $TCID_{50}$ concentration of the virus administered in each group of mice. Days 14-21 post infection are not included in the graphs.

Appendix 6.7. *The copy number and titre of MVEV strains in the homogenates of mice brain inoculated with MVE viruses from different genotypes.*

Viruses	Dose (Log_{10} $TCID_{50}$)	Copy Number /g of mouse brain (Log_{10})	Titration/g of mouse brain (Log_{10} $TCID_{50}$)
Genotype 1 isolates			
G1-P0	1	13.03	9.20
G1-P0	2	12.89	9.40
G1-P0	2	13.51	9.00
G1-P0	2	12.93	9.20
G1-P0	2	12.39	9.40
G1-C10	1	13.47	9.80
G1-C10	1	12.65	9.40
G1-C10	1	12.94	9.20
G1-C10	1	13.43	9.40
G1-C10	1	13.05	9.40
G1-C10	2	13.45	9.80
G1-C10	2	12.58	9.60
G1-C10	2	13.02	9.00
G1-C10	2	12.80	9.80
G1-C10	2	13.46	9.40
G1-C10	2	12.81	9.20
G1-A16	2	13.14	9.20
Genotype 2 Isolates			
G2-P0	2	12.58	8.60
G2-P0	2	11.19	7.60
G2-P0	3	12.56	8.40
G2-P0	4	11.44	7.20
G2-P0	4	12.19	8.00
G2-C10	2	12.58	8.00
G2-C10	2	11.19	8.20
G2-C10	3	12.56	8.40
G2-C10	3	11.44	8.60
G2-C10	4	12.19	7.20

Appendix 6.8. *The result of Immunofluorescence Assays of G1 and G2 isolates.*

Virus	Dose ($TCID_{50}$)	IFA Results
		Positive
Genotype 1 isolates		
G1-P0	0	6/6 (100%)
G1-P0	1	5/5 (100%)
G1-P0	2	2/2 (100%)
G1-C10	0	6/6 (100%)
G1-C10	1	1/1 (100%)
G1-C10	2	N/A
G1-D10	0	6/6 (100%)
G1-D10	1	6/6 (100%)
G1-D10	2	6/6 (100%)
G1-D10	3	6/6 (100%)
G1-D10	4	6/6 (100%)
G1-D10	5	6/6 (100%)
G1-D10	6	6/6 (100%)
G1-A16	0	5/6 (83.3%)
G1-A16	1	5/5 (100%)
G1-A16	2	6/6 (100%)
G1-A16	3	6/6 (100%)
G1-A16	4	6/6 (100%)
Genotype 2 Isolates		
G2-P0	2	4/4 (100%)
G2-P0	3	5/5 (100%)
G2-P0	4	4/4 (100%)
G2-C10	2	4/4 (100%)
G2-C10	3	4/4 (100%)
G2-C10	4	5/5 (100%)
G2-D10	3	5/6 (83.3%)
G2-D10	4	6/6 (83.3%)
G2-D10	5	6/6 (100%)
G2-D10	6	6/6 (100%)
G2-A16	3	5/6 (83.3%)
G2-A16	4	6/6 (100%)
G2-A16	5	6/6 (100%)
G2-A16	6	6/6 (100%)

* Denominator is the number of mice that survived the infection.

Appendix 6.9. *Observed substitutions in the complete genome of CDV OR156 compared to the complete genome of CDV-1-51.*

G/R	G/C	G/R	G/C	G/R	G/C	G/R	G/C
C	K18R	E	S461T	NS3	A106V	NS4B	A188S
C	M66I	E	P462Q	NS3	I107V	NS4B	V189L
C	K101N	E	V474I	NS3	A119T	NS4B	I195V
C	T110A			NS3	I131T	NS4B	D214E
C	V112A	NS1	I21V	NS3	I147V	NS4B	A220V
C	M114L	NS1	I29V	NS3	K215R	NS4B	M233I
C	L115A	NS1	K33R	NS3	A356P		
C	I116G	NS1	R105K	NS3	S445G	NS5	S26N
C	I120V	NS1	G167V	NS3	D515E	NS5	V71I
		NS1	T176S	NS3	I538L	NS5	A91T
E	A15V	NS1	H192Y	NS3	K562R	NS5	M116T
E	V21I	NS1	E271D	NS3	I583T	NS5	I156V
E	L55V	NS1	K293R	NS3	K590R	NS5	I164V
E	V68I					NS5	K194R
E	S72A	NS2A	A37T	NS4A	A17V	NS5	T274S
E	N89S	NS2A	R98K	NS4A	V62I	NS5	K277R
E	N123S	NS2A	T124S	NS4A	M71L	NS5	R278K
E	A126T	NS2A	I150M	NS4A	V88A	NS5	T374I
E	T157S	NS2A	I177V	NS4A	A90V	NS5	Q526K
E	V165A	NS2A	V197I			NS5	A587T
E	T205S	NS2A	I220V	NS4B	T11D	NS5	V623I
E	A229S			NS4B	R14K	NS5	I640V
E	E232D	NS2B	D59N	NS4B	S20P	NS5	V721L
E	I238V	NS2B	G65E	NS4B	N25T	NS5	V769L
E	V240M	NS2B	D84N	NS4B	V27A	NS5	T834M
E	S275T	NS2B	V99I	NS4B	P30S	NS5	S836A
E	S276G	NS2B	R113W	NS4B	F32L	NS5	N873Y
E	T330A			NS4B	P40R	NS5	V878I
E	V352I	NS3	K11R	NS4B	I115A	NS5	V88M
E	A367V	NS3	R33S	NS4B	M116L	NS5	Q889L
E	A369S	NS3	N71S	NS4B	V121I	NS5	T897A

G/R: Genomic region; **G/C:** Genetic changes

Substitutions are based on the amino acid sequence of the structural and non-structural proteins. Numbers indicate the position of the amino acid from the beginning of each protein.