MOLECULAR AND BIOLOGICAL CHARACTERISATION OF ORTHOBUNYAVIRUSES

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Submitted in fulfilment of the requirements for the Degree of
Doctor of Philosophy in Molecular Virology

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May 2016

Abstract

Orthobunyaviruses are the largest genus within the *Bunyaviridae* family, with over 170 named viruses classified into 18 serogroups (Elliott and Blakqori, 2001; Plyusnin *et al.,* 2012). Orthobunyaviruses are transmitted by arthropods and have a tripartite negative sense RNA genome, which encodes 4 structural proteins and 2 non-structural proteins. The non-structural protein NSs is the primary virulence factor of orthobunyaviruses and potent antagonist of the type I interferon (IFN) response. However, sequencing studies have identified pathogenic viruses that lack the NSs protein (Mohamed *et al.,* 2009; Gauci *et al.,* 2010).

The work presented in this thesis describes the molecular and biological characterisation of divergent orthobunyaviruses. Data on plaque morphology, growth kinetics, protein profiles, sensitivity to IFN and activation of the type I IFN system are presented for viruses in the Anopheles A, Anopheles B, Capim, Gamboa, Guama, Minatitlan, Nyando, Tete and Turlock serogroups. These are complemented with complete genome sequencing and phylogenetic analysis. Low activation of IFN by Tete serogroup viruses, which naturally lack an NSs protein, was also further investigated by the development of a reverse genetics system for Batama virus (BMAV). Recombinant viruses with mutations in the virus nucleocapsid protein amino terminus showed higher activation of type I IFN *in vitro* and data suggests that low levels of IFN are due to lower activation rather than active antagonism. The anti-orthobunyavirus activity of IFN-stimulated genes IFI44, IFITMs and human and ovine BST2 were also studied, revealing that activity varies not only within the orthobunyavirus genus and virus serogroups but also within virus species. Furthermore, there was evidence of active antagonism of the type I IFN response and ISGs by non-NSs viruses.

In summary, the results show that pathogenicity in man and antagonism of the type I IFN response *in vitro* cannot be predicted by the presence, or absence, of an NSs ORF. They also highlight problems in orthobunyavirus classification with discordance between classical antigen based data and phylogenetic analysis.

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Acknowledgements

To my lab family and the boss, Professor Richard M. Elliott, thank you for making me a graduate of the Elliott lab, it has been an honour and a privilege, hopefully I will do you proud.

Ingeborg van Knippenberg and Ruth Jarrett, there were times when I wasn't sure this would happen, thank you for looking out for me and getting me past the final hurdle.

I would like to thank my assessors and secondary supervisors Rupert Russell and David Jackson, University of St Andrews, and Robert Gifford, Sam Wilson, and Margaret Hosie, University of Glasgow, for helpful discussions of my work. I am also indebted to Thomas Briese, Centre for Infection and Immunity, New York, for supporting the sequencing collaboration and for his continued guidance and encouragement. I would also like to thank previous colleagues in NHS Scotland, with special thanks to Dave Yirrell and the microbiology department at Ninewells Hospital, Dundee, you taught me how science is done and to have confidence in my abilities.

To my family, it has been a long road filled with many, many flat moves and you have been there for everyone. Your support, encouragement and delivery of home cooked meals through locked metal gates has helped me do things I never thought I would. A special thank you must also go to the mini-Macs; Hannah, Ruaridh and Lucy, it's hard to worry about work when chasing the naughty tup around the Pinky Ponk, thank you for letting me escape with you.

Lastly, to C. David Owen, it has been an inconceivable journey from St Andrews to Glasgow, New York, Australia and Sierra Leone, but it is done and a new adventure begins.

Declaration

I, Gillian Sinclair Slack, declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: 

Printed name: G SLACK

List of Abbreviations

General Abbreviations

aa	amino acid
AHVLA	Animal Health and Veterinary Laboratories Agency
ArboCAT	International Catalog of Arboviruses
bp	base pairs
BSA	bovine serum albumin
BST2	bone marrow stromal cell antigen 2 (tetherin)
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid (antigenome)
CDS	coding sequence
CFR	case fatality ratio
CFT	complement fixation test
CT	cytoplasmic tail
CII	Center for Infection and Immunity
CO_2	carbon dioxide
CPE	cytopathic effect
d p.i.	days post infection
DEFRA	Department for Environment, Food and Rural Affairs
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme linked immune assays
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FCS	fetal calf serum
GFP	green fluorescent protein
GIMAP2	GTPase of immunity associated proteins 2
GMEM	Glasgow minimum essential medium
Gn/Gc	glycoproteins Gn and Gc
GTP	guanosine triphosphate
h	hours
h p.i.	hours post infection
hBST2	human bone marrow stromal cell antigen 2
HF	haemorrhagic fever
HFRS	haemorrhagic fever with renal syndrome

HI	haemagglutination inhibition
Ho	homologous
HPS	hantavirus pulmonary syndrome
HRP	horseradish peroxidase
Ht	heterologous
HTS	high-throughput sequencing
ICTV	International Committee on Taxonomy of Viruses
IFI44	interferon induced protein 44 (also known as MTAP44)
IFITM	interferon stimulated transmembrane protein
IFN	interferon
IRSE	internal ribosome entry site
ISG	interferon stimulated gene
ISRE	interferon-stimulated regulatory elements
ITAM	immunoreceptor tyrosine-based activation motif
L	L protein (viral polymerase) or large genomic segment
M	medium genomic segment
min	minutes
M-MLV	Moloney murine leukemia virus
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MTAP44	Microtubule-associated protein 44 (also known as IFI44)
N	nucleoprotein
NC	negative control
NEAA	non-essential amino acids
NSm	non-structural protein encoded on M segment
NSs	non-structural protein encoded on S segment
nt	nucleotide
NT	neutralisation test
oBST2	ovine bone marrow stromal cell antigen 2
OAS	oligoadenylate synthetases
ORF	open reading frame
OSBP	oxysterol-binding protein
OTU	ovarian tumour domain
p	virus passage number
PC	positive control
p.f.u	plaque forming units
PABP	poly-A binding protein
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein disruption buffer
PEG	polyethylene glycol

PKR	protein kinase R
PRNT	plaque reduction neutralisation test
PRR	pathogen recognition receptor
PVDF	polyvinylidene fluoride
r	recombinant
RACE	rapid amplification of cDNA ends
RdRp	RNA-dependent RNA-polymerase
RER	rough endoplasmic reticulum
RFP	red fluorescent protein
rIFN	relative interferon
RIG-I	retinoic acid inducible gene I
RLU	relative light units
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RNP	ribonucleocapsid protein
RT	reverse transcriptase
sp.	species
ss	single-stranded
S	small genomic segment
SAP30	Sin3A associated protein 30
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
T7RNAP	T7 RNA polymerase
TLR	toll-like receptor
Tm	melting temperature
TPB	tryptose phosphate broth
UK	United Kingdom
USA	United States of America
UTMB	University of Texas Medical Branch
UTR	untranslated region
VAPA	vesicle-membrane-protein-associated protein A
vRNA	viral ribonucleic acid
wt	wild type

Virus Abbreviations in Main Text

57389	Anopheles A virus ColAn 57389
AKAV	Akabane virus
ANDV	Andes virus
ANAV	Anopheles A virus, original 1940 isolate
ANBV	Anopheles B virus

ANDV	Andes virus
BAHV	Bahig virus
BAKV	Bakau virus
BCCV	Black Creek Canal virus
BMAV	Batama virus
BORV	Boraceia virus
BUCV	Buffalo Creek virus
BUNV	Bunyamwera virus
BWAV	Bwamba virus
CAPV	Capim virus
CCHFV	Crimean-Congo haemorrhagic fever virus
CPEV	Caraipe virus
CMV	cytomegalovirus
DGUV	Dugbe virus
EMCV	encephalomycocarditis virus
EREV	Eretmapodites virus
GAMV	Gamboa virus
GJAV	Guajara virus
GMAV	Guama virus
HCoV	human coronavirus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HTNV	Hantaan virus
IAV	influenza A virus
INKV	Inkoo virus
JCV	Jamestown Canyon virus
JDV	Juan Diaz virus
LACV	La Crosse virus
LEAV	Leanyer virus
LEDV	Lednice virus
JACV	Jamestown Canyon virus
KETV	Ketapang virus
KOOV	Koongol virus
KSHV	Kaposi’s sarcoma-associated herpesvirus
LUKV	Lukuni virus
LMV	Las Maloyas virus
MERS-CoV	Middle East respiratory syndrome coronavirus
MHV	Mahogany Hammock virus
MNTV	Minatitlan virus
MORV	Moriche virus
MPKV	Maprik virus
MPOV	M’Poko virus

MTRV	Matruh virus
NDV	Nyando virus
NOLAV	Nola virus
NSDV	Nairobi sheep disease virus
OLIV	Olifantsvlei virus
OROV	Oropouche virus
PGAV	Pongola virus
PLSV	Palestina virus
PTV	Punta Toro virus
RVFV	Rift Valley fever virus
SARS	severe acute respiratory syndrome
SBV	Schmallenberg virus
SEDV	Sedlec virus
SEOV	Seoul virus
SNV	Sin Nombre virus
SFSTV	severe fever with thrombocytopenia syndrome virus
SJV	San Juan virus
SNV	Sindbis virus
SSHV	Snowshoe hare virus
TAHV	Tahyna virus
TCMV	Tacaiuma virus
TETEV	Tete virus
TFV	Telok Forest virus
TRMV	Trombetas virus
TRV	Tanjong Rabok virus
TSUV	Tsuruse-like virus
TSWV	tomato spotted wilt virus
TUCV	Tucurui virus
TULV	Tula virus
TURV	Turlock virus
UMBV	Umbre virus
UUKV	Uukuniemi virus
VSV	vesicular stomatitis virus
WELV	Weldona virus
WONV	Wongal virus
WYOV	Wyeomyia virus

Chapter 1: General Introduction

1.1 Bunyaviruses

1.1.1 Classification

The *Bunyaviridae* family was first recognised by the International Committee on Taxonomy of Viruses (ICTV) in 1975 (Porterfield *et al.*, 1975; Fenner, 1976). It is named after the prototype virus species, Bunyamwera virus (BUNV), which was first described in 1946 (Smithburn *et al.*, 1946; Fenner, 1976). The *Bunyaviridae* family now contains over 350 named virus isolates which are classified into 5 genera (*Orthobunyavirus*, *Hantavirus, Phlebovirus*, *Nairovirus*, and *Tospovirus*) based on serological cross-reactivity, conserved terminal sequences, morphological and biochemical characteristics (Plyusnin *et al.*, 2012). Bunyaviruses are enveloped viruses 80 nm - 120 nm in diameter with tripartite single-stranded negative, or ambisense, RNA genomes, with virus replication occurring exclusively in the cell cytoplasm (Holmes, 1971; Porterfield *et al.*, 1975; Plyusnin *et al.*, 2012). Bunyaviruses have a global distribution and, with the exception of hantaviruses, are arthropod-borne viruses. A number of bunyaviruses are of clinical and economic significance causing disease in humans, livestock and commercial crops (Table 1-1). It is important to note that the classification of bunyaviruses is currently under review as genome sequencing continues to identify novel virus species with little homology to currently recognised species or genera (Figure 1-1, Table 1-2) (Galkina *et al.*, 2005; Lanciotti *et al.*, 2013; Marklewitz *et al.*, 2013; Li *et al.*, 2015a).

1.1.2 Bunyavirus Genera

1.1.2.1 Orthobunyavirus

The *Orthobunyavirus* genus is the largest genus within the *Bunyaviridae* family, with 48 ICTV-recognised virus species and over 170 named virus isolates that are classified into 18 serogroups (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012). Orthobunyaviruses have a global distribution and are transmitted by a wide range of arthropods including ticks, midges, mosquitoes, biting flies and bedbugs.

Table 1-1: Examples of Clinically and Economically Significant Bunyaviruses

Virus	Geographic location	Primary Vector	Host: Disease
Orthobunyavirus			
La Crosse virus	North America	Mosquito	Human: fever, headache, encephalitis
Oropouche virus	South America	Midge	Human: fever, myalgia, arthralgia, encephalitis
Schmallenberg virus	Europe	Midge	Ruminant: abortion, foetal malformation, decreased milk production
Tahyna virus	Asia, Europe	Mosquito	Human: fever, pneumonia, encephalitis
Hantavirus			
Hantaan virus	Asia, Europe	Field mouse	Human: HF with renal syndrome
Puumala virus	Europe	Bank vole	Human: nephropathia epidemica
Seoul virus	Americas, Asia, Europe	Brown rat	Human: HF with renal syndrome
Sin Nombre virus	North America	Deer mouse	Human: hantavirus pulmonary syndrome
Phlebovirus			
Rift Valley fever virus	Africa, Arabia	Mosquito	Human: encephalitis, HF, retinitis Ruminant: abortion, haemorrhage
SFTSV	Asia	Tick	Human: fever, thrombocytopenia, leukocytopenia
Toscana virus	Europe	Sandfly	Human: conjunctivitis, fever, myalgia
Nairovirus			
CCHFV	Africa, Asia, Europe	Tick	Human: HF
NSDV	Africa		Sheep and goats: Haemorrhagic gastroenteritis, abortion
Tospovirus			
Tomato-spotted wilt virus	Global	Thrips	Plants: necrotic spotting, wilting, death

This table is by no means exhaustive and only serves to demonstrate the wide geographical distribution and possible severity of bunyavirus infection. Geographical location and vector information as listed in The International Catalog of Arboviruses (ArboCAT) (Berge, 1975). SFTSV, Severe fever with thrombocytopenia syndrome virus; CCHFV, Crimean Congo Haemorrhagic fever virus; NSDV, Nairobi sheep disease virus; HF, haemorrhagic fever.

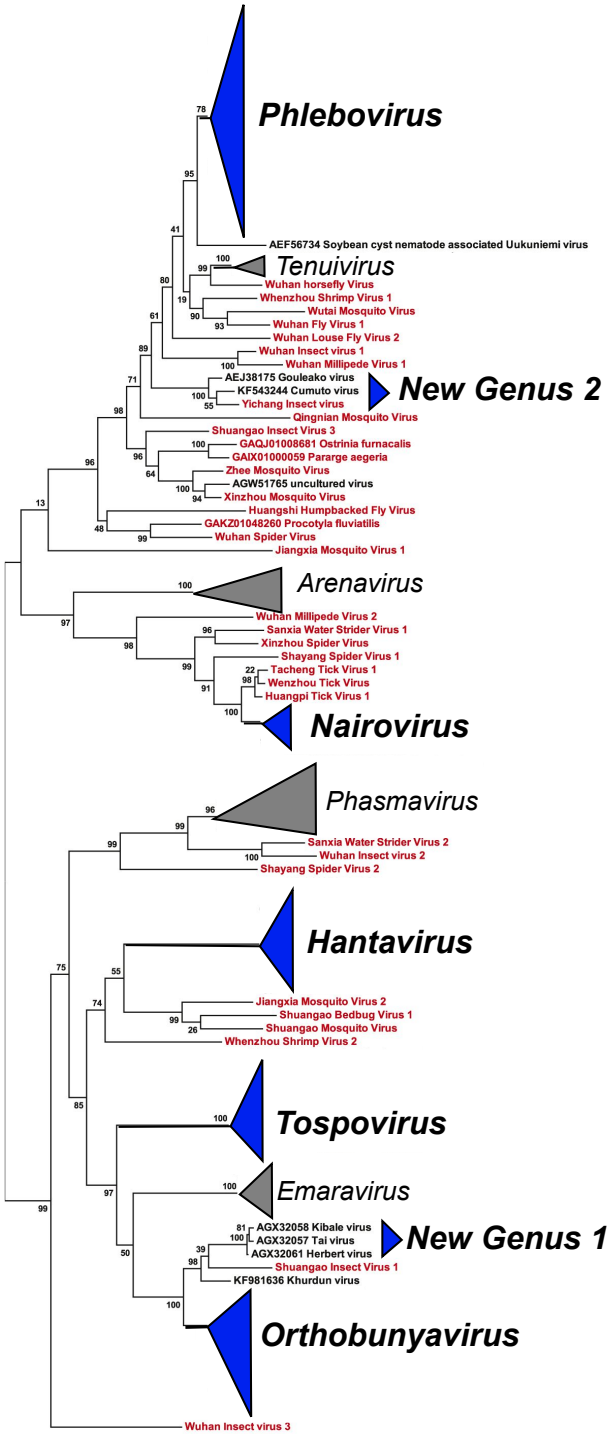


Figure 1-1: Phylogenetic Relationships of Bunya-like Viruses

Figure adapted from Li *et al.,* 2015a showing phylogeny of bunya-like viruses by RNA-dependent RNA polymerase alignments (412 aa) with statistical support. Bunyavirus genera are compressed and coloured in blue, with genus names in bold. Non-bunyavirus genera are compressed and coloured in grey. Novel viruses sequenced by Li *et al.* (2015a) that do not fall within current recognised genera are shown in red.

Table 1-2: Unclassified Bunyaviruses

Virus	Geographic location	Primary Vector	Human Disease	Virus Identification
New Genus 1				
Herbert virus	Africa	Mosquitoes	N, insect only	Marklewitz *et al.,* (2013)
Kibale virus	Africa	Mosquitoes	N, insect only	Marklewitz *et al.,* (2013)
Taï virus	Africa	Mosquitoes	N, insect only	Marklewitz *et al.,* (2013)
New Genus 2				
Cumuto virus	Americas	Mosquitoes	NR	Auguste *et al.,* (2014)
Gouléako virus	Africa	Mosquitoes	N, insect only	Marklewitz *et al.,* (2011)
KF298274 virus	Europe	Mosquitoes	NR	Cook *et al.,* (2013)
Unclassified Group 1				
Antequera virus	South America	Mosquitoes	NS	Calisher *et al.,* (1985)
Barranqueras virus	South America	Mosquitoes	NS	Calisher *et al.,* (1985)
Resistencia virus	South America	Mosquitoes	NS	Calisher *et al.,* (1985)
Unclassified Group 2				
Okola virus	Africa	Mosquitoes		Brottes *et al*., (1966)
Tanga virus	Africa	Mosquitoes	Nt Ab	Woodall and Williams (1967)
Unclassified No Group				
Bangui virus	Africa	Unknown*	Y, fever/rash	Digoutte *et al.,* (1973)
Belem virus	South America	Unknown†	Nt Ab	Berge (1975)
Belmont virus	Australia	Mosquitoes	NR	McPhee and Westaway (1981)
Bobaya virus	Africa	Unknown†	NR	Mekki *et al.,* (1979)
Caddo Canyon virus	North America	Ticks	NR	ICTV unclassified list
Hissar virus¥	Asia	Ticks	NR	Gordeeva *et al.,* (1990)
Pacora virus	North America	Mosquitoes	NR	Berge (1975)
Para virus	South America	Mosquitoes	NR	Berge (1975)
Santarem virus	South America	Mosquitoes	NR	Berge (1975)
Sunday Canyon virus	North America	Ticks	NR	Yunker *et al.,* (1977)
Tataguine virus	Africa	Mosquitoes	Y, febrile illness	Salaun *et al.,* (1968)
Wanowrie virus	Asia	Ticks	NR	Dandawate *et al.,* (1970)
Witwatersrand virus	Africa	Mosquitoes	Nt Ab	Monath *et al.,* (1972)
Yacaaba virus	Australia	Mosquitoes	NR	Marshall *et al.,* (1980)

List of unclassified bunyaviruses and possible new genus classifications (Personal communication, Prof Richard M. Elliott). Unless stated vector, geographic location and human disease are as listed in The International Catalog of Arboviruses (ArboCAT) (Berge, 1975). * isolated from man only, † isolated from birds only. ¥ also referred to as Gissar virus. Human disease is classified as (NR) not reported, if limited samples have been tested, (NS) not studied, if no human samples tested, (Nt Ab) if neutralising antibodies have been detected, and (Y) yes, if samples positive for virus isolation and clinical symptoms reported.

Orthobunyaviruses with clinical and economic significance include La Crosse virus (LACV), Oropouche virus (OROV), Schmallenberg virus (SBV), and Akabane virus (AKAV) (Table 1-1). LACV is the second most common, domestically-acquired arbovirus in the United States of America (USA), and a leading cause of viral encephalitis in children < 16 years of age (McJunkin *et al.*, 2001; Lindsey *et al.*, 2014). OROV is widespread in South America, with > 30 documented outbreaks in Brazil, Peru, Panama and Trinidad and Tobago in 1960 – 2009, during which over 500 000 people are estimated to have been infected (Pinheiro *et al.*, 1981; Mourãão *et al.*, 2009). OROV causes a self-limiting febrile illness frequently associated with headache, myalgia, arthralgia and rash and it is increasingly recognised as a major public health issue (Azevedo *et al.*, 2007; Mourãão *et al.*, 2009). In contrast to OROV and LACV, which cause clinical disease in man, SBV and AKAV are associated with foetal malformation, still birth, and abortion in ruminants (Coverdale *et al.,* 1978; van den Brom *et al.*, 2012). The prototype species of the orthobunyavirus genus, and *Bunyaviridae* family, BUNV, is a cause of disease in both ruminants, equines and humans (Kokernot *et al.*, 1958; Spence and Downs, 1968; Edwards *et al.*, 1989; Tauro *et al.*, 2012; Tauro *et al.*, 2015).

1.1.2.2 Hantavirus

There are currently 23 ICTV-recognised hantavirus species and a further 30 named virus isolates that are probable, but unclassified, members of the genus (Plyusnin *et al.*, 2012). Hantaviruses are unique amongst the *Bunyaviridae* family as their life cycle does not contain an arthropod vector, instead they are maintained in nature through rodent, bat and insectivore species (Jonsson *et al.*, 2010; Gu *et al.*, 2014; Sabino-Santos *et al.*, 2015). In rodents hantaviruses cause persistent asymptomatic infections, during which they are shed into the environment in rodent excreta. As the excreta dry they can become aerosolised, spreading to new hosts via inhalation, or occasionally via a bite. In humans hantaviruses cause acute infections, with human to human transmission only documented for Andes virus (ANDV) during outbreaks in Argentina and Chilli (Wells *et al.*, 1997; Padula *et al.*, 1998; Martinez-Valdebenito *et al.*, 2014). Human hantavirus infections are associated with 2 classical clinical presentations: haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Avšič-

Županc *et al.*, 2013). HFRS is traditionally associated with Old World hantaviruses such as Hantaan virus (HNTV), Dobrava-Belgrade virus, Puumala virus and Seoul virus (SEOV) (Chumakov *et al.*, 1981; Tkachenko *et al.*, 1982; Lee *et al.*, 1982a; Lee *et al.,* 1982b; Avšič-Županc *et al.*, 2013). HPS, which normally presents as a non-specific prodromal febrile illness that rapidly progresses to acute pulmonary oedema with cardiogenic shock, is associated with the New World hantaviruses which include Andes virus (ANDV), Black Creek Canal virus (BCCV), Bayou virus and Sin Nombre virus (SNV) (Nichol *et al.*, 1993; Duchin *et al.*, 1994; Zaki *et al.*, 1995; MacNeil *et al.,* 2011; Núñez *et al.*, 2014).

1.1.2.3 Phlebovirus

The *Phlebovirus* genus currently contains 9 ICTV-recognised virus species, or antigenic complexes, and a further 35 tentative virus species pending classification (Bouloy, 2011; Plyusnin *et al.*, 2012). The genus is divided into 2 main subgroups, the sandfly fever group contains viruses transmitted by mosquitoes, phlebotomines and ceratopogonids, whilst the Uukuniemi group contains viruses transmitted by ticks (Bouloy, 2011; Plyusnin *et al.*, 2012). The type species of the genus, Rift Valley fever virus (RVFV), a member of the sandfly fever group, causes severe disease in livestock and humans. Major epidemics usually follow periods of excessive rain, as flooding creates fresh water reservoirs and mosquito populations boom (Andriamandimby *et al.*, 2010; Bouloy, 2011). In ruminants RVFV causes fever, hepatitis and/or abortion, regardless of the stage of pregnancy, with 'abortion storms' a hallmark of RVFV outbreaks. In humans RVFV infections are frequently asymptomatic or restricted to a self-limiting febrile illness; however, severe and potentially fatal disease can occur with encephalitis, retinitis, and hepatitis associated with haemorrhagic fever (Imam *et al.,* 1979; McIntosh *et al.*, 1980; Alrajhi *et al.*, 2004; Bouloy, 2011). Other viruses in the sandfly fever group, such as Toscana virus (TOSV), are also associated with human infection, although disease is limited to a self-limiting febrile illness with occasional meningeal involvement, with no recorded fatalities (Charrel *et al.*, 2005; Nougairede *et al.*, 2013). Tick-borne phleboviruses were, until recently, associated only with sub-clinical infections. However, in 2007 a novel tick-borne phlebovirus, severe fever with thrombocytopenia syndrome virus (SFTSV), emerged in South East Asia (Bao *et al.,*

2011; Yu *et al.*, 2011). SFTSV infection was characterised by gastrointestinal symptoms, arthralgia, myalgia, thrombocytopaenia and haemorrhage with a case fatality rate (CFR) of 2% - 15% (Guo *et al.*, 2015a; Li, 2015b). This was quickly followed by the isolation of Heartland virus from patients in the USA suffering from fever, gastrointestinal symptoms and thrombocytopenia (Xing *et al.*, 2013; Nasci *et al.,* 2014). Recent molecular studies have also identified viruses within the unclassified Bhanja virus antigenic complex as tick-borne phleboviruses associated with human disease (Matsuno *et al.*, 2013; Palacios *et al.*, 2013).

1.1.2.4 Nairovirus

Nairoviruses are primarily transmitted by ticks, although occasional transmission by mosquitoes has been reported (Sudeep *et al.,* 2009; Frias-Staheli *et al.,* 2011). Transovarial transmission in ticks ensures maintenance in the environment, whilst nairovirus infection of vertebrate hosts plays an important amplifying role in the virus life cycle. The *Nairovirus* genus currently contains 36 named virus isolates classified into 7 virus species, or antigenic complexes (Frias-Staheli *et al.,* 2011; Plyusnin *et al.*, 2012; Lasecka and Baron, 2014). Several members of the genus cause severe disease (Table 1-1). Crimean-Congo haemorrhagic fever virus (CCHFV) is endemic in tick populations in parts of Africa, Asia and Southern and Eastern Europe (Papa *et al.*, 2009; Gergova *et al.,* 2012; Tekin *et al.*, 2012; Messina *et al.*, 2015). CCHFV causes asymptomatic infection in livestock and wild herbivores; however, in humans it can cause severe morbidity, developing into a haemorrhagic fever with < 30% CFR (Ergönül, 2006). Conversely, Nairobi sheep disease virus (NSDV), which is also referred to as Ganjam virus, is associated with a benign febrile illness in man but severe gastroenteritis and abortion in sheep and goats, with a CFR close to 90% (Morrill *et al.*, 1990; Davies, 1997; Marczinke and Nichol, 2002; Sudeep *et al.,* 2009).

1.1.2.5 Tospovirus

Tospoviruses are pathogenic plant viruses and there are currently 8 ICTV-recognised species with 13 additional virus isolates pending classification (Kormelink, 2011;

Plyusnin *et al.*, 2012). Tospoviruses infect ornamental plants and economically important crops with annual crop losses estimated at > $ 1 billion (Goldbach and Peters, 1994). The type species of the genus, Tomato spotted wilt virus (TSWV), infects > 800 plant species, with disease presentation including wilted leaves, necrotic spotting, reduced vegetative output and plant death (Kormelink, 2011). Tospoviruses are transmitted and propagated by 13 species of thrips, which have a global distribution. Thrips are infected as larvae, and the virus then undergoes a period of latency and replication prior to transmission during the adult stages of the thrips life cycle (Kormelink, 2011). The only exception to this is *Frankliniella occidentalis* (Western flower thrips), which have been shown to transmit the virus from second larval instars stage onwards (Wijkamp *et al.,* 1995). The viruses are transmitted to naïve plants during feeding, with virus particles injected into the host cell cytoplasm. Interestingly adult thrips that feed on infected plants do not propagate the virus, as the virus particles are retained in the thrips midgut (Ullman *et al.*, 1992; Nagata *et al.*, 1999).

1.1.2.6 Unclassified Bunyaviruses

There are a number of serologically distinct bunyaviruses isolated during arbovirus surveillance that have yet to be assigned to a genus or virus species (Table 1-2). Furthermore, the number of unclassified bunyaviruses is rising as high-throughput sequencing (HTS) screens of insect pools and clinical samples identify probable bunyaviruses that challenge current classification methods (Table 1-2, Figure 1-1) (Li *et al.,* 2015a). For example, Brazoran virus, isolated from mosquitoes in North America appears most similar to viruses in the orthobunyavirus genus (Lanciotti *et al.*, 2013). However, it employs a unique coding strategy, in which the NSs ORF precedes that of the N protein (Lanciotti *et al.*, 2013). An increasing number of insect-only viruses with limited homology to either the *Phlebovirus* or *Orthobunyavirus* and *Tospovirus* genera have also been discovered and it is proposed that they may form 2 new genera within the *Bunyaviridae* family (Marklewitz *et al.*, 2011; Marklewitz *et al.,* 2013). Many of the serologically characterised viruses lack sequencing information, and similarly, many of the new viruses identified by sequencing lack serological data. Sequencing

serologically classified viruses will aid virus classification and help unravel the complex relationships and evolutionary history of bunya-like viruses.

1.1.3 Virion Structure

Bunyaviruses have pleomorphic or spherical enveloped virus particles 80 nm - 120 nm in diameter when observed by cryo-electron microscopy (Holmes, 1971; Murphy *et al.,* 1973; Porterfield *et al.*, 1975; Plyusnin *et al.*, 2012). Glycoprotein projections of 5 nm to 10 nm, can be seen protruding from the host derived lipid bilayer, which is 5 - 7 nm thick (Figure 1-2) (Holmes, 1971; Talmon *et al.*, 1987; Huiskonen *et al.*, 2009; Battisti *et al.*, 2010; Bowden *et al.*, 2013). The glycoproteins are present in a 1:1 molar ratio and arranged in heterodimers, the exact arrangement of which varies between the different bunyavirus genera. Hantavirus Gn/Gc heterodimers are arranged in tetramers that have 4-fold symmetry and are unevenly distributed across the virion surface (Battisti *et al.*, 2010; Huiskonen *et al.*, 2010). In contrast, phlebovirus Gn/Gc heterodimers form round, closely packed capsomeres that display T=12 icosahedral symmetry (Freiberg *et al.*, 2008; Huiskonen *et al.*, 2009; Sherman *et al.*, 2009). The glycoprotein heterodimers of orthobunyaviruses, tospoviruses and nairoviruses were thought to be disordered; however, high resolution cryo-electron microscopy of BUNV has recently identified a tripodal spike (Figure 1-2B) (Bowden *et al.*, 2013). The virus genome is enclosed within the lipid envelope. Each RNA genome segment is encapsidated by the virus nucleocapsid protein (N) to form ribonucleocapsid protein (RNP) complexes that associate with small amounts of virus L protein. Bunyavirus RNPs lack helical symmetry and appear as non-condensed, imperfect circles due to base-pairing of complementary terminal sequences present in each genome segment (Figure 1-2A).

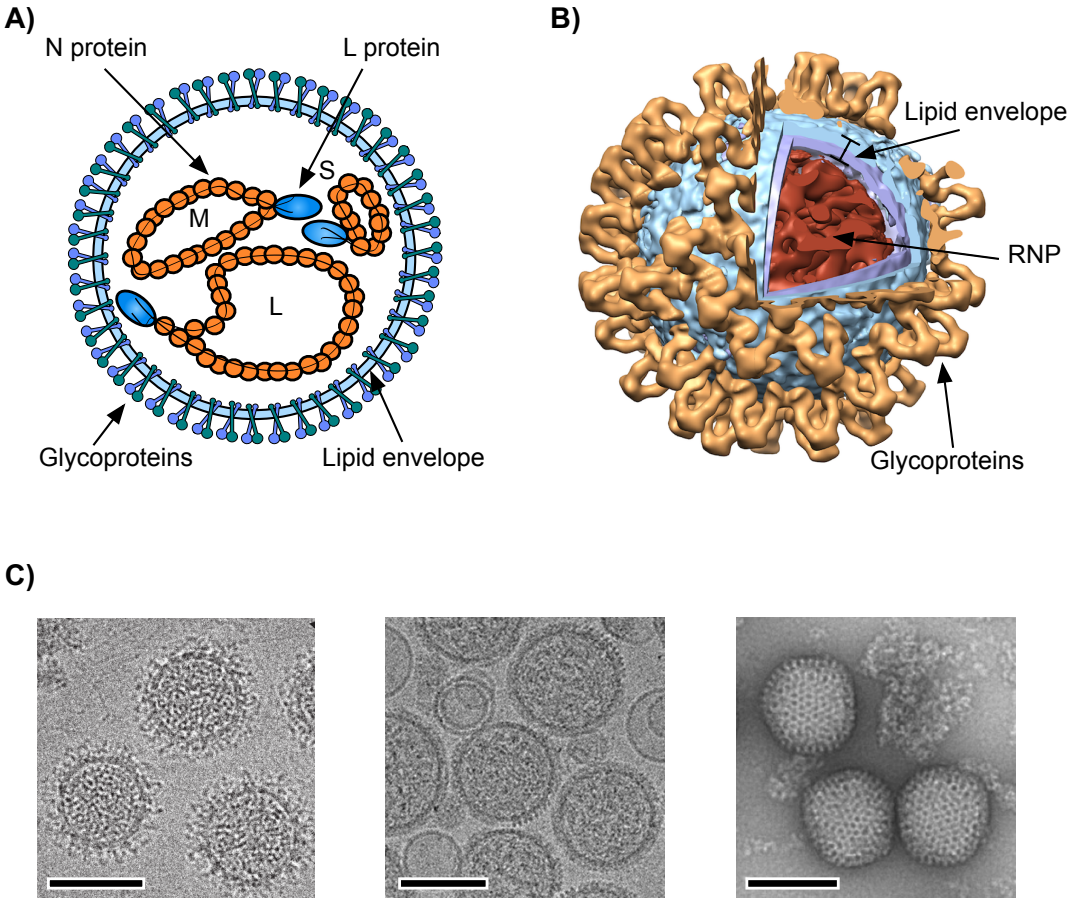


Figure 1-2: Bunyavirus Particle Structure

A) Schematic representation of a typical bunyavirus particle showing host derived lipid envelope with projections of the virus glycoproteins, Gn and Gc, which are present as heterodimers. The lipid envelope surrounds the virus genome segments, S, M and L, which are encapsidated by virus nucleocapsid protein (N), forming ribonucleocapsid protein (RNP) complexes. Each RNP complex is associated with small amounts of the virus polymerase (L) protein and forms a panhandle structure due to complementarity in terminal untranslated regions (UTRs). **B)** Computational model of a Bunyamwera virus particle created by mapping averaged glycoprotein structures onto a tomographic reconstruction. Image taken from Bowden *et al.,* 2013. **C)** Cryo-electron micrographs of Bunyamwera virions, Hantaan virions embedded in vitreous ice and Rift Valley fever virions. Scale bars represent 100 nm. Images were taken from Bowden *et al*., 2013, Battisti *et al.,* 2010 and Huiskonen *et al*., 2009, respectively.

1.1.4 Genome Organisation and Coding Strategy

Bunyaviruses have tripartite negative, or ambisense, single-stranded (ss) RNA genomes. Each genome segment comprises a coding region flanked by terminal 3' and 5' untranslated regions (UTRs) of varying lengths. The RNA genome segments have genus-specific 3' and 5' terminal sequences (Table 1-3) (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012). These sequences have inverted complementarity, allowing base pairing and the formation of the characteristic panhandle structure.

The three genome segments, termed small (S), medium (M) and large (L), encode 4 structural proteins; the S segment encodes the N protein, the M segment encodes glycoproteins Gn and Gc, and the L segment encodes the L protein, an RNA-dependent RNA polymerase (RdRp) (Table 1-3, Figure 1-3). In addition to the conserved structural proteins S and M genome segments may encode accessory proteins. These proteins lack conservation differing in their polarity, genome location and presence/absence (Figure 1-3) (Plyusnin *et al.,* 2011). The S segments of phleboviruses, tospoviruses, hantaviruses and orthobunyaviruses may encode a non-structural protein, NSs. The NSs proteins of hantaviruses and orthobunyaviruses are encoded in an overlapping reading frame that occurs within the N protein ORF. In contrast, phleboviruses and tospoviruses have an ambisense coding strategy with a negative sense ORF encoding the virus N protein and a separate positive sense ORF encoding the NSs protein (Figure 1-3). The M segments of orthobunyaviruses, sandfly fever group phleboviruses, and tospoviruses also encode a non-structural protein, NSm. Tospovirus NSm proteins are encoded in a separate ambisense ORF whilst orthobunyavirus and phlebovirus NSm proteins are encoded as part of the M polyprotein (Figure 1-3).

Table 1-3: Molecular Characteristics of the *Bunyaviridae*

Genus	Size of RNA Segments (kb)	Size of Structural Proteins (kDa)	3' and 5' consensus sequences
Orthobunyavirus	L 6.9 M 4.5 S 1.0	L 250 Gc, Gn 110, 35 N 25	3' UCAUCACAUGA... ...UCGUGUGAUGA 5'
Hantavirus	L 6.5 M 3.6 S 1.7	L 250 Gc,Gn 55, 70 N 50	3' AUCAUCAUCUG... .....AUGAUGAU 5'
Phlebovirus	L 6.4 M 3.5 S 1.7	L 250 Gc, Gn 65, 55-70 N 30	3' UGUGUUUC... ...GAAACACA 5'
Nairovirus	L 12.2 M 4.9 S 1.7	L 450 Gc, Gn 75, 35 N 50	3' AGAGUUUCU... ...AGAAACUCU 5'
Tospovirus	L 8.9 M 4.8 S 2.9	L 330 Gc, Gn 75, 45 N 30	3' UCUCGUUAAG... ....CUAACGAGA 5'

Molecular characteristics of each bunyavirus genus are given. RNA sizes of genome segments small (S), medium (M) and large (L) rounded to the nearest 100 bases highlight genus specific features such as the large nairovirus L segment which encodes an L protein that is over 100 kDa larger than that of viruses in other genera. Genus-specific terminal consensus sequences are also shown. Table adapted from Elliott and Blakqori, 2011.

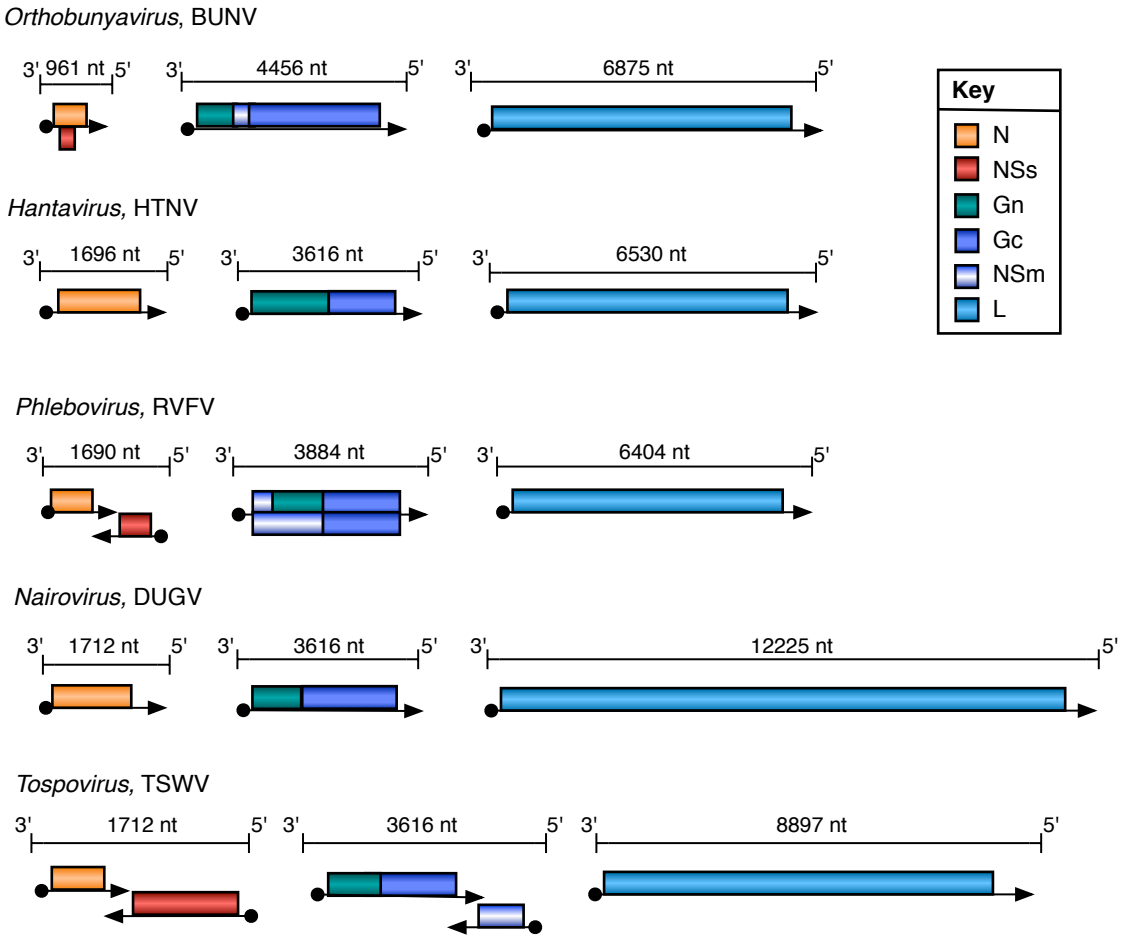


Figure 1-3: Coding Strategy of Type Species

Schematic representation of the virus genomes of Bunyamwera virus (BUNV), Hantaan virus (HTNV), Rift Valley fever virus (RVFV), Dugbe virus (DGUV) and tomato spotted wilt virus (TSWV), the type species of each bunyavirus genus. Viral genome RNA (vRNA) is depicted by capped lines, with size in nucleotides (nt) (S segments scaled at 1.5). Arrowed lines represent 5' capped mRNA with the arrow indicating the direction of transcription. Coloured boxes represent virus proteins: nucleocapsid protein (N), non-structural proteins (NSs, NSm), glycoproteins (Gn, Gc) and viral polymerase (L). Figure adapted from Plyusnin *et al*., 2011.

1.1.5 Bunyavirus Gene Products

1.1.5.1 Nucleocapsid Protein

The N protein is the most abundant viral protein during virus replication, reflecting the multifactorial role of this protein in the virus life cycle (Elliott and Blakqori, 2011; Plyusnin *et al.,* 2011). Bunyavirus N proteins range from 25 kDa – 50 kDa in size and although there is little sequence homology the proteins share common functions, the most well-studied of which is the encapsidation of nascent vRNA and cRNA to form RNPs. Bunyavirus RNPs are the template for genome replication and transcription by the virus L protein (Kolakofsky and Hacker, 1991). Cryo-electron microscopy shows RNPs as imperfect string-like circles that are wider than monomeric N protein, suggesting that the base unit for RNA encapsidation is N multimers (Figure 1-4). This has recently been confirmed by x-ray crystallography, with orthobunyavirus N proteins forming tetramers in the presence of RNA that are of corresponding diameter to native RNPs (Figure 1-4) (Ariza *et al.,* 2013; Reguera *et al.*, 2013; Niu *et al.*, 2013; Dong *et al.*, 2013b). In contrast, hantavirus and phlebovirus N proteins form stable trimers and hexamers, although due to inconsistencies with native RNP diameter the repeating unit of these RNPs is still debated (Mir and Panganiban, 2004; Raymond *et al.*, 2010; Raymond *et al.*, 2012; Guo *et al.*, 2015b). Encapsidation of viral RNA by the N protein has been mapped to 5’ sequences in both the vRNA and cRNA, and 5’ vRNA sequences have been shown to be both sufficient and necessary to induce N protein binding (Osborne and Elliott, 2000; Severson *et al.,* 2001; Ogg and Patterson, 2007).

X-ray crystallography coupled with targeted mutagenesis has allowed detailed mapping of N protein functions, with distinct sites identified for N-N interactions, vRNA encapsidation and mRNA cap binding (Eifan and Elliott, 2009; Mir *et al.*, 2010; Walter *et al.*, 2011; Reguera *et al.*, 2013). For example, hantavirus N proteins have been shown to interact with ribosomal protein S19, which forms part of the 40S subunit, functionally replacing the cellular cap binding complex eIF4F and initiating translation of viral mRNAs (Cheng *et al*., 2011; Mir and Panganiban, 2008). They have also been shown to sequester 5’mRNA caps stored in P bodies, enhancing viral transcription (Mir *et al.,* 2008).

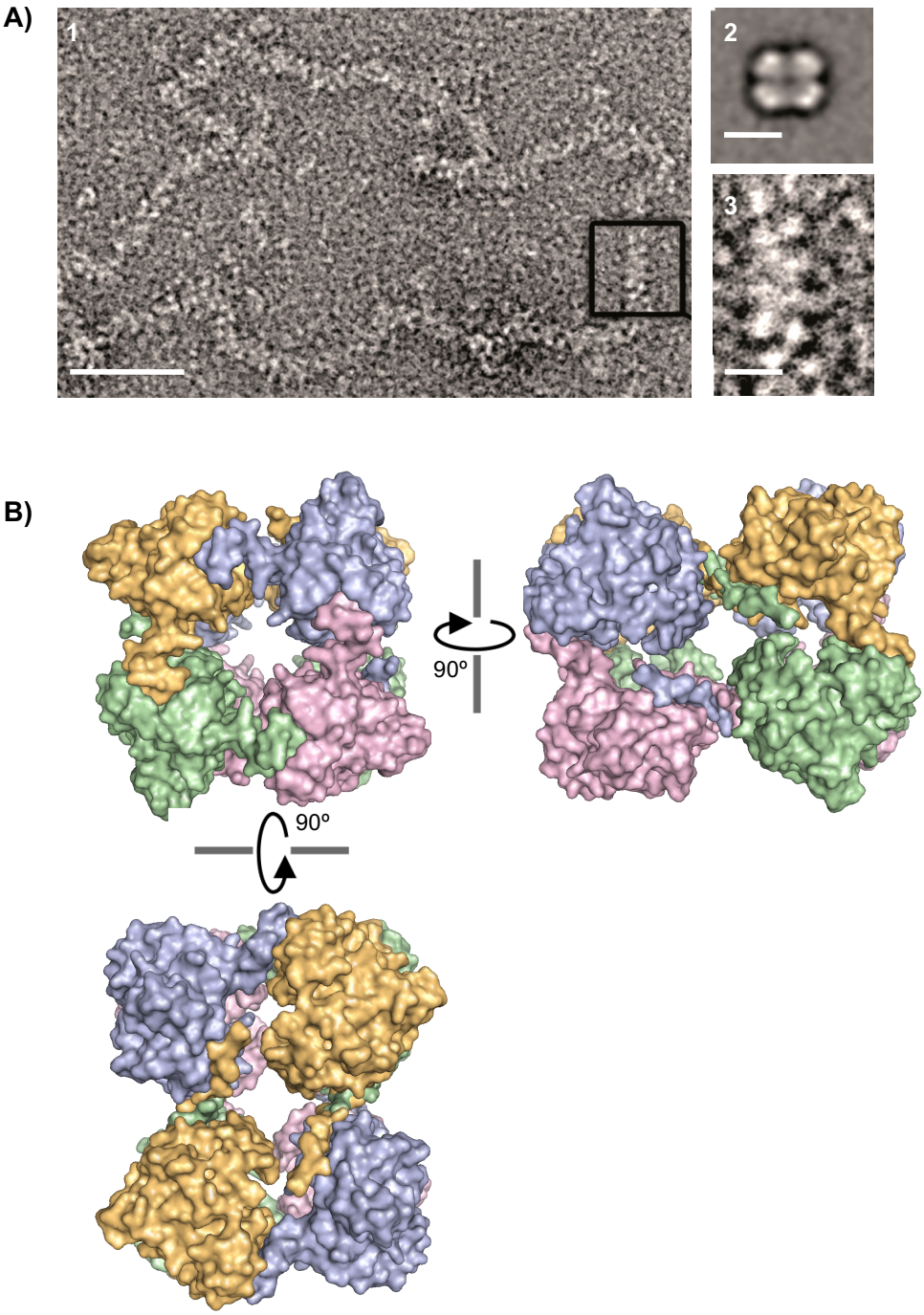


Figure 1-4: Bunyavirus RNP and N Protein Formation

A) Electron micrographs of negative-stained Bunyamwera virus (BUNV) RNPs. 1) Purified (native) RNP from BUNV virions, scale bar represents 50 nm, 2) A single tetramer bound to synthetic 48-mer RNA, scale bar represents 10 nm. 3) Close-up image of purified (native) virion RNP highlighted by a black box in image 1, scale bar represents 10 nm. Images taken from Ariza *et al*. (2013). **B)** Crystal structure of tetrameric BUNV N protein, PDB 3ZLA (Ariza *et al.*, 2013), images prepared in PyMol.

In addition to the functions described above, bunyavirus N proteins have been reported to interact with virus glycoproteins and the virus polymerase; however, the exact mechanisms and function of these interactions remain undefined.

1.1.5.2 Glycoproteins

Bunyaviruses encode 2 glycoproteins, Gn and Gc (previously known as G1 and G2), which are type I integral transmembrane proteins (Elliott and Blakqori, 2011). The glycoproteins are encoded as part of a polyprotein that is translated by ribosomes at the rough endoplasmic reticulum (RER). Full length polyprotein has never been detected in infected cells and the polyprotein is thought to undergo co-translational processing in the lumen of the ER (Fazakerley *et al.*, 1988). The precise sites of polyprotein cleavage remain unknown for many bunyaviruses although sequence analysis identifies motifs that could be recognised by host cell, signal peptide peptidases (Elliott and Blakqori, 2011; Frias-Staheli *et al.,* 2011; Spiropoulou, 2011; Bouloy, 2011). Cleavage of hantavirus M polyproteins has been shown to occur at the conserved pentapeptide WAASA motif, and nairovirus CCHFV Gn protein is cleaved at an RRLL tetrapeptide sequence in the amino terminus of Gn by SKI-1 proteases (Löber *et al.*, 2001; Sanchez, Vincent and Nichol, 2002; Vincent *et al.*, 2003).

During virus replication Gn and Gc accumulate at the Golgi and the Golgi targeting/retention signal has been mapped to the transmembrane domain of Gn, with Gc requiring Gn for Golgi localisation (Andersson *et al.*, 1997; Gerrard and Nichol, 2002; Shi *et al.,* 2004a; Snippe *et al.*, 2007). N-glycosylation and disulphide bond formation also play important roles in ensuring correct protein folding and trafficking (Shi *et al.,* 2004b; Shi *et al.,* 2005). Gn and Gc are N-glycosylated at asparagine residues and the position of cysteine residues is highly conserved within each genus, despite otherwise low sequence homology.

1.1.5.3 Polymerase

The bunyavirus L protein acts as transcriptase, replicase and endonuclease, replicating the virus genome and transcribing viral mRNAs. Transcription is initiated by a 'cap-snatching' mechanism in the host cell cytoplasm and the L protein acts as an endonuclease cleaving 5' capped oligonucleotides from host mRNAs to prime viral mRNA transcription (Bishop *et al.,* 1983; Patterson and Kolakofsky, 1984; Ihara *et al.,* 1985; Jin and Elliott, 1993; Reguera *et al.,* 2010). Endonuclease activity maps to the amino terminal PD-(D/E)xK motif, which is conserved throughout the *Bunyaviridae* family (Reguera *et al.,* 2010). Structure-based sequence alignments also indicate that similar motifs are present in the RdRp N terminus of all segmented negative sense RNA viruses, suggesting a common origin for the cap-snatching mechanism (Reguera *et al.,* 2010).

Bunyavirus L proteins also contain the conserved 'polymerase module', a group of 6 motifs (designated pre-motif A, A, B, C, D, and E) present in all segmented RNA virus polymerases (Poch *et al.*, 1989; Müller *et al.*, 1994; Roberts *et al.*, 1995). The critical role of the polymerase module in BUNV replication was confirmed by mutagenic studies, and the SDD motif present in domain C, which catalyses nucleotide polymerisation, shown to be essential for polymerase activity (Jin and Elliott, 1992). A seventh conserved region downstream of domain E has also been detected in bunyaviruses, the function of which has yet to be elucidated (Aquino *et al.,* 2003).

Nairoviruses encode larger L proteins than other bunyaviruses and, in addition to the conserved motifs already discussed, they contain an ovarian tumour (OTU) domain in their amino-terminal region (Honig *et al.,* 2004; Kinsella *et al.*, 2004). The OTU domain is part of a superfamily of ubiquitin-deconjugating cysteine proteases found in prokaryotes, eukaryotes, and viruses (Makarova *et al.,* 2000). In nairoviruses the L protein OTU domain has been shown to have protease activity *in vitro* and it is hypothesised that it may play an anti-viral role, targeting the ubiquitin pathway to deactivate host proteins (Frias-Staheli *et al.*, 2007; van Kasteren *et al.*, 2012).

1.1.5.4 S Segment Non-structural Protein

In addition to encoding the N protein, the S segment of phleboviruses, tospoviruses, and some members of the hantavirus and orthobunyavirus genera encode a non-structural protein NSs. Two coding mechanisms are utilised; hantavirus and orthobunyavirus NSs proteins are encoded in overlapping reading frames downstream of N with transcription initiated by a leaky-scanning mechanism, whilst phlebovirus and tospovirus NSs proteins are encoded in a separate ambisense ORF (Figure 1-2). Cellular localisation of NSs proteins varies, RVFV NSs protein accumulates and forms filaments inside the nucleus whilst hantavirus NSs proteins display granular cytoplasmic localisation (ANDV) and perinuclear localisation (Tula virus) (Struthers and Swanepoel, 1982; Yadani *et al.*, 1999; Virtanen *et al.*, 2009; Vera-Otarola *et al.*, 2012). Orthobunyavirus NSs proteins are predominantly found in the cell cytoplasm but intranuclear inclusions have been detected in cells transfected with FLAG-tagged BUNV NSs expression plasmids (Weber *et al.*, 2001; Thomas, 2004).

Despite differing coding strategies, cellular localisation, and a lack of sequence homology, the primary role of all NSs proteins is thought to be that of a non-essential virulence factor. Tospovirus NSs proteins have been shown to suppress antiviral RNA silencing mechanisms in plants, with TSWV NSs protein inhibiting Dicer-mediated cleavage of long dsRNA *in vitro* (Takeda *et al.*, 2002; Bucher *et al.*, 2003; Schnettler *et al.*, 2010). Phlebovirus RVFV clone 13, which has a large in-frame deletion in the NSs gene, replicates well in mosquitoes and Vero E6 cells, although unlike wild type (wt) virus it is a strong inducer of type I IFN (Bouloy *et al.,* 2001). Similarly, recombinant orthobunyaviruses lacking an NSs protein show growth attenuation in IFN-competent cell lines and induce high levels of IFN compared to wt NSs encoding viruses. There is, as yet, no reverse genetics system for hantaviruses thus limiting studies into NSs function. However, hantaviruses encoding an NSs protein survive for more passages in IFN-competent cell lines than naturally occurring NSs deficient viruses (Jääskeläinen *et al.*, 2008). Transient expression of hantavirus NSs proteins has also been shown to antagonise IFN-β promoter activity and IFN synthesis *in vitro*, albeit to a lesser degree than those of BUNV or RVFV (Jääskeläinen *et al.*, 2007). The role of NSs as a virulence factor and the mechanisms of NSs interactions with the type I IFN system are discussed in greater detail in section 1.1.7.

1.1.5.4 M segment Non-structural Proteins

The M segment encodes the virus glycoproteins Gn and Gc and, in phleboviruses, tospoviruses and orthobunyaviruses, the non-structural protein NSm (Figure 1-3). M segment non-structural proteins have key roles in protein maturation, virion assembly and release. The orthobunyavirus NSm protein is encoded between Gn and Gc as part of the M polyprotein and is thought to be cleaved by a furin-like enzyme due to the presence of a conserved RxxR motif in the C terminus of Gn (Briese *et al*., 2004; Fazakerley *et al*., 1988; Gerbaud *et al.*, 1992). In contrast, sandfly fever group phleboviruses encode NSm pre-Gn and in RVFV alternate use of inframe AUG codons determines synthesis of either NSm1-Gc polyprotein, or NSm2-Gn-Gc polyprotein, which encode 78 kDa and 14 kDa NSm proteins respectively (Bouloy, 2011; Suzich *et al.,* 1990). Phlebovirus and orthobunyavirus NSm proteins are thought to play an important role in virus assembly, acting as scaffolding proteins and localising to the Golgi independently of Gn and Gc (Fontana *et al*., 2008; Lappin *et al,*. 1994; Nakitare and Elliott, 1993; Shi *et al*., 2006). Tospovirus NSm proteins are encoded in an ambisense ORF and play a key role in cell-to-cell spread of virus RNPs in plant hosts, interacting directly with virus N protein (Soellick *et al.*, 2000; Leastro *et al.*, 2015).

The M segments of nairoviruses encode 2 additional proteins, glycoprotein GP38 and a mucin-rich protein, the functions of which remain undefined. The additional proteins are located at the N terminal region, pre-Gn (Sanchez *et al.,* 2002).

1.1.6 Replication in Mammalian Cells

The bunyavirus replication cycle comprises 4 main stages: virus attachment and cell entry; primary transcription and translation; genome replication; and virion assembly, maturation and release (Figure 1-5).

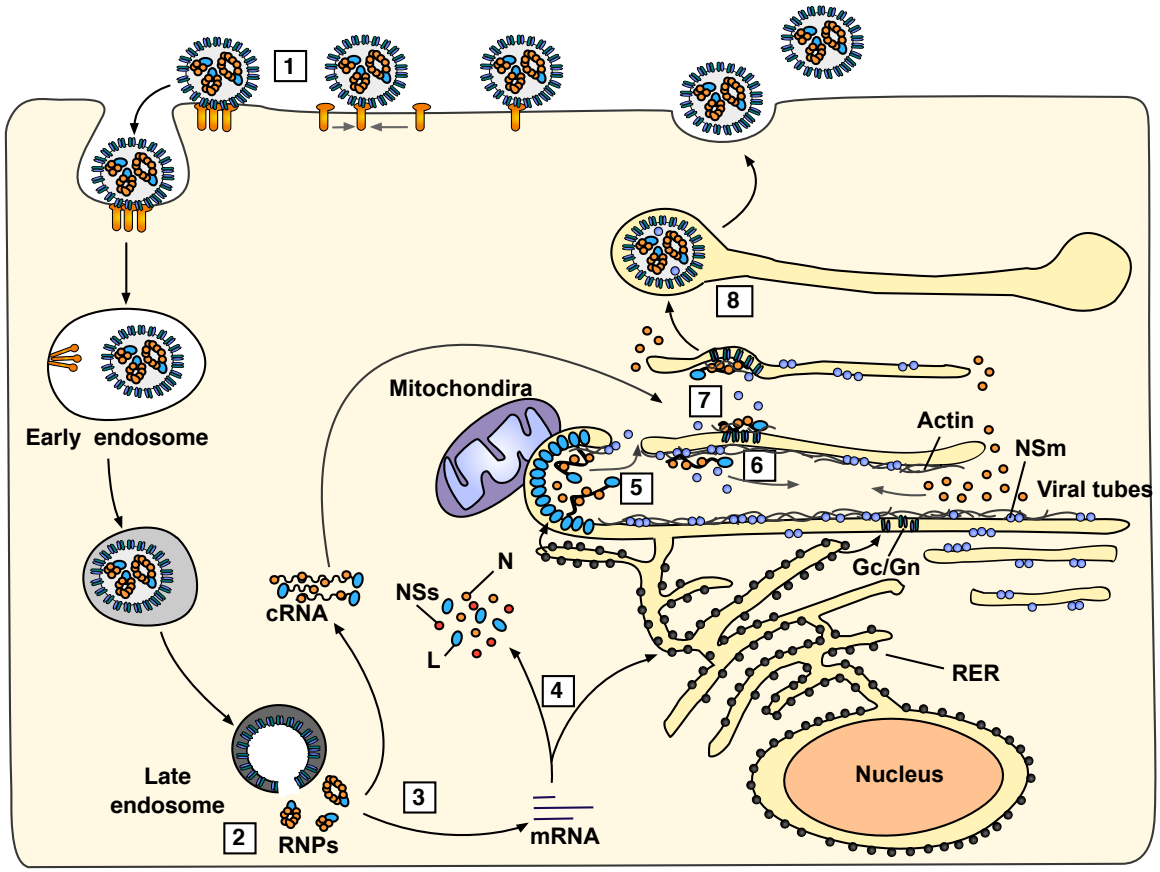


Figure 1-5: Bunyavirus Replication Cycle

Schematic representation of bunyavirus replication in mammalian cells. **1)** Infectious virus particles attach to cell surface receptors inducing receptor clustering and receptor-mediated endocytosis. **2)** Virus particles dissociate from host cell receptors and following late endosomal acidification membrane fusion occurs releasing RNPs into the cytoplasm. **3)** Virus replication begins with virion-associated L protein synthesising viral mRNAs and cRNAs. **4)** S and L segment mRNAs are translated at free ribosomes into N, NSs (when encoded) and L proteins whilst the M segment mRNA is translated into viral glycoproteins and NSm (when encoded) by ribosomes at the rough endoplasmic reticulum (RER). **5)** As viral replication continues viral tubes linked to the RER and mitochondria form in association with Golgi stacks. The L protein concentrates in the globular domain of viral tubes, synthesising viral mRNA transcripts and full length cRNAs and vRNA which associate with trimeric N. **6)** N is present in abundance inside viral tubes and around the Golgi, facilitating RNP movement through the cytoplasm with assistance from actomyosin based motors. **7)** RNPs associate with the cytoplasmic domain of Gn/Gc heterodimers which are concentrated in Golgi membranes due to the presence of the Golgi retention signal in Gn. **8)** Once associated with Gc/Gn, RNP packaging occurs and new virus particles form and mature through budding. Figure adapted from Fontana *et al.*, 2008 and Walter and Barr, 2011.

1.1.6.1 Cell Attachment and Cell Entry

The key mediators of cell attachment are viral surface glycoproteins, Gn and Gc, which interact with host cell surface receptors (Shi *et al*., 2010). It has been proposed that hantavirus cell entry is mediated through interactions with integrins, and more specifically that pathogenic hantaviruses utilise β3 integrins (CD61), whilst non-pathogenic hantaviruses use β1 integrin (Gavrilovskaya *et al.*, 1998; Gavrilovskaya *et al.*, 2002; Mou *et al.*, 2006). However, pathogenic hantavirus ANDV exhibits robust entry in β3 integrin deficient cells and CD55 and gC1qR/p32 have also been implicated in HTNV cell entry (Choi *et al.*, 2008; Krautkrämer and Zeier, 2008; Ray *et al.*, 2010). Phlebovirus cell entry may be mediated through DC-SIGN, a C-type lectin shown to interact with high mannose N-glycans on phlebovirus glycoproteins Gn and Gc (Lozach *et al.*, 2011; Hofmann *et al.*, 2013). High mannose N-glycans are also present in nairovirus, hantavirus and orthobunyavirus glycoproteins and it is postulated that DC-SIGN mediated entry may occur (Schmaljohn *et al.*, 1986; Sanchez *et al.*, 2002; Shi *et al.,* 2005). However, bunyaviruses infect a range of tissues that exceeds the cellular distribution of DC-SIGN, and additional receptors must therefore exist (Lozach *et al.*, 2011). In addition to varying receptor specificity, the polarity of cell attachment varies across the *Bunyaviridae*, for example, in polarised epithelial cells CCHFV shows basolateral entry, BCCV apical entry, whilst RVFV and ANDV exhibit bidirectional entry (Ravkov *et al.,* 1997; Gerrard *et al.,* 2002; Rowe and Pekosz, 2006; Connolly-Andersen *et al.,* 2007).

Following attachment, bunyaviruses undergo receptor-mediated endocytosis in a clathrin-dependent, caveolin-independent process (Jin *et al.*, 2002; Santos *et al.*, 2008; Simon *et al.,* 2009; Lozach *et al.*, 2011; Hollidge *et al.*, 2012). However, some bunyaviruses, e.g. ANDV, utilise an alternative and undefined mechanism as both clathrin and caveolin inhibitors chlorpromazine and filipin fail to prevent virus entry (Ramanathan and Jonsson, 2008). Following internalisation, virus containing vesicles undergo acidification, inducing membrane fusion and releasing viral RNPs into the cytoplasm (Figure 1-5). Studies with LACV predict that a 22 amino acid (aa) hydrophobic domain in Gc (residues 1066-1087) acts as a class II fusion peptide undergoing pH-dependent conformational change associated with membrane fusion

(Plassmeyer *et al.*, 2005; Plassmeyer *et al.*, 2007). This region is conserved among other members of the *Bunyaviridae* and mutational analysis supports this hypothesis (Plassmeyer *et al.*, 2007; Shi *et al.*, 2009). However, studies with BUNV have also shown that the cytoplasmic tail of Gn also plays a pivotal role in membrane fusion (Shi *et al.*, 2007).

1.1.6.2 Primary Transcription and Translation

Following cell entry, RNP complexes are released into the host cell cytoplasm, shielding the negative sense RNA genome from exposure to the host cell and initiating viral replication (Figure 1-5).

Virion associated L protein initiates transcription of functional viral mRNAs via a 'cap-snatching' mechanism whereby L exhibits endonuclease activity cleaving mature host cellular mRNAs present in the cytoplasm to generate short primers of 5' capped mRNA (Figure 1-6) (Bishop *et al.,* 1983; Patterson and Kolakofsky, 1984; Ihara *et al.,* 1985; Jin and Elliott, 1993; Kukkonen *et al.,* 2004; Reguera *et al.,* 2010). Fully transcribed viral mRNAs are shorter than their genome counterparts and termination signals with conserved pentanucleotide motifs have been identified in the 5' UTR of orthobunyavirus S and L segments and phlebovirus S and M segments (Barr *et al.,* 2006; Ikegami *et al.*, 2007; Albariño *et al.,* 2007). However, termination signals have yet to be characterised in the orthobunyavirus M segment, phlebovirus L segment or the hantavirus genera.

Translation of viral mRNAs occurs concurrently with transcription and is required to prevent premature termination of transcription (Figure 1-6C) (Abraham and Pattnaik, 1983; Barr, 2007; Lara *et al.*, 2011). It is postulated that ribosome translocation blocks termination signals present in the coding region of viral mRNAs, disrupting RNA-RNA interactions and facilitating the progression of L (Barr, 2007). It is also important to note that, excluding the M segment of SNV, bunyavirus mRNAs are not polyadenylated; translation is therefore not enhanced via poly-A binding protein (PABP) and knockdown of PABP does not inhibit translation of viral mRNAs (Abraham and Pattnaik, 1983; Hutchinson *et al.,* 1996; Blakqori *et al.,* 2009).

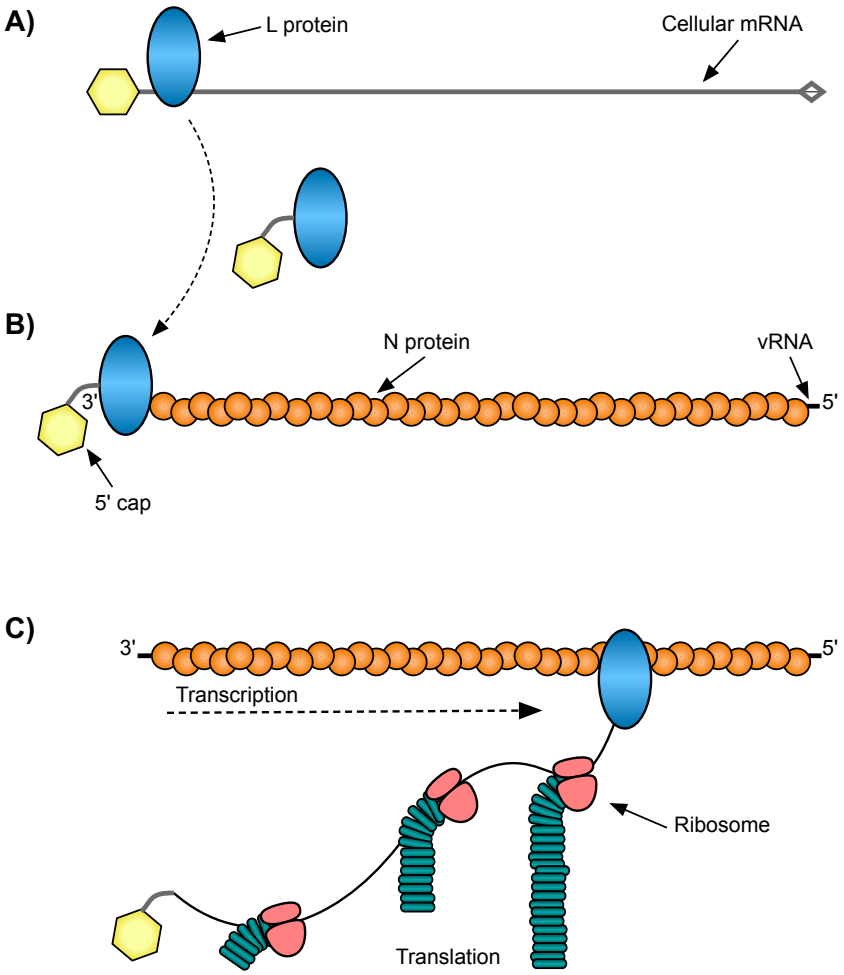


Figure 1-6: Bunyavirus Transcription and Translation
Schematic representation of bunyavirus mRNA transcription initiation and subsequent translation. **A)** The viral L protein, an RNA-dependent RNA polymerase, exhibits endonuclease activity, cleaving 5' capped oligonucleotides from host cellular mRNAs. **B)** Cleaved 5' capped oligonucleotides are used to prime transcription of viral mRNAs from the virus genome (vRNA). **C)** Translation occurs concurrently with transcription.

However, reduced levels of eukaryotic translation initiation factor (eIF) 4G has a marked effect on viral translation indicating a PABP-independent, eIF4G-dependent mechanism of translation (Blakqori *et al.,* 2009).

1.1.6.3 Genome Replication

It is unclear what triggers the switch from transcription to replication in infected cells although increasing levels of N may play a role (Elliott and Blakqori, 2011). Replication and synthesis of the cRNA (also known as the antigenome) is initiated via 5' nucleoside triphosphates, therefore unlike viral mRNAs the cRNA lacks a 5' primer extension (Kolakofsky and Hacker, 1991). The cRNA represents a full length copy of the viral genome, extending to the 5' terminus of the genomic RNA template (Figure 1-7) (Kolakofsky and Hacker, 1991). The mechanism by which the polymerase reads through mRNA transcription termination signals remains undefined; it may be that interactions with host proteins alter its function or that encapsidation of nascent antigenome prevents RNA RNA interactions, blocking mRNA termination signals (Elliott and Blakqori, 2011).

1.1.6.4 Virion Assembly, Maturation and Release

Bunyavirus maturation typically occurs at smooth membranes in the Golgi, the site of virus glycoprotein retention and accumulation, although maturation at the plasma membrane has been observed with New World hantaviruses and RVFV (Holmes, 1971; Zeller *et al.*, 1989; Novoa *et al.*, 2005; Fontana *et al.*, 2008; Shi *et al.*, 2010; Plyusnin *et al.*, 2012). Glycoproteins Gc and Gn are cysteine rich and following cotranslational processing form a heterodimer in the ER prior to localisation at the Golgi due to a targeting sequence in Gn (Matsuoka *et al.,* 1994; Matsuoka *et al.*, 1996; Andersson *et al.*, 1997; Gerrard and Nichol, 2002; Shi *et al.,* 2004a; Snippe *et al.*, 2007; Huiskonen *et al.*, 2009).

It is at the Golgi that glycoproteins associate with RNPs. Scanning mutagenesis of the phlebovirus Uukuniemi virus (UUKV) identified aa 76 - 81 in the Gn cytoplasmic tail

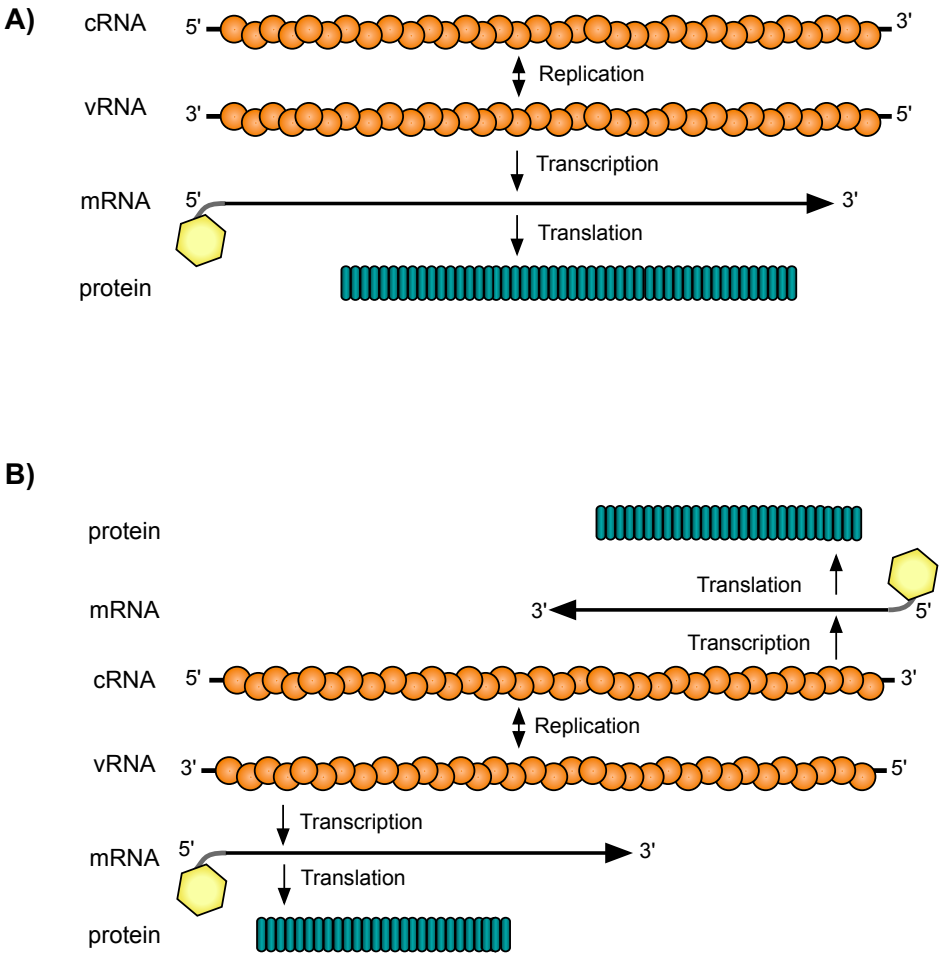


Figure 1-7: Translation and Replication

Schematic representation of bunyavirus replication, transcription and translation. **A)** 5' capped mRNA is transcribed from the viral genome (vRNA), whereas the virus genome replicates via full length complementary copies (cRNA), which is also referred to as the antigenome. **B)** Transcription and translation in viruses with an ambisense coding strategy. Transcription of the ambisense ORF occurs from the cRNA and not vRNA.

(CT) as essential for RNP binding and packaging (Overby *et al.*, 2007). Furthermore, studies with RVFV indicate that Gn is sufficient for RNP packaging and hantavirus Gn CT has been shown to directly interact with N, supporting the hypothesis that the Gn CT of glycoprotein multimers interacts with N in RNP packaging (Wang *et al.*, 2010; Piper *et al.,* 2011). RNPs form throughout the replication cycle, and encapsidation is thought to be concomitant with RNA synthesis (Figure 1-5). Within RNPs the signal for encapsidation maps to the 5' termini of vRNAs and cRNAs, with this region both necessary and sufficient for encapsidation of viral and non-viral RNAs (Osborne and Elliott, 2000; Severson *et al.,* 2001; Ogg and Patterson, 2007). Furthermore, the 5'UTR of the M segment is key in ensuring the co-packaging of all 3 RNA segments, although interactions between the 3 RNAs are also involved (Elliott and Blakqori, 2011; Terasaki *et al.*, 2011).

Once RNPs are encapsidated and associated with viral glycoproteins, immature precursors assemble into dense intermediate virus particles at the Golgi stacks with glycoproteins becoming partially resistant to endo-B-*N*-acetylglucosaminidase H digestion prior to budding from the cell (Gerbaud *et al.*, 1992; Novoa *et al.*, 2005). In UUKV Gn protein aa 21 - 25 and 46 - 50 are key for the successful initiation of virus particle budding and trafficking from the ER to the Golgi, respectively. From the Golgi immature virus particles are transported within vacuoles to the cell surface via the exocytic pathway where fusion of the vacuole and plasma membrane releases the virus into the extracellular environment (Figure 1-5). The polarity of virion release varies and following release from the host cell bunyaviruses undergo a final stage of maturation and defined glycoprotein spikes become visible on the virion surface (Pekosz and González-Scarano, 1996; Gerrard *et al.,* 2002; Rowe and Pekosz, 2006; Elliott and Blakqori, 2011).

1.1.7 Bunyavirus Interaction With The Host Cell

1.1.7.1 Activation of the Innate Immune Response

The innate immune system is the first line of defence against pathogens, with pathogen recognition receptors (PRRs) recognising foreign particles through pathogen

association molecular patterns (PAMPs). The toll like receptors (TLRs) are a subset of PRRs and the expression of TLR3, an endosomal receptor which recognises dsRNA, has been shown to alter the pathogenesis of bunyavirus infection (Gowen *et al.*, 2008; Jeong and Lee, 2011) (Figure 1-8). In Hantaan virus infections TLR3 is required for activation of MxA, and expression of TLR3 is linked with an attenuation in virus titre (Handke *et al.*, 2009). TLR3 also plays a key role in the immune-modulated pathogenesis of phlebovirus Punta Toro virus (PTV) with TLR3-mediated upregulation of IL-6 and inflammatory mediators contributing to disease severity in mouse models (Gowen *et al.*, 2006). TLR3 recognition of dsRNA is independent of RNA sequence, and once bound to dsRNA TLR3 forms dimers which act as a signalling complex, triggering activation of the type I IFN response (Alexopoulou *et al.*, 2001; Doyle *et al.*, 2002; Leonard *et al.*, 2008).

Bunyavirus RNA may also be recognised by cytoplasmic PRRs, RIG-I (retinoic acid inducible gene I) and PKR (protein kinase R) (Streitenfeld *et al.,* 2003; Nallagatla *et al.*, 2007; Weber *et al.*, 2013). RIG-I recognises dsRNA and ssRNA with polyuridine or 5'triphosphate sequences and bunyavirus RNPs with 5' triphosphorylated panhandle structures have been shown to activate RIG-I *in vitro* (Figure 1-8) (Weber *et al.*, 2013). Hantaviruses and nairoviruses process their viral RNA to form 5'monophosphorylated genomes, which in theory should minimise RIG-I activation (Habjan *et al.*, 2008; Wang *et al.*, 2011). Yet, Lee et al. (2011) demonstrated that expression of HTNV N ORF stimulates RIG-I and it is postulated that this may be due secondary structures within the vRNA. PKR, a serine-threonine kinase, also recognises dsRNA and ssRNA with 5'triphosphate groups and activates NFκB. In addition to acting as a PRR, PKR acts as an ISG. PKR-mediated suppression of mRNA translation is discussed in section 1.1.7.2.

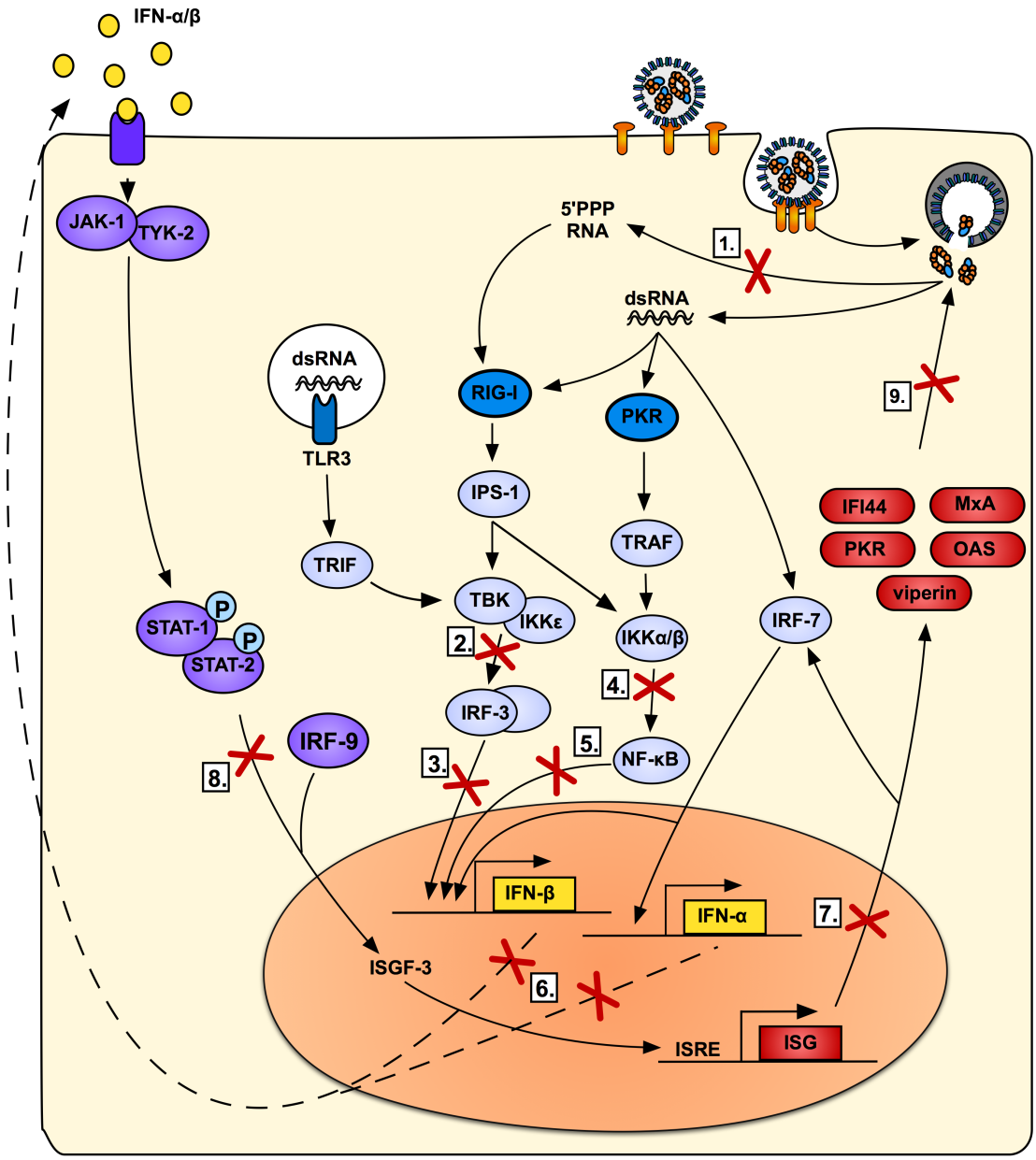


Figure 1-8: Interactions with Type I Interferon Response

Schematic representation of bunyavirus interaction with type I interferon (IFN) response. Bunyavirus particles are recognised by pathogen recognition receptors including RIG-I, PKR and TLR3. Red crosses mark bunyavirus mechanisms to antagonise and evade the type I IFN system. 1) CCHFV has a 5'monophosorylated genome that is not recognised by RIG-I. 2, 3) Hantavirus Gn inhibits IKKε and TBK-1 kinases, preventing dimerisation and nuclear translocation of IRF-3. 4) The viral ovarian tumour domain in CCHFV L protein inhibits activation of NFκB. 5) Hantavirus N protein binds importin-alpha, blocking nuclear translocation of NFκB. 6,7) NSs proteins inhibit RNA polymerase II, blocking transcription of cellular mRNAs. 8,9) RVFV NSs protein activates SOCS-1a, a negative regulator of JAK-STAT signalling pathway, and induces PKR degradation. Figure adapted from Haller *et al.*, 2006 with bunyavirus evasion and antagonism overlaid.

1.1.7.2 Evasion and Antagonism of the Type I IFN Response

The type I IFN system is a powerful component of the innate immune response, activated by PRRs and capable of regulating hundreds of IFN-stimulated genes (ISGs). Many bunyaviruses suppress activation of this system. For example, orthobunyavirus and phlebovirus NSs proteins antagonise the type I IFN response at the transcriptional level, blocking RNA polymerase II (RNAPII) and inhibiting the transcription of cellular mRNAs (Weber *et al.*, 2002; Billecocq *et al.*, 2004; Thomas, 2004). This blockade has a profound effect on the IFN-β promoter and recombinant viruses lacking NSs show increased sensitivity to type I IFNs (Bridgen *et al.*, 2001; Blakqori *et al.*, 2007). RVFV NSs has also been shown to interact with SAP30 (Sin3A associated protein 30), a cellular protein forming part of a Sin3A/NCoR/HDAC-3 co-repressor complex which binds to the IFN-β promoter (Le May *et al.*, 2008). The binding of NSs to this complex inhibits histone acetylation and the recruitment of co-activator CREB binding protein and YY1 at the -122 site, preventing promoter activation (Le May *et al.*, 2008). The importance of this interaction was shown by Le May *et al.* (2008) who created recombinant virus recZHD210-230 in which NSs lacked the SAP30 binding domain; this virus was unable to antagonise IFN-β expression and was avirulent in a mouse model. RVFV NSs has also been shown to activate SOCS-1, a negative regulator of the JAK-STAT pathway inhibiting STAT activation and subsequent induction of ISGs (Figure 1-8) (Haller *et al.,* 2006).

Hantavirus NSs proteins only partially inhibit IFN synthesis, and some pathogenic bunyaviruses do not encode an NSs protein, strongly suggesting additional mechanisms of IFN suppression (Jääskeläinen *et al.*, 2007; Mohamed *et al.,* 2009). Both pathogenic and non-pathogenic hantaviruses inhibit the IFN signalling pathway, suppressing phosphorylation and nuclear translocation of STAT-1 and 2 (Spiropoulou *et al.*, 2007). However, pathogenic hantaviruses induce ISGs later than non-pathogenic hantaviruses in primary endothelial cells and IRF-3 dimerisation and nuclear translocation is also down-regulated (Kraus *et al.*, 2004). The Gn proteins of pathogenic hantaviruses contain conserved ITAM (immunoreceptor tyrosine-based activation motif) sequences and have been shown to inhibit IKKe and TBK-1 kinases, suppressing IRF-3 phosphorylation and nuclear translocation (Figure 1-8) (Geimonen *et al.*, 2003; Alff *et*

al., 2006; Alff *et al.,* 2008). The N proteins of Old World hantaviruses have also been shown to be involved in IFN suppression, binding importin-α and blocking nuclear translocation of NFκB (Taylor *et al.*, 2009).

In addition to suppressing IFN activation and signalling, bunyaviruses antagonise and evade ISG products. ISG products known to have anti-bunyavirus activity include MxA, the 2'-5' OAS system, viperin, and IFI44 (MTAP44). Human MxA protein is a large (76 kDa) GTPase that accumulates in the cytoplasm of IFN-treated cells and acts as a defence against RNA viruses (Kochs, 2002; Haller and Kochs, 2002; Bridgen *et al.*, 2004). Members of the orthobunyavirus, hantavirus, nairovirus and phlebovirus genera have been shown to be sensitive to MxA (Frese *et al.*, 1996; Kanerva *et al.*, 1996; Andersson *et al.*, 2004; Bridgen *et al.*, 2004). Studies with LACV have found that MxA binds N, forming MxA/N copolymers that localise at the perinuclear compartment, inhibiting transport of N to the Golgi and reducing N protein availability at replication sites (Reichelt *et al.*, 2004; Kochs and Haller, 2010). A decrease in viral N protein and viral RNA genome synthesis have also been shown to occur in Dugbe virus (DGUV) infected cells expressing MxA (Bridgen *et al.*, 2004). However, not all bunyaviruses are sensitive to MxA, and it has little effect on BUNV (Bridgen *et al.*, 2004).

The type I IFN response upregulates PKR expression which, in addition to acting as a PRR and activating NKκB pathways, inhibits translation of cellular and viral mRNAs. On binding RNA PKR forms homo-dimers which phosphorylate the α subunit of eukaryotic translation initiation factor eIF-2, inhibiting mRNA translation (Goodbourn *et al.,* 2000; Nallagatla *et al.*, 2007). PKR is activated in bunyavirus infection, yet it has limited anti-bunyavirus activity (Streitenfeld *et al.,* 2003). In RVFV infections this may be attributed to NSs mediated PKR degradation. However, this activity is specific to RVFV NSs, and is not shared with other phleboviruses, or the orthobunyaviruses LACV and BUNV (Streitenfeld *et al.,* 2003; Habjan *et al.*, 2009; Ikegami *et al.,* 2009).

The 2'-5' OAS system is a group of enzymes that recognise and process dsRNAs, producing a series of short 2'-5'oligoadenylates that bind and activate monomeric RNase L, inducing formation of the active RNase L homodimer (Stark *et al.*, 1998). Activated RNase L catalyses cleavage of ssRNA and 28S ribosomal RNA, inhibiting

viral replication and levels of Oasl2 are significantly raised in BALB/cByJ mouse embryonic fibroblasts infected with RVFV (do Valle *et al.*, 2010). However, RNase L does not seem to have activity against BUNV as there was no difference in disease progression in wild type and RNase L -/- mice inoculated intracerebrally with BUNV (Streitenfeld *et al.,* 2003).

Additional ISG products with anti-bunyavirus activity include viperin and IFI44 (MTAP44) (Carlton-Smith and Elliott, 2012). Viperin localises at the ER membrane and impairs the formation of lipid rafts which are needed by some viruses for replication or budding (Weber and Mirazimi, 2008). IFI44 is a cytoplasmic protein previously associated with HCV infection (Kitamura *et al.*, 1994). IFI44 contains a GTP binding site but lacks homology to GTPases or G proteins and it has been proposed that IFI44 may sequester intracellular GTP, preventing extracellular signal regulated kinase signalling, leading to cell cycle arrest inducing an antiproliferative state *in vitro* (Hallen *et al.*, 2007).

Polyubiquitin chains also play an important role in the type I IFN response, degrading IκB and activating RIG-I. The amino terminus of nairovirus L proteins contain a functional OTU domain capable of hydrolysing poly and non-linear ubiquitin chains (Frias-Staheli *et al.*, 2007; van Kasteren *et al.*, 2012). Moreover, CCHFV L protein has been shown to suppress activation of the NFκB responsive promoter in a dose-dependent manner and to hydrolyse ISG15 conjugates and precursors (Figure 1-8) (Frias-Staheli *et al.*, 2007). ISG15 is an antiviral ubiquitin-like protein that is thought to act co-translationally to inhibit viral proteins.

1.1.7.3 Host Cell Shut Off

As mentioned previously, orthobunyavirus NSs proteins antagonise the IFN response at the transcriptional level, blocking RNAPII and inhibiting host cell transcription. For example, the C terminus of BUNV NSs protein interacts with MED8 and strongly inhibits the phosphorylation of serine 2 in the RNAPII C terminal domain heptapeptide repeat YSPTSPS, blocking transcription elongation, whilst LACV NSs induces degradation of RNAPII subunit RPBI (Thomas, 2004; Léonard *et al.*, 2006;

Verbruggen *et al.*, 2011). Phleboviruses also antagonise RNAPII, and RVFV NSs protein has been shown to prevent formation of TFIIH, sequestering p44 and promoting degradation of p62 (Billecocq *et al.*, 2004; Le May *et al.*, 2004; Kalveram *et al.,* 2011).

As a consequence of NSs inhibiting host cell mRNA transcription, cellular mRNA levels fall leading to a decrease in translation. This is exacerbated by bunyavirus L and N proteins, which have been shown to decrease reporter gene expression independently, although not as extensively as NSs. L cleaves host cell mRNAs to generate 5'capped leader sequences to prime viral transcription, inducing cellular mRNA instability and further reducing availability of cellular mRNAs (Raju and Kolakofsky, 1988). BUNV N protein has also been shown to bind and colocalise with PABP in the cytoplasm before PABP is redistributed to the nucleus. PABP N protein binding is enhanced in the presence of NSs and it inhibits the translation of polyadenylated cellular mRNAs (Blakqori *et al.,* 2009).

1.1.7.4. Host Cell Apoptosis and Persistent Infections

The ability to induce apoptosis in mammalian cells varies across the *Bunyaviridae* family and within each genus, for example, LACV is capable of inducing apoptosis whilst BUNV is not (Kohl *et al.*, 2003; Blakqori and Weber, 2005). The NSs proteins of California serogroup orthobunyaviruses have been shown to bind apoptotic regulator Scythe and to trigger cytochrome C release and caspase 3 activation inducing apoptosis. Conversely, in BUNV infections deletion of NSs is required to induce apoptosis (Colón-Ramos *et al.*, 2003; Kohl *et al.*, 2003). In addition to NSs, NSm proteins have also been shown to influence the induction of apoptosis *in vitro*. RVFV NSm displays anti-apoptotic properties *in vitro* and deletion of NSm triggers apoptosis via caspases 3, 8 and 9 (Won *et al.*, 2007). Virus yields are not significantly affected by cell death and blocking apoptosis through over-expression of anti-apoptotic factors does not prevent eventual cell death due to extensive cytopathic effect. It therefore appears that apoptosis of mammalian cells is not essential for virus replication but rather a consequence of replication and inhibition of the type I IFN response (Blakqori *et al.*, 2007).

In contrast to the acute infections observed in mammalian cells, a key aspect of the bunyavirus life cycle is the establishment of persistent infections in arthropod and rodent vectors. It is thought that regulatory T cells may mediate hantavirus persistence in rodents, during which there is a selective reduction of the pro-inflammatory response in the lung, limiting host pathology and perhaps facilitating continued virus replication (Schountz *et al.*, 2007). However, the underlying mechanisms of persistence remain unclear in both rodent and arthropod vectors. It has been shown that in arthropod vectors viral proteins can have different functions as NSs proteins fail to block host cell protein synthesis and the strength of viral promoters changes (Kohl *et al.*, 2004).

1.2 Orthobunyaviruses

1.2.1 Orthobunyavirus Classification

As discussed previously, orthobunyaviruses are the largest genus within the *Bunyaviridae* with 18 serogroups containing over 170 named virus isolates and a rising number of unclassified viruses (Table 1-4). The current gold standard for orthobunyavirus classification is the PRNT assay (Plaque Reduction Neutralisation Test), which detects neutralising antibodies targeting viral glycoproteins. To be classified as a distinct virus species viruses should have > 4-fold difference in heterologous (Ht) versus homologous (Ho) titres (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012). Complement fixation tests (CFTs), which detect anti-N antibodies, are also often used, although due to the occurrence of reassortant viruses, they can give conflicting and sometimes confusing results (Figure 1-9) (Whitman and Shope, 1962; Casals, 1963; Zarate *et al.*, 1968; Berge, 1975). Classification methods have also recently been updated to consider sequencing data, and when available, N protein aa sequences should be > 10% divergent for viruses to be considered as distinct virus species and not part of the same replicating lineage (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012).

Table 1-4: Orthobunyavirus Species List

Orthobunyavirus Serogroups, Virus species and Named Isolates

Anopheles A serogroup
Anopheles A virus
Anopheles A virus (ANAV)
Arumateua virus (ARTV)
Caraipé virus (CPEV)
ColAn 57389 (CA57389V)
Las Maloyas virus (LMV)
Lukuni virus (LUKV)
Trombetas virus (TRMV)
Tucuruí virus (TUCV)
Tacaiuma virus
Tacaiuma virus (TCMV)
CoAr 1071 virus (CA1071V)
CoAr 3627 virus (CA3626V)
Virgin River virus (VRV)

Capim serogroup
Acara virus
Acara virus (ACAV)
Moriche virus (MORV)
Benevides virus
Benevides virus (BVSV)
Bushbush virus
Benfica virus
Bushbush virus (BSBV)
Juan Diaz virus (JDV)
Capim virus
Capim virus (CAPV)
Guajara virus
Guajara virus (GJAV)

Gamboa serogroup
Alajuela virus
Alajuela virus (ALJV)
San Juan virus (SJV)
Gamboa virus
Gamboa virus (GAMV)
Pueblo Viejo virus (PVV)

Guama serogroup
Bertioga virus
Bertioga virus (BERV)
Cananeia virus (CNAV)
Guaratuba virus (GTBV)
Itimirim virus (ITIV)
Mirim virus (MIRV)
Bimiti virus
Bimiti virus (BIMV)
Catu virus
Catu virus (CATUV)
Guamá virus
Ananindeua virus (ANUV)
Guamá virus (GMAV)
Mahogany Hammock virus (MHV)
Moju virus (MOJUV)
Timboteua virus
Timboteua virus (TBTV)

Tete serogroup
Batama virus
Batama virus (BMAV)
Tete virus
Bahig virus (BAHV)
Matruh virus (MTRV)

Anopheles B serogroup
Anopheles B virus
Anopheles B virus (ANBV)
Boracéia virus (BORV)

Bunyamwera serogroup
Bunyamwera virus
Anadyr virus (ANADV)
Batai virus (BATV)
Birao virus (BIRV)
Bozo virus (BOZOV)
Bunyamwera virus (BUNV)
Cache Valley virus (CVV)
Fort Sherman virus (FSV)
Germiston virus (GERV)
Iaco virus (IACOV)
Ilesha virus (ILEV)
Lokern virus (LOKV)
Maguari virus (MAGV)
Mboke virus (MBOV)
Ngari virus (NRIV)[R]
Northway virus (NORV)
Playas virus (PLAV)
Potosi virus (POTV)
Santa Rosa virus (SARV)
Shokwe virus (SHOV)
Stanfield virus
Tensaw virus (TENV)
Tlacotalpan virus (TLAV)
Tucunduba virus (TUCV)
Xingu virus (XINV)
Guaroa virus
Guaroa virus (GROV)
Kairi virus
Kairi virus (KRIV)
Main Drain virus
Main Drain virus (MDV)
Unclassified
Abbey Lake virus (Ab-BUNV)

Kongool serogroup
Koongol virus
Koongol virus (KOOV)
Wongal virus (WONV)

Minatitlan serogroup
Minatitlan virus
Minatitlan virus (MNTV)
Palestina virus (PLSV)

Nyando serogroup
Kaeng Khoi virus
Kaeng Khoi virus (KKV)

Nyando virus
Nyando virus (NDV)
Eretmapodites virus (EREV)
Unclassified
Mojuí dos Campos virus (MDCV)

Olifantsvlei serogroup
Botambi virus
Botambi virus (BOTV)
Olifantsvlei virus
Bobia virus (BIAV)

Bakau serogroup
Bakau virus
Bakau virus (BAKV)
Ketapang virus (KETV)
Nola virus (NOLAV)
Tanjong Rabok virus (TRV)
Telok Forest virus (TFV)

Bwamba serogroup
Bwamba virus
Bwamba virus (BWAV)
Pongola virus (PGAV)

California serogroup
California encephalitis virus
California encephalitis virus (CEV)
Khatanga virus (KHATV)[R]
Inkoo virus (INKV)
Jamestown Canyon virus (JCV)
Jerry Slough virus
Keystone virus (KEYV)
Khatanga virus
La Crosse virus (LACV)
Lumbo virus (LUMV)
Melao virus (MELV)
Morro Bay virus (MBV)
San Angelo virus (SAV)
Serra do Navio virus (SDNV)
snowshoe hare virus (SSHV)
South River virus (SORV)
Tahyña virus (TAHV)
Trivittatus virus (TVTV)

Group C serogroup
Caraparú virus
Apeú virus (APEUV)
Bruconha virus (BRUV)
Caraparú virus (CARV)
Ossa virus (OSSAV)
Vinces virus (VINV)
Madrid virus
Madrid virus (MADV)
Marituba virus
Gumbo Limbo virus (GLV)
Marituba virus (MTBV)
Murutucú virus (MURV)
Nepuyo virus (NEPV)
Restan virus (RESV)
Zungarococha virus (ZUNV)
Oriboca virus
Itaquí virus (ITQV)
Oriboca virus (ORIV)

Patois serogroup

Patois virus
Abras virus (ABRV)
Babahoya virus (BABV)
Pahayokee virus (PAHV)
Patois virus (PATV)
Shark River virus (SRV)
Zegla virus
Zegla virus (ZEGV)

Tete virus (TETEV)
Tsuruse virus (TSUV)
Weldona virus (WELV)

Simbu serogroup
Akabane virus
Akabane virus (AKAV)
Sabo virus (SABOV)
Tinaroo virus (TINV)
Yaba-7 virus (Y7V)
Manzanilla virus
Buttonwillow virus (BUTV)
Cat Que virus
Ingwavuma virus (INGV)
Inini virus (INIV)
Manzanilla virus (MANV)
Mermet virus (MERV)
Oropouche virus
Facey's Paddock virus (FPV)
Iquitos virus (IQTV)
Madre de Dios virus
Oropouche virus (OROV)
Perdões virus
Pintupo virus
Utinga virus (UTIV)
Utive virus (UVV/UTVEV)
Sathuperi virus
Douglas virus (DOUV)
Sathuperi virus (SATV)
Simbu virus
Simbu virus (SIMV)
Shamonda virus
Peaton virus (PEAV)
Sango virus (SANV)
Shamonda virus (SHAV)
Shuni virus
Aino virus (A(I)NOV)
Kaikalur virus (KAIV)
Shuni virus (SHUV)
Thimiri virus
Thimiri virus (THIV)
Unclassified
Leanyer virus (LEAV)
Jatobal virus (JATV)[R]
Schmallenberg virus (SBV)[R]

Dabakala virus (DABV)
Olifantsvlei virus (OLIV)
Oubi virus (OUBIV)

Turlock serogroup
M'Poko vius
M'Poko virus (MPOV)
Yaba-1 virus (Y1V)
Turlock virus
Lednice virus (LEDV)
Turlock virus (TURV)
Umbre virus (UMBV)

"Sedlec serogroup"
Sedlec virus
I612045 virus
Oyo virus (OYOV)
Sedlec virus (SEDV)

No Probable Serogroup
Brazoran virus (BRAZV)
Estero Real virus (ERV)
Enseada virus (ENSV)
Khurdun virus (KHURV)
Kowanyama virus (KOWV)
Marambaia virus
Pacui virus (PACV)
Rio Preto da Eva virus (RPEV)
Shuangao insect virus 1
Tapirapé virus (TPPV)
Termeil virus (TERV)
Wuhan louse fly virus 1

"Mapputta serogroup"
Buffalo Creek virus (BUCV)
Gan Gan virus (GGV)
Mapputta virus (MAPV)
Maprik virus (MPKV)
Murrumbidgee virus (MURBV)
Salt Ash virus (SASHV)
Trubanaman virus (TRUV)

"Wyemoyia serogroup"
Wyeomyia virus
Anhembi virus (AMBV)
BeAr 328208 virus (BAV)
Cachoeira Porteira virus (CPOV)
Iaco virus (IACOV)
Macaua virus (MCAV)
Rio Pracupi virus
Sororoca virus (SORV)
Taiassui virus (TAIAV)
Tucunduba virus (TUCV)
Wyeomyia virus (WYOV)

Orthobunyavirus serogroups (bold), ICTV-recognised species (italics) and classified named isolates (indented) are listed. Recently proposed and not yet ICTV-approved serogroups are indicated by quotation marks. The Wyeomyia group currently forms part of the Bunyamwera serogroup, whilst Mapputta and Sedlec viruses branch alone on phylogenetic analysis (Chowdhary *et al.*, 2012; Bakonyi *et al.*, 2013; Gauci *et al.*, 2015). (R) indicates reassortant virus. Virus abbreviations are also listed. Table adapted from Elliott and Blakqori (2011).

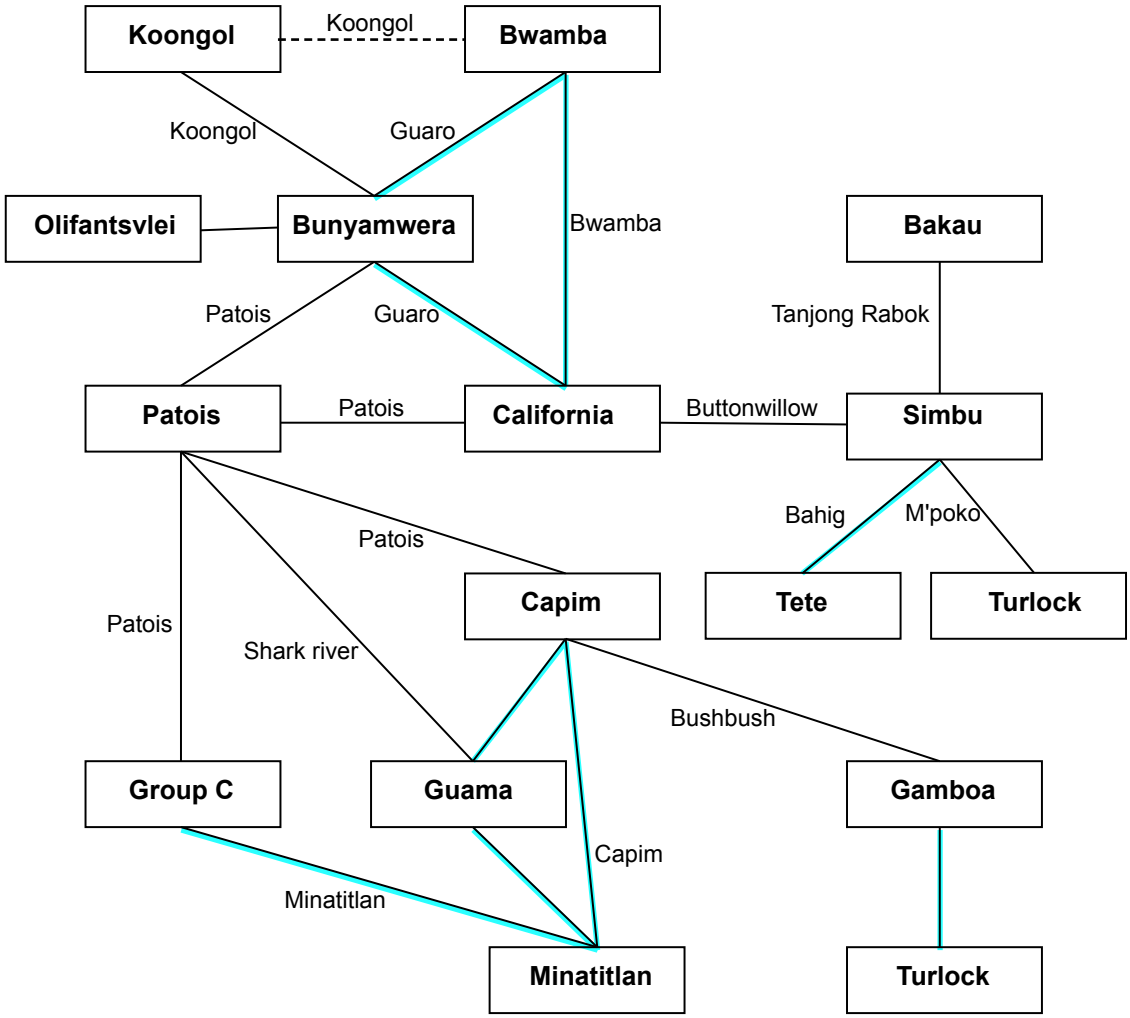


Figure 1-9: Antigenic Relationships between Orthobunyaviruses

Adapted from Calisher 1988, with additional data from Mohamed 2007, Shope and Whitman 1962, Zarate *et al.,* 1968, Zeller *et al.,* 1989 and Berge, 1975. Orthobunyavirus serogroups are highlighted in boxes and those linked by cross-reaction in either neutralisation or complement fixation tests are joined by a black line with an example of a virus with cross-reaction. The dotted line indicates questionable cross-reaction. Cross-reactions that have subsequently been supported by sequencing and phylogenetic analysis are highlighted in turquoise.

1.2.2 Orthobunyavirus Evolution

The driving factors behind bunyavirus evolution are genetic drift and genetic shift (Figure 1-10). Genetic drift occurs through the accumulation of mutations in the virus genome during virus replication as the virus polymerase, an RdRp, has an intrinsic error rate and lacks proof reading capabilities (Figure 1-10A). It is postulated that the low fidelity of viral RdRps may play a key role in the virus life cycle as it leads to the rapid accumulation of mutations and the formation of quasispecies. Quasispecies represent a cloud of sequences genetically linked by mutations (Schneider and Roossinck, 2001; Lauring and Andino, 2010; Ojosnegros *et al.*, 2011). Cloud size is a key property of quasispecies, occurring when the level of diversity in a replicating population reaches equilibrium (Lauring and Andino, 2010). Quasispecies are thought to be vital in the virus life cycle, providing a source of diversity to overcome bottlenecks in virus replication such as host switching. Although the error rate of orthobunyavirus RdRp has not yet been determined, the error rate for hantavirus S segments is approximately 10^{-3}, which falls within the range estimated for other RNA viruses (10^{-3} - 10^{-5}) (Plyusnin *et al.*, 1995; Plyusnin *et al.*, 1996).

The second driver in orthobunyavirus evolution is genetic shift, or genetic reassortment. Reassortment occurs during mixed infections when genome segments from different parental strains are co-packaged to form progeny with mixed ancestry (Figure -10B). In dual orthobunyavirus infections 8 progeny may therefore be formed, 6 reassortant viruses and 2 viruses matching the parental strains (Figure 1-10B). Reassortment occurs only between closely related viruses and may be considered when forming species classification, as distinct virus species should not be able to form recombinant progeny (Elliott and Blakqori, 2011). Reassortant orthobunyaviruses were first identified in the 1970s when co-infection experiments yielded progeny viruses with phenotypic profiles that did not match the parental strains, providing evidence that orthobunyavirus genomes may be segmented (Gentsch and Bishop, 1976; Gentsch *et al.*, 1977). Naturally occurring reassortant viruses have now been documented in the Bunyamwera, Simbu, California, Group C and Patois serogroups (Briese *et al.,* 2013).

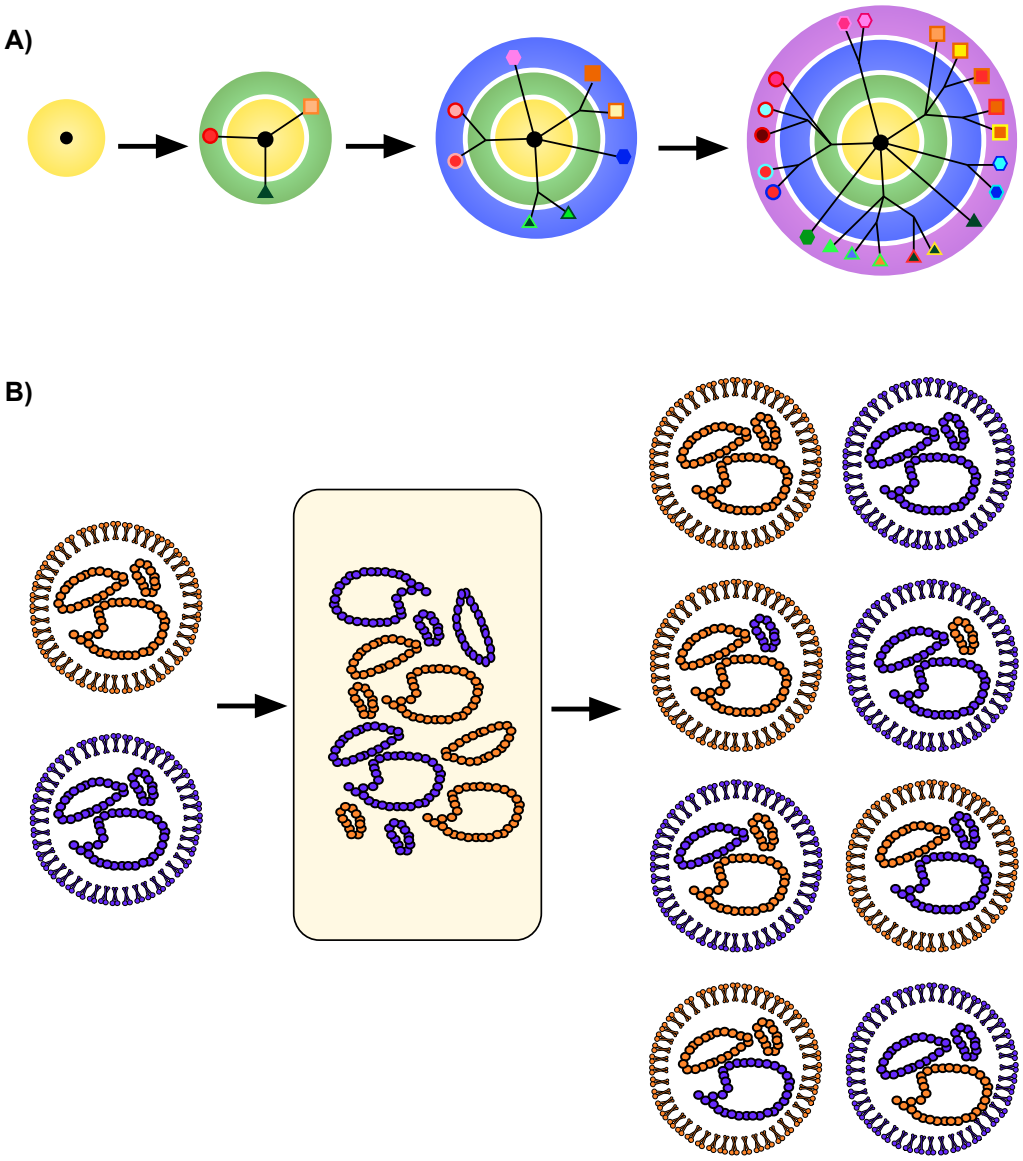


Figure 1-10: Genetic Drift and Shift

A) Genetic Drift. A schematic representation of quasispecies development during virus replication. Rounds of replication are represented by concentric circles and black lines connect variants linked by a point mutation. Although this schematic over simplifies quasispecies it represents how quickly the accumulation of mutations can lead to a diverse but related population of viruses. Adapted from Lauring *et al.,* 2010. **B)** Genetic Shift. A schematic representation of virus reassortment during dual infection. Two distinct viruses (orange and purple) infect a host cell and during virus replication genome segments from either virus may be incorporated into mature virus particles, resulting in 8 possible progeny. In nature bunyavirus reassortment appears restricted with most reassortant viruses sharing a common ancestor for S and L segments.

As more reassortant viruses are identified there appears to be a genetic preference for co-inheritance of S and L segments, although this does not occur in California serogroup viruses, or when the parental strains are themselves reassortants (Gentsch *et al.*, 1977; Gentsch *et al.,* 1979). The molecular barrier for virus reassortment has not yet been determined, although the compatibility of the RNP and viral polymerase presumably plays a role.

1.2.3 Orthobunyavirus Ecology

Orthobunyaviruses are transmitted and maintained in the environment through arthropod vectors. Orthobunyaviruses have been isolated from a diverse range of arthropods, including midges, mosquitoes, biting flies, bed bugs and hard and soft bodied ticks. Arthropod vectors become infected by taking a blood meal on a viraemic host or through transovarial transmission (Figure 1-11). Transovarial transmission has been recorded in ticks, mosquitoes and midge species and is thought to be a key factor in the orthobunyavirus life cycle, sustaining orthobunyaviruses through winter months and acting as a reservoir of infection come Spring (Converse *et al.*, 1974; Mclean, 1975; Labuda and Kozuch, 1986; Dutary *et al.*, 1989; Reese *et al.*, 2010). Moreover, transovarial transmission can dramatically increase the virus reservoir as multiple infected progeny arise from a single adult female. Transovarial transmission has not been documented for all orthobunyaviruses but it is postulated that negative field studies may be skewed by an overall low incidence of infection in the test population (Allingham and Standfast, 1990; Scholte *et al.*, 2014).

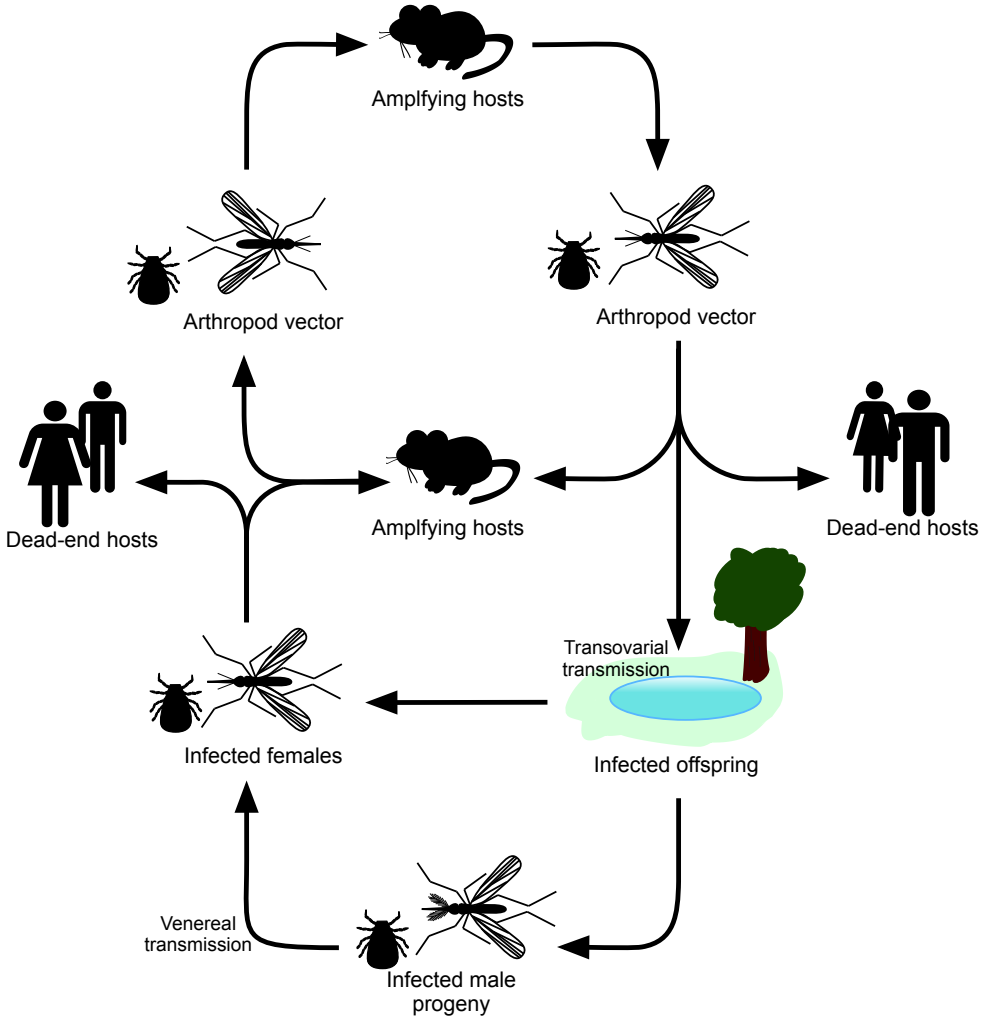


Figure 1-11: Epizoonotic Life Cycle of Orthobunyaviruses

Schematic of the epizoonotic life cycle of orthobunyaviruses. Arthropod vectors include midges, mosquitoes, hard and soft bodied ticks, bedbugs and biting flies. Amplifying hosts include small and large mammals, marsupials and birds. Transovarial transmission of orthobunyaviruses has been reported in ticks, mosquitoes and midge species. Humans and other hosts may be incidentally infected and due to low levels of viraemia do not transmit the virus to non-infected biting arthropods.

In addition, a third, less documented, mode of transmission may occur. LACV has been shown to be transmitted horizontally from male to female *Ae. triseriatus* mosquitoes (Figure 1-11) (Thompson and Beaty, 1977). The male mosquitoes are infected transovarially and then as mature adults transmit the virus to female mosquitoes during copulation (Figure 1-11) (Thompson and Beaty, 1977).

As arboviruses environmental conditions related to vector abundance and activity, such as elevation, host availability, changes in land use and meteorological conditions influence orthobunyavirus distribution and infection rates (Vasconcelos *et al.*, 2001; Bessell *et al.*, 2013). For example, wind-borne introduction of SBV-infected *Culicoides* midges from mainland Europe to the UK in 2011 is thought to account for the emergence of SBV in the UK, and the spread of OROV in Brazil has been linked with the construction of the Belem-Brasilia highway (Figure 1-12) (Vasconcelos *et al.*, 2001; Sedda and Rogers, 2013; Kluiters *et al.,* 2015).

Orthobunyaviruses tend to have a wide host range, which again facilitates virus spread and maintenance. However, some hosts are incidental, or dead-ends; this occurs when the level and/or duration of viraemia is not conducive to onward transmission of the virus to subsequent biting arthropods (Figure 1-11) (Calisher, 1994).

A summary of current knowledge regarding orthobunyavirus vectors, geographic location, hosts and disease is detailed, by serogroup, in sections 1.2.3.1 to 1.2.3.19.

1.2.3.1 Anopheles A Serogroup

The Anopheles A serogroup contains 2 virus species, the Anopheles A virus species (sp.) and the Tacaiuma virus sp. (Table 1-4) (Calisher *et al.*, 1973; Calisher *et al.*, 1980; Calisher *et al.*, 1985). Anopheles A virus sp. have been isolated from *Anopheles* and *Aedes* mosquitoes and equines in South America (Calisher *et al.*, 1973; Calisher *et al.*, 1980; Calisher *et al.*, 1985). Tacaiuma virus sp. have a slightly larger geographical

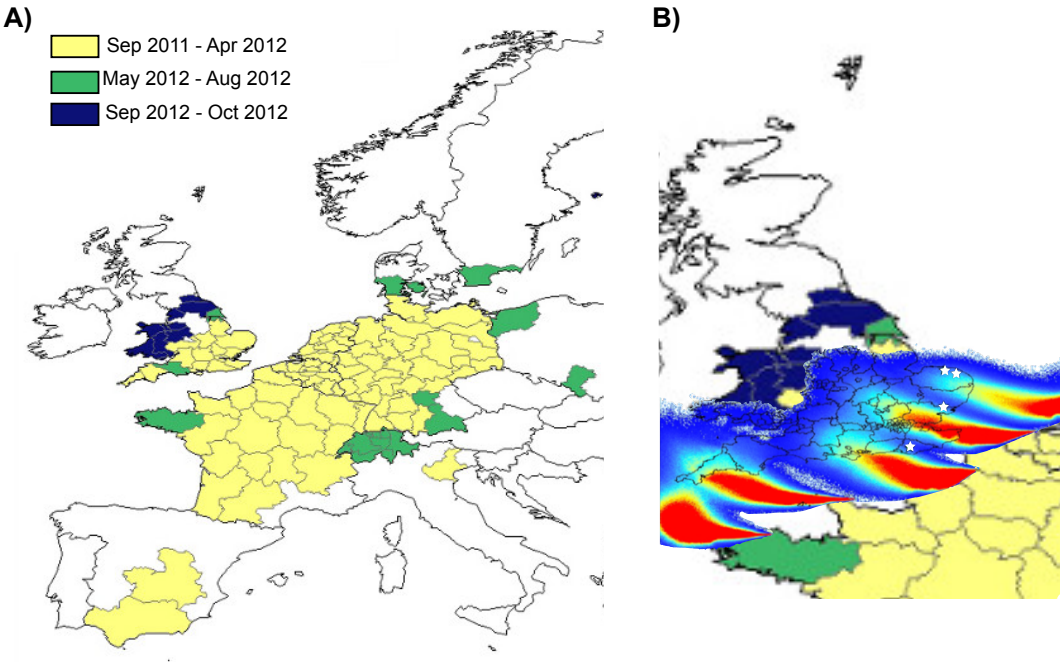


Figure 1-12: Spread of Schmallenberg virus in Europe

Schematic representation of geographical spread of Schmallenberg virus (SBV) in Europe. **A)** Location of confirmed SBV infection in large animals, image from Doceul *et al.*, 2013. Dates of first isolations are summarised into three categories; September (Sep) 2011 - April (Apr) 2012, May 2012 - August (Aug) 2012 and September (Sep) 2012 - October (Oct) 2012. **B**) Image from DEFRA and AHVLA depicting high risk wind for midge imports, overlaid on SBV map by Doceul *et al.,* 2013. White stars indicate the first cases of UK SBV infection in ruminants.

distribution and have been isolated from *Anopheles* and *Haemagogus* mosquitoes in South and North America (Calisher *et al.*, 1973; Berge, 1975; Calisher *et al.*, 1980, Calisher *et al.,* 1985). Named virus isolate TCMV has also been isolated from non-human primates and is associated with a benign febrile illness in man (Berge, 1975; Calisher *et al.*, 1980). Interestingly, S segment sequencing of ANAV, LUKV and TCMV revealed viruses in this serogroup lack a full length NSs ORF (Mohamed *et al.,* 2009; Shchetinin *et al.*, 2015).

1.2.3.2 Anopheles B Serogroup

The Anopheles B serogroup has 1 recognised virus species, the Anopheles B virus sp., which contains 2 named virus isolates Anopheles B virus (ANBV) and Boraceia virus (BORV) (Table 1-4) (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012). ANBV and BORV have been isolated from Anopheles mosquitoes in South America and neutralising antibodies to BORV have been found in cattle, dogs, equines, chickens, rodents and man (de Souza Lopes *et al.*, 1966; de Souza Lopes and de Abreu Sacchetta, 1974; Berge, 1975). BORV has not yet been linked with clinical disease as 16 subjects that underwent seroconversion during a short study period did not report symptoms (de Souza Lopes and de Abreu Sacchetta, 1974). Complete S segment sequences for ANBV and BORV were determined in 2009, and identified ANBV and BORV as viruses lacking a functional NSs ORF (Mohamed *et al.,* 2009).

1.2.3.3 Bakau Serogroup

The Bakau serogroup comprises 1 virus species, the Bakau virus sp., which contains 5 named virus isolates: Bakau virus (BAKV); Ketapang virus (KETV); Tanjong Rabok virus (TRV); Telok Forest virus (TFV); and Nola virus (NOLAV) (Table 1-4) (Elliott and Blakqori, 2011). BAKV, KETV and TFV virus have been isolated from *Culex* mosquitoes and soft bodied ticks (BAKV only) in Malaysia (Berge, 1975). Neutralising antibodies to these viruses have also been detected in bats, birds, rodents, primates and man. Although no live virus has been isolated from man, TFV and TRV have been isolated from macaques (Berge, 1975). Interestingly, NOLAV appears to bridge the

Bakau and Simbu serogroups, as although it is listed as a member of the Bakau serogroup by Elliott and Blakqori (2011) it is classified as a member of the Simbu antigenic group in the ArboCAT (Berge, 1975). In neutralisation assays NOLAV had a Ht/Ho titre of 640 with Simbu group (pooled) hyperimmune ascitic fluid and a Ht/Ho titre of 40/1024 with Manzanilla antibody (Berge, 1975). However, with Bakau serogroup antibodies NOLAV had Ht/Ho titres of 40/320 in neutralisation assays, and 16/1024 in CFTs with TFV antigen (Zeller *et al.*, 1989). Perhaps most convincingly in enzyme linked immune assays (EIA) NOLAV had Ht/Ho titres of 1600/3200 to 3200/3200 with BAKV and TFV respectively (Zeller *et al.*, 1989). Interestingly, NOLAV has a different geographic distribution to other members of the Bakau virus sp., with the sole reported isolation of NOLAV from *Culex* mosquitoes in the Central African Republic (Berge, 1975).

1.2.3.4 Bunyamwera Serogroup

The Bunyamwera serogroup contains 4 virus species, the Bunyamwera, Guaroa, Kairi and Main Drain virus sp. (Table 1-4) (Plyusnin *et al.*, 2012). The Bunyamwera virus sp., contains a large lineage of replicating isolates with 22 named virus isolates, whilst Guaroa, Kairi and Main Drain remain the sole named viruses in their species (Table 1-4) (Elliott and Blakqori, 2011). Bunyamwera group viruses are predominantly transmitted by mosquitoes, although Lokern virus (Bunyamwera sp.) and Main Drain virus (Main Drain sp.) have also been isolated from biting flies (Berge, 1975). Bunyamwera group viruses have a wide geographical distribution with isolations in South and North America, Africa, Asia and Europe (Berge, 1975; L'vov *et al.*, 1989; Lambert *et al.*, 2014). Bunyamwera virus sp. are documented causes of abortion and foetal malformation in ruminants, abortion and encephalitis in equines, and febrile illness in man (Okuno, 1961; Chippaux *et al.*, 1969; Chung *et al.*, 1990; Chung *et al.*, 1991; Campbell *et al.*, 2006; Tauro *et al.*, 2012; Nguyen *et al.*, 2013; Odhiambo *et al.*, 2015; Tauro *et al.*, 2015). Ngari virus, a reassortant within the Bunyamwera virus sp., was first isolated in 1979, and caused a large outbreak of HF in East Africa in 1997 and 1998 (Zeller *et al.*, 1996; Bowen *et al.*, 2001; Gerrard *et al.*, 2004). Guaroa virus sp. have also been documented as a cause of a self-limiting febrile illness in man in South America, whilst Main Drain and Kairi virus sp. have been isolated from equines with

encephalitis (Emmons *et al.*, 1983; Calisher *et al.*, 1988; Tauro *et al.,* 2009; Aguilar *et al.*, 2010).

1.2.3.5 Bwamba Serogroup

The Bwamba serogroup contains Bwamba virus (BWAV) and Pongola virus (PGAV), which, due to high level of cross-reactivity in serological tests, were assigned to a single virus sp., Bwamba virus sp. (Table 1-4) (Smithburn *et al.,* 1941; Kokernot *et al.*, 1957). However, recent sequencing data show that BWAV and PGAV share comparatively low levels of sequence identity and clearly diverge on phylogenetic analysis, supporting their re-classification into distinct virus sp. (Groseth *et al.*, 2014). BWAV and PGAV are mosquito-borne viruses and serological surveys indicate BWAV and PGAV are widespread in Africa (Smithburn *et al.,* 1941; Moore *et al.*, 1975; Berge, 1975; Lutwama *et al.*, 1999). BWAV is associated with Bwamba fever, a non-specific febrile illness commonly associated with exanthema and occasionally meningeal involvement (Smithburn *et al.,* 1941; Moore *et al.*, 1975; Johnson *et al.*, 1978). However, BWAV can cause severe disease with haemorrhagic manifestations and 14 fatal cases were recorded during an outbreak among Rwandan refugees (Lutwama *et al.*, 2002). PGAV has also been linked with human infection, with high levels of seropositivity in many African countries, and virus isolation from a febrile patient with myalgia and headache in Uganda (Berge, 1975). PGAV neutralising antibodies have been found in livestock and donkeys (Berge, 1975). Complete genome sequencing and phylogenetic analysis reveals Bwamba serogroup isolates are most closely related to the California serogroup, a relationship supported by antigenic cross-reactions (Figure 1-9) (Groseth *et al.*, 2014).

1.2.3.6 California Serogroup

The California serogroup contains 13 named viruses, all of which have been assigned to the California encephalitis virus sp. (Table 1-4). Viruses within this serogroup are predominantly vectored by mosquitoes although Jamestown Canyon virus (JACV) and Snowshoe hare virus (SSHV) have been isolated from biting flies (Berge, 1975;

Hubalek *et al.*, 2014; Putkuri *et al.*, 2014; Westby *et al.*, 2015). California serogroup viruses are a recognised cause of febrile encephalitis in man, with infections occurring in North America (e.g. LACV, JACV, SSHV, Trivittatus virus), Europe (e.g. Inkoo virus (INKV), Tahyna virus (TAHV)), Africa and China (e.g. SSHV, TAHV) (Centers for Disease Control and Prevention (CDC), 2011; Li *et al.*, 2014; Sonnleitner *et al.*, 2014; Pastula *et al.*, 2015). California serogroup viruses have also been isolated in Siberia and, although no clinical cases have been reported, neutralising antibodies were detected in human sera (L'vov *et al.*, 1989; Mitchell *et al.*, 1993; L'vov *et al.*, 1998).

1.2.3.7 Capim Serogroup

Capim serogroup viruses have been isolated from *Culex* mosquitoes, rodents and marsupials in South America (Spence *et al.*, 1967; Berge, 1975; Calisher *et al.*, 1983a). The serogroup contains 4 virus species, Capim, Benevides, Acara and Bushbush (Table 1-4) (Spence *et al.*, 1967; Jonkers *et al.*, 1968; Calisher *et al.*, 1983a; Elliott and Blakqori, 2011). There is no evidence of infection in man with > 500 sera collected from areas with virus isolation in rodents, mosquitoes and marsupials negative by neutralisation assay for Capim virus (CAPV) (Berge, 1975).

Capim serogroup viruses have been documented to cross-react with Guama group viruses in neutralisation and haemagglutination inhibition (HI) assays, but not CFT assays (Figure 1-9).

1.2.3.8 Gamboa Serogroup

The Gamboa serogroup contains 4 named isolates classified into 2 virus species, Alajuela virus sp. and Gamboa virus sp. (Table 1-4) (Calisher, Gutierrez, *et al.*, 1983; Mitchell *et al.*, 1987; Elliott and Blakqori, 2011). Gamboa virus sp., have been isolated from Aedeomyia mosquitoes, eggs and larvae in South America (Calisher, Gutierrez, *et al.*, 1983; Mitchell *et al.*, 1987; Dutary *et al.*, 1989). Evidence for infection of vertebrate hosts is limited, with > 2200 sentinel mice collected from locations with

Gamboa virus (GAMV) infected mosquitoes testing negative by neutralisation assays, although an association with migratory birds has been reported (Berge, 1975).

1.2.3.9 Group C Serogroup

The Group C serogroup derives its name from the historical classification of viruses and its distinctive serological profile compared with group A (*Togaviridae*) and group B (*Flaviviridae*) viruses (Casals and Whitman, 1961; Calisher *et al.*, 1983b). The Group C serogroup currently contains 4 recognised species, namely Caraparu, Madrid, Marituba and Oriboca, each of which contains a number of named isolates (Table 1-4) (Elliott and Blakqori, 2011). Group C viruses are predominantly vectored by mosquitoes in South America and Mexico, and are associated with a self-limited Dengue-like illness in man (Casals and Whitman, 1961; De Rodaniche *et al.,* 1964; Iversson *et al.*, 1987; Turell *et al.*, 2005; Hontz *et al.*, 2015). Deforestation and changes in land use throughout South America have been associated with increasing isolations of Group C viruses in man and the burden of disease is believed to be underestimated (Walder *et al.,* 1984).

1.2.3.10 Guama Serogroup

The Guama serogroup contains 4 virus species, Bimiti, Bertioga, Catu and Guama (Table 1-4). In addition to the named species isolate, the Bertioga virus sp. contains Cananeia virus, Guaratuba virus, Itimirim virus, and Mirim virus, whilst Ananindeua virus, Moju virus and Mahogany Hammock virus (MHV) have been assigned to the Guama virus sp. (Whitman and Casals, 1961; Coleman *et al.,* 1969; de Souza Lopes *et al.*, 1975; Calisher *et al.*, 1983b). Aside from MHV, which was isolated in Florida, USA, all Guama group viruses have been isolated in South America (Coleman *et al.,* 1969; Berge, 1975). Guama group viruses have been isolated from diverse species of mosquitoes, marsupials, bats, rodents and sentinel primates (Whitman and Casals, 1961; Coleman *et al.,* 1969; Berge, 1975; Calisher *et al.*, 1983b). Two viruses, Catu virus and Guama virus, are associated with a self-limiting febrile illness with myalgia and headache, and have been isolated from febrile patients (Berge, 1975). Guama

serogroup viruses have been linked, by cross-reaction in serological assays, to viruses in the Capim and Minatitlan serogroups (Figure 1-9) (Zarate *et al.*, 1968; Berge, 1975). Recent sequencing studies and phylogenetic analysis reveal close branching of Capim and Guama serogroup clusters but there are as yet no published sequences for Minatitlan serogroup viruses (Shchetinin *et al.*, 2015).

1.2.3.11 Koongol Serogroup

The Koongol serogroup contains 1 virus species, Koongol virus sp., to which both Koongol virus (KOOV) and Wongal virus (WONV) belong (Table 1-4). KOOV and WONV were isolated from *Culex annulirostris* mosquitoes in Australia, and KOOV has subsequently been identified in New Guinea (Doherty *et al.*, 1963; Berge, 1975; Wright *et al.*, 1981; Marshall *et al.,* 1982). A study into the plaque morphology of arboviruses found KOOV and WONV formed plaques ≤ 1 mm in diameter 15 and 10 d p.i., respectively, on Vero cells. KOOV formed slightly larger plaques (≤ 2 mm 10 d p.i.) on an additional cell line, LLC-MK_2, whilst WOOV failed to plaque on these cells (Stim, 1968). Complete coding sequences for KOOV were published during preparation of this manuscript and phylogenetic analysis showed KOOV branching with Umbre virus, a Turlock serogroup virus (Shchetinin *et al.*, 2015). The host range of Koongol serogroup viruses remains unclear. Sera from cattle, marsupials and birds has tested low-level positive by HI assays for KKOV and WONV; however, results were rarely confirmed by neutralisation tests and no clinical disease has been reported (Doherty *et al.*, 1970).

1.2.3.12 Minatitlan Serogroup

To date the Minatitlan serogroup contains 2 named isolates, Minatitlan virus (MNTV) and Palestina virus (PLSV), both of which belong to the Minatitlan virus species (Table 1-4). MNTV and PLSV have been isolated from *Culex* mosquitoes and sentinel hamsters in Mexico, Ecuador and Guatemala (Calisher *et al.*, 1983a). Cross-reaction in serology based assays has been documented for the Minatitlan serogroup with both MNTV and PLSV cross-reacting with Capim, Guama and Group C serogroup viruses

in CFTs and Guama serogroup viruses in PRNT assays (Figure 1-9) (Berge, 1975; Calisher *et al.*, 1983a).

1.2.3.13 Nyando Serogroup

Nyando serogroup viruses have been isolated from mosquitoes in Africa (Williams *et al.,* 1965; Ardoin and Simpson, 1965; Lee and Moore, 1972; Lutwama *et al.*, 1999). There is currently 1 recognised virus sp., the Nyando virus, which contains both Eretmapodites virus (EREV) and Nyando virus (NDV) isolates (Table 1-4) (Ardoin and Simpson, 1965; Berge, 1975; Elliott and Blakqori, 2011). There are limited documented isolations of NDV from febrile patients although serological surveys indicate a high prevalence in Kenya, Uganda and Senegal (Digoutte *et al.*, 1972; Groseth *et al.*, 2014). Frequent isolations from mosquitoes also indicate that the prevalence and disease burden of NDV may be underestimated (Berge, 1975; Groseth *et al.*, 2014).

1.2.3.14 Olifantsvlei Serogroup

The Olifantsvlei serogroup contains 2 virus species: Botambi virus and Olifantsvlei virus (Table 1-4) (Plyusnin *et al.*, 2012). The Olifantsvlei virus species contains 4 named viruses, Olifantsvlei virus (OLIV), Bobia virus, Dabakala virus and Oubi virus, whilst Botambi virus remains the sole member of its species (Elliott and Blakqori, 2011). Olifantsvlei serogroup viruses have been isolated from *Culex* mosquitoes in Africa (Berge, 1975; McIntosh, 1978). There is little published information on this group of viruses although Stim (1968) reported virus preparations of OLIV were fatal to new born mice despite failing to form plaques on Vero or LLC-MK_2 cell lines.

1.2.3.15 Patois Serogroup

The Patois serogroup contains 2 virus species, Zegla virus sp. and Patois virus sp., which contains 5 named virus isolates, Patois virus, Abras virus, Babahoya virus, Pahayokee virus and Shark River virus (Table 1-4) (Calisher *et al.*, 1983a). Patois serogroup viruses have been predominately isolated from *Culex* mosquitoes and small rodents in South America, although Shark River virus was isolated in the Florida Everglades, USA, and from 1 pool of *Anopheles* mosquitoes (Berge, 1975). Antibodies to Patois virus and Zegla virus have been found in man, although there are no reports of clinical disease or virus isolation (Scherer *et al.*, 1972). Viruses in the Patois serogroup cross-react with viruses in the Bunyamwera, California, Capim, Group C and Guama serogroups (Figure 1-9) (Ushijima *et al.,* 1981).

A sixth, currently unclassified virus, Estero Real virus has nominally been placed in the Patois serogroup (Shchetinin *et al.*, 2015). However, Estero Real virus did not cross-react with Abras, Babahoyo, Pahayokee, Patois, Zegla or Shark River virus in immunofluorescence tests (Zeller *et al.*, 1989). In CFT assays Estero Real virus failed to cross-react with all Patois serogroup viruses except Zegla virus, which gave a Ht/Ho CFT titre of 32/1024 (Zeller *et al.*, 1989). Moreover, Estero Real virus appears divergent from other Patois serogroup viruses as it was isolated from soft bodied ticks, and not mosquitoes, in South America (Málková *et al.*, 1985).

1.2.3.16 Simbu Serogroup

The Simbu serogroup contains 8 ICTV-recognised species, Akabane, Shamonda, Shuni, Thimiri, Manzanilla, Oropouche, Sathuperi and Simbu virus, with each species representing a large lineage of named viruses (Table 1-4). Simbu serogroup viruses are vectored by both mosquitoes and midge sp. and are widely distributed with isolations in Africa, Asia, Australia, Europe and South and North America. The Simbu serogroup contains a number of clinically and economically important pathogens, such as OROV, Shuni virus, AKAV and SBV (Table 1-1). The emergence and rapid spread of SBV in Europe in 2011 exemplified the devastating impact emerging arboviruses can have (Gibbens, 2012).

1.2.3.17 Tete Serogroup

The Tete serogroup contains 1 recognised species, the Tete virus sp., to which Tete, Bahig (BAHV), Matruh (MTRV), Tsuruse (TSUV) and Weldona (WELV) viruses have been assigned (Table 1-4) (Elliott and Blakqori, 2011). Tete serogroup viruses have been isolated from wild and domestic birds in Europe, Africa, Asia and North America, and there are no documented cases of human infection (Berge, 1975). The vectors of Tete serogroup viruses vary; MTRV and BAHV have been isolated from hard bodied ticks, whilst WELV has been isolated from *Culicoides* flies, and the vectors of TSUV and TETEV are, as yet, undetermined (Converse *et al.*, 1974; Moussa *et al.*, 1974; Berge, 1975; Calisher *et al.*, 1990).

The Batama virus sp., although currently listed as unclassified, is widely accepted as a member of the Tete serogroup and although no vector has been identified it has been isolated from diverse bird species (Table 1-4) (Berge, 1975; Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012).

1.2.3.18 Turlock Serogroup

Turlock serogroup viruses are associated with *Culex* mosquitoes and birds, and have a wide geographical distribution with isolations in Africa, Asia, Europe, South America, and North America (Berge, 1975; Calisher, Gutierrez, *et al.*, 1983; Mitchell *et al.*, 1987). There are currently 2 virus species, Turlock virus sp. which contains Turlock (TURV), Lednice (LEDV) and Umbre (UMBV) viruses, and the M'Poko virus sp., which contains M'Poko virus (MPOV) and Yaba-1 virus (Table 1-4) (Calisher *et al.*, 1984). Neutralising antibodies to UMBV and MPOV have been found in man in Malaysia and the Central African Republic, respectively, although no virus isolations have been made (Berge, 1975). Moreover, experimental infection of non-human primates with LEDV led to the recovery of antibodies in regional lymph nodes but no viraemia or clinical signs (e.g. raised temperature) were detected (Málková *et al.*, 1976).

1.2.3.19 New Serogroups: Wyeomyia, Mapputta and Sedlec

Three new serogroups have been proposed: Wyeomyia, Mapputta and Sedlec (Newton *et al.,* 1983; Chowdhary *et al.,* 2012; Bakonyi *et al.,* 2013). The Wyeomyia serogroup includes viruses originally classified as members of the Wyeomyia virus sp. in the Bunyamwera serogroup (Chowdhary *et al.*, 2012). Found in South America, Wyeomyia serogroup viruses are primarily vectored by mosquitoes and virus hosts remain largely undetermined with infrequent isolations in birds and rodents (Aitken *et al.*, 1968; Berge, 1975; de Souza Lopes *et al.*, 1975). Neutralising antibodies to Wyeomyia group viruses have been found in man and Wyeomyia virus (WYOV) has been documented as the aetiological agent in 2 cases of self-limiting febrile illness (Sirhongese and Johnson, 1965; Aitken *et al.*, 1968; de Souza Lopes *et al.*, 1975). Evidence that the Wyeomyia virus sp. should be considered a distinct complex from the Bunyamwera serogroup came from the complete genome sequencing of 8 named isolates belonging to the Wyeomyia virus sp. (Chowdhary *et al.*, 2012). Phylogenetic analysis revealed that these isolates form a clade that is distinct from the Bunyamwera serogroup (Chowdhary *et al.,* 2012). Additionally, Wyeomyia viruses lack a full length NSs ORF (Mores *et al.*, 2009; Chowdhary *et al.*, 2012).

The Mapputta serogroup is a complex of antigenically distinct bunyaviruses that are currently unassigned within the *Bunyaviridae* family (Newton *et al.*, 1983; Plyusnin *et al.*, 2012). Mapputta serogroup viruses are vectored by mosquitoes, circulate in Australia and are associated with an acute febrile illness with polyarthritis (Doherty *et al.*, 1963; Doherty *et al.*, 1970; Gard *et al.,* 1973; Wier, 2002). Recent complete genome sequencing of Mapputta serogroup viruses has revealed that these viruses lack a full length NSs ORF and form a distinct clade on phylogenetic analysis that lies within the orthobunyavirus genus (Gauci *et al.*, 2015; Shchetinin *et al.*, 2015).

The Sedlec serogroup was proposed in 2013 following partial sequencing of Sedlec virus (SEDV) (Bakonyi *et al.*, 2013). Originally isolated from a wild bird in the Czech Republic, SEDV did not cross-react with other bunyaviruses in CFTs but morphological characteristics placed it within the *Bunyaviridae* family (Hubalek *et al.*, 1990). Sequencing then revealed that SEDV clustered with 2 unclassified viruses

I612045 and Oyo virus on L segment analysis and branched with Simbu serogroup viruses on S segment analysis (Bakonyi *et al.*, 2013).

1.2.4 Orthobunyavirus Reverse Genetics

Reverse genetics exploits recombinant DNA technology, utilising cloned DNA copies of virus genomes to create infectious recombinant virus particles (Figure 1-14). This allows mutagenesis of the virus genome via plasmid DNA clones and the correlation between genotype and phenotype to be studied. The first reverse genetics system for a segmented negative sense RNA virus was developed for BUNV (Bridgen and Elliott, 1996). BUNV virus segments were cloned as full length cDNA copies into transcription plasmids under the control of a T7 promoter. Transcription of cDNA clones into vRNA was then achieved by transfecting cells expressing bacteriophage T7 RNA polymerase (T7RNAP) and recombinant BUNV proteins (Bridgen and Elliott, 1996). T7RNAP was provided by pre-infecting cells with vTF7-3, a recombinant vaccinia virus which expresses T7RNAP, and BUNV proteins from the transfection of BUNV N, M and L ORF expression plasmids (Jin and Elliott, 1991; Nakitare and Elliott, 1993; Dunn *et al.*, 1995; Bridgen and Elliott, 1996). Rescue supernatants were then passaged in mosquito cells to remove vTF7-3 and enrich recombinant BUNV particles (Bridgen and Elliott, 1996). As reverse genetics systems have progressed the provision of T7RNAP by vTF7-3 has been replaced by transfection of a T7RNAP expression plasmid or by using cell lines that constitutively express T7RNAP, such as the BHK-21 derived cell line BSR-T7/5 (Buchholz *et al.,* 1999; Lowen *et al.*, 2004).

T7RNAP is often the expression system of choice as it can be expressed in a wide range of cell types and localises to the cytoplasm, the site of bunyavirus replication. However, T7RNAP activity is enhanced by a guanosine residue +1 from the 3' end of the T7 promoter sequence, which is incorporated into the vRNA transcript. Although many recombinant viruses tolerate the presence of an additional terminal nucleotide, it can affect recognition by the bunyavirus L proteins, with activity levels differing from non-recombinant viruses. Furthermore, transcription termination by T7RNAP is not precise, although the addition of self-trimming hepatitis-δ ribozyme sequences at the 3' terminus of viral cDNAs can be used to maintain exact terminal sequences and

counteract problems associated with T7RNAP transcription. Alternatively, a parallel nuclear-based reverse genetics system which utilises cellular DNA-dependent RNA-polymerase (Pol-I) to transcribe vRNA copies can be used (Ogawa *et al.*, 2007). Pol-I is more precise than T7RNAP. However, it requires promoter sequences to be optimised for each species of cell line used and helper plasmids containing cell-specific Pol-II promoters for expression of virus N and L proteins.

Orthobunyavirus reverse genetics systems have now been created for a number of viruses, including BUNV, LACV, AKAV and OROV. The technology has allowed recombinant viruses lacking NSs proteins to be extensively studied, unravelling molecular mechanisms behind virus pathogenesis. It has also enabled monitoring of the replication of fluorescently tagged viruses in real-time, and the creation of recombinant viruses with deletions or alanine substitutions has helped elucidate the role of NSm, Gn and Gc proteins in virus particle assembly and maturation. Furthermore, reverse genetics has enabled the function of UTR sequences in virus replication to be analysed and has provided a platform for the creation of chimeric orthobunyavirus vaccine candidates (Bennett *et al.*, 2012).

Chapter 2: Materials

2.1 Cell Lines

Table 2-1: Cell Lines

Cell Line	Description and Cell Maintenance
293FT	Fast growing variant of the 293T cell line, maintained in DMEM supplemented with 10% (v/v) FCS, 1X NEAA and 500 μg ml^{-1} G418 (Graham *et al.*, 1977; Gama-Norton *et al.*, 2011).
293T	Derived from primary human embryo kidney cells transformed with sheared human adenovirus type 5 DNA and SV40 T antigen, maintained in DMEM with 10% (v/v) FCS and 1X NEAA (Graham *et al.*, 1977).
A549	Derived from adenocarcinomic human alveolar basal epithelial cells and maintained in DMEM with 10% (v/v) FCS
A549-NPro	Derivative of A549 cells, which express the NPro protein of bovine viral diarrhea virus (BVDV). NPro induces proteasome-mediated degradation of IRF-3, abolishing IFN-β production (Hilton *et al.*, 2006). A549-NPro cells were maintained in DMEM with 10% (v/v) FCS and 2 μg ml^{-1} puromycin.
A549-IFITM	Derived from the A549 cell line. As part of this study A549 cells were transduced with lentiviral vectors expressing either IFITM1, IFITM2, IFITM3 or empty SCRPSY vector. A549 IFITM cell lines were maintained in DMEM with 10% (v/v) FCS and 2 μg ml^{-1} puromycin to ensure selection of transduced cells.
BHK-21 clone 13	Derived from baby hamster kidney fibroblast cells, maintained in GMEM with 10% (v/v) NCS and 10% (v/v) TPB (MacPherson and Stoker, 1962).
BSR-T7/5	BHK-21-derived cell line that constitutively expresses T7RNAP (Buchholz, Finke and Conzelmann, 1999). BSR-T7/5 cells were maintained in GMEM with 10% (v/v) FCS, 10% (v/v) TPB and 1 mg ml^{-1} G418 to maintain selection of cells expressing T7RNAP.
Flp-In™ T-REx™ 293 MTAP44 and CAT	Flp-In™ T-REx™ 293 cells co-transfected with pOG44 and pcDNA5/FRT/ΔCAT containing FLAG-tagged full length MTAP44 (microtubule associated protein 44, also known as IFI44) or untagged chloramphenicol CAT (chloramphenicol acetyltransferase) cDNA forming stable cells with tetracycline inducible expression of FLAG-tagged MTAP44 or CAT (Jiang *et al.*, 2008). Cells were maintained in DMEM with 10% (v/v) Tet System Approved FCS, 250 μg ml^{-1} hygromycin and 5 μg ml^{-1} blasticidin.
HEK-293	Human embryonic kidney cells, maintained in DMEM with 10% FCS (v/v).
HeLa	Derived from human epithelial cervical cancer cells, maintained in DMEM with 10% FCS (v/v) (Scherer *et al.,* 1953).
Huh7	Derived from human hepatic tumourigenic cells and maintained in DMEM with 10% FCS (v/v) and 1X NEAA.
Huh7-Lunet T7	Huh7 cells transduced to constitutively over-express T7RNAP, maintained in DMEM with 10% FCS (v/v), 1X NEAA and 5 μg ml^{-1} zeocin (Appel *et al.*, 2005; Koutsoudakis *et al.*, 2006).
Vero E6	Derived from African Green Monkey kidney cells and maintained in DMEM with 10% FCS (v/v).

A description of cell lines used in this study is given. DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; NEAA, non-essential amino acids; GMEM, Glasgow minimum essential medium; TPB, tryptose phosphate broth; NCS, newborn calf serum.

2.2 Virus Strains

- Bunyamwera virus (BUNV), the type species of the Bunyamwera serogroup and orthobunyavirus genus as a whole, was kindly provided by Prof Richard M. Elliott (University of Glasgow).

- rBUNdelNSs2, a recombinant BUNV that does not express NSs protein, was kindly provided by Dr Ingeborg van Knippenberg (University of Glasgow) (Bridgen *et al.*, 2001; Hart, Kohl and Elliott, 2009; van Knippenberg, Carlton-Smith and Elliott, 2010).

- Encephalomycocarditis virus (EMCV) is an IFN-sensitive virus that was used to test for the presence of biological IFN in infected cell supernatants.

- 49 other orthobunyaviruses, as detailed in Table 2-2, were kindly provided by Dr Robert Tesh and the late Dr R.E. Shope, both University of Texas Medical Branch, Galveston, Texas.

2.3 Bacterial strains and culture

- Plasmids stocks were grown and maintained in *Escherichia coli* strain JM109: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk – mk +), *rel*A1, *sup*E44, Δ*prolac*AB), [F' *tra*D36, *pro*AB, *laq*IqZΔM15].

- Transformations were carried out with competent JM109 cells, which were created using Mix & Go *E. coli* Transformation Kit & Buffer Set (Zymo Research).

- Bacteria were cultured in liquid phase using 2.5% (w/v) LB Broth Miller (Formedium) and in solid phase using 4% (w/v) LB Agar Miller (Formedium). Dilutions were made with deionised water and transformants selected by the addition of 100 μg/ml ampicillin (Sigma-Aldrich). In addition to ampicillin selection, pGEM®-T easy recombinants were selected by blue/white screening in the presence of IPTG/X-gal.

Table 2-2: Virus Isolates

Virus Name (ABBV)	Serogroup	Strain Information	Date of freeze-dried isolate	Date of first isolation
Anopheles A (ANAV)	Anopheles A	-	DD.MM.1984	DD.MM.1940
Anopheles A (ColAn57389)	Anopheles A	ColAn 57389	13.10.1981	-
Anopheles A (CoAr1071)	Anopheles A	CoAr 1071	05.04.1985	25.03.1985
Anopheles A (CoAr3624)	Anopheles A	CoAr 3624	22.09.1966	12.09.1966
Arumateua (ARTV)	Anopheles A	BeAr 437811	08.12.1986	-
Caraipe (CPEV)	Anopheles A	BeAr 428793	24.10.1984	-
Las Maloyas (LMV)	Anopheles A	AG 80-14	10.09.1981	-
Lukuni (LUKV)	Anopheles A	TR 10076	07.06.1965	-
Tacaiuma	Anopheles A	Sp-Ar 2317	26.05.1971	30.04.1971
Tacaiuma (TCMV)	Anopheles A	BeAn73	21.07.2001	DD.MM.1955
Trombetas (TRMV)	Anopheles A	BeAn 306771	21.09.1981	-
Tucurui (TUCRV)	Anopheles A	BeAR 422535	20.11.1985	-
Virgin River (VRV)	Anopheles A	743-366	13.10.1981	-
Anopheles B (ANBV)	Anopheles B	DMC 1537	15.02.1963	-
Boraceia (BORV)	Anopheles B	SP Ar395	06.12.1965	DD.MM.1962
Bakau (BAKV)	Bakau	MM2325	09.05.1980	DD.MM.1956
Ketapang (KETV)	Bakau	MM2549	15.05.1970	-
Bwamba (BWAV)	Bwamba	M 459	DD.03.1980	-
Pongola (PGAV)	Bwamba	-	17.03.1971	-
Acara (ACAV)	Capim	BeAn 27639	26.06.1966	-
Benevides (BVSV)	Capim	BeAn 153564	26.10.2011	-
Benfica (BENV)	Capim	BeAn 84381	DD.MM.1966	-
Bushbush (BSBV)	Capim	Tr. 26668 #22602	18.01.1985	21.12.1984
Capim (CAPV)	Capim	-	26.06.1966	DD.MM.1961
Guajara (GJAV)	Capim	BeAn 10615	27.03.1968	-
Juan Diaz (JDV)	Capim	Maru 8563	12.06.2001	-
Moriche (MORV)	Capim	-	17.11.1966	15.11.1966
Gamboa (GAMV)	Gamboa	GML 438524	17.12.1990	-
San Juan (SJV)	Gamboa	78V 241	04.12.1981	-
Bertioga (BERV)	Guama	-	28.09.1966	04.01.1966
Guama (GMAV)	Guama	BeAn 277	13.01.1992	-
Koongol (KOOV)	Koongol	MRM 31	20.06.1966	-
Wongol (WONV)	Koongol	MRM 168	02.06.1966	06.06.1966
Minatitlan (MNTV)	Minatitlan	70439 #168	30.04.1974	25.04.1974
Palestina (PLSV)	Minatitlan	76V 1565	31.08.1982	-
Eretmapodites (EREV)	Nyando	E147V	10.07.1966	-
Nyando (NDV)	Nyando	-	11.10.2003	-
Olifantsvlei (OLIV)	Olifantsvlei	SA Ar 5133	28.07.1964	-
Botambi (BOTV)	Olifantsvlei	BA937	08.06.1970	-
Pahayokee (PAHV)	Patois	-	25.09.1968	14.09.1968
Patois (PATV)	Patois	-	22.12.1966	-
Bahig (BAHV)	Tete	Eg B-90 #22607	07.01.1985	21.12.1984
Batama (BMAV)	Tete	DAK AnB1292	15.12.1984	DD.MM.1970
Matruh (MTRV)	Tete	EgAn 1047-61	12.07.1985	17.06.1985
Tete (TETEV)	Tete	SA An3518	15.12.1984	DD.MM.1959
Tsuruse-like (TSUV)	Tete	Magpie 271580	03.11.1999	-
Weldona (WELV)	Tete	76V 21935	30.04.2009	-
M'Poko (MPOV)	Turlock	BA365	10.03.2003	-
Turlock (TURV)	Turlock	MP847-32	09.02.1959	-

Viruses used in this study that were obtained from the World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, Texas, USA. Virus name (abbreviation), serogroup, and where available, strain information and isolate history are given. Dates are formatted as DD.MM.YYYY.

2.4 Plasmids

- Plasmids were purified using the PureYield™ Plasmid Miniprep System (Promega, A1223) and NucleoBond® Xtra Midi kit (Macherey-Nagel, 74041.50).

- Expression plasmids, listed in Table 2-3, were used in 5 plasmid rescue systems, luciferase reporter assays, lentivirus construction and protein expression.

- Transcription plasmids, described in Table 2-4, were used in viral rescue systems. Viral segments requiring sequence confirmation following HTS and *de novo* assemblies were also cloned into the pTvT7 backbone, as this would facilitate future development of rescue systems if needed.

- pGEM®-T Easy vector was supplied as part of the pGEM®-T Easy Vector System I (Promega, A1360) and was used for sequence confirmation of viral fragments. The plasmid contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning site located within the α-peptide coding region of the β-galactosidase enzyme, which undergoes insertional inactivation allowing the selection of recombinants by blue/white screening. The plasmid is supplied as linearised plasmid with single thymidine overhangs at both ends of the insertion site.

2.5 Oligonucleotides

Oligonucleotides were purchased from Integrated DNA technologies (IDT) at 25 nmoles scale with standard desalt purification. Primer sequences are listed in Appendix 1 and split into the following categories:

A) Primers for RACE-PCR and UTR sequencing

B) Primers for sequence confirmation (PCR products, pGEM-T easy)

C) Primers for sequence confirmation (pTvT7 clones)

D) Primers for BMAV reverse genetics and structural studies

E) Primers for cloning virus ORFs into pCMV-empty

Table 2-3: Expression Plasmids

Plasmid	Description	Source/Reference
pTM1	The pTM1 plasmid contains bacteriophage T7 promoter followed by encephalomyocarditis (EMCV) internal ribosome entry site (IRES), cloning site and a T7 terminator sequence. Transcripts contain the EMCV IRES element upstream of the cloned ORF to allow expression in mammalian cells lines expressing T7RNAP.	Moss *et al*., 1990
pTM1-BMAV pTM1-BMAV M pTM1-BMAV L	pTM1 backbone containing the complete coding sequence for BMAV N, M and L proteins respectively, under the control of the T7 promoter and EMCV IRES.	This study
pIFΔ(-125) lucter	Firefly luciferase under the control of the IFN-β promoter.	Prof R.M Elliott, UoG
phRL CMV	Humanised Renilla luciferase under the control of a CMV immediate early promoter.	Promega
pCMV	The coding sequence for humanised Renilla luciferase was removed from the phRL CMV plasmid by excision PCR to create a pCMV vector which could be used to drive expression of viral proteins in mammalian cells.	This study
pCAGGS-2CARD	pCAGGS expression plasmid encoding 2 tandem amino-terminal caspase activation and recruitment domains (CARDs) of the Retinoic-acid-inducible gene-I (RIG-I).	Dr Ben Hale, UoG
pCMV-BUNVN pCMV-BUNVNSs	pCMV backbone containing the complete coding sequence for BUNV N and NSs proteins respectively, under control of the CMV promoter.	This study
pCMV-27N pCMV-27M pCMV-27L	pCMV backbone containing the complete coding sequence for BMAV N, M and L proteins respectively, under control of the CMV promoter.	This study
pCMV-22N pCMV-22NSs	pCMV backbone containing Weldona virus N and NSs ORFs.	This study
pCMV-20ORF	pCMV containing a predicted ORF in MTRV S segment (cRNA 465 – 665 bp).	This study
pCMV-34NSs	pCMV backbone containing Eretmapodites virus NSs ORF.	This study
pCMV-29NSs	pCMV backbone containing Nyando virus NSs ORF.	This study
pCMV-48NSs	pCMV backbone containing M'Poko virus NSs ORF.	This study
pCMV-36NSs	pCMV backbone containing Gamboa virus NSs ORF.	This study
pEHISTEV	Expression vector derived from pET used for soluble protein expression for structural biology studies. The vector contains a multiple cloning site flanked by an amino terminal hexahistidine tag, and tobacco etch virus protease cleavage site.	Liu and Naismith, 2009
pSCRPSY-IFITM	HIV-1 based gateway-compatible lentiviral vector that uses the endogenous splicing machinery of HIV-1 to co-express TagRFP, the puromycin resistance gene and either IFITM-1, -2 or -3 from a Tag-RFP-IFITM-Puro cassette, which is downstream of a CMV promoter and EMCV IRES site.	Schoggins *et al.*, 2012
pCMVR8.91	Packaging plasmid used in lentivirus construction, which expresses HIV-1 gag and pol proteins	Zufferey *et al.*, 1997
pVSV-G	Expression plasmid which encodes VSV-G (vesicular stomatitis virus envelope G protein) under a CMV promoter.	Burns *et al.*, 1993

A description of expression plasmids used in this thesis is given. Unless stated, plasmids are ampicillin resistant in bacteria. University of Glasgow (UoG).

Table 2-4: Transcription Plasmids

Plasmid	Description	Source
pTvT7R(0,0)	pTvT7R(0,0) plasmid is a T7 RNA polymerase transcription plasmid with an additional G in the +1 position upstream of the T7 promoter to facilitate efficient T7 transcription. The insert site is immediately followed by a hepatitis Delta ribozyme sequence and the T7 terminator sequence.	Johnson *et al.,* 2000
pTvT7-ANAVSS	pTvT7R(0,0) plasmid containing full length Anopheles A virus antigenomic S segment sequence under control of the T7 promoter.	This study
pTvT7-CA5739S	pTvT7R(0,0) plasmid containing full length Anopheles A ColAn 57389 antigenomic S segment sequences respectively, under control of the T7 promoter.	This study
pTvT7-E147VS pTvT7-E147VM pTvT7-E147VL	pTvT7R(0,0) plasmid containing full length Eretmapodites virus antigenomic S, M and L segment sequences respectively, under control of the T7 promoter.	This study
pTvT7-JDVS	pTvT7R(0,0) plasmid containing full length Juan Diaz virus antigenomic S segment sequences under control of the T7 promoter.	This study
pTvT7-LMVS pTvT7-LMVM	pTvT7R(0,0) plasmid containing full length Las Maloyas virus antigenomic S and M segment sequences respectively, under control of the T7 promoter.	This study
pTvT7-MTRVS	pTvT7R(0,0) plasmid containing full length Matruh virus antigenomic S segment sequences under control of the T7 promoter.	This study
pTvT7-TCMVS	pTvT7R(0,0) plasmid containing full length Tacaiuma virus antigenomic S segment sequences under control of the T7 promoter.	This study
pTvT7-TETEVS	pTvT7R(0,0) plasmid containing full length Tete virus antigenomic S segment sequences under control of the T7 promoter.	This study
pTvT7-TRMV	pTvT7R(0,0) containing full length Trombetas virus antigenomic S segment sequence under control of the T7 promoter.	This study
pTvT7-TSUVS pTvT7-TSUVM	pTvT7R(0,0) plasmid containing full length Tsuruse-like antigenomic S and M segment sequences respectively, under control of the T7 promoter.	This study
pTvT7-WELVS	pTvT7R(0,0) containing full length Weldona virus antigenomic S segment sequence under control of the T7 promoter.	This study
pTvT7-BMAVS pTvT7-BMAVM pTvT7-BMAVL	pTvT7R(0,0) plasmid containing full length Batama virus antigenomic S, M and L segment sequences respectively, under control of the T7 promoter.	This study
rBMAVBu25N	Modified pTvT7-BMAVS in which the N terminal 25 amino acids of the N protein have been modified to mimic the termini of BUNV N protein.	This study
rBMAVBu29N	Modified pTvT7-BMAVS in which the N and C terminal amino acids of the N protein have been modified to mimic the termini of Bunyamwera virus N protein.	This study
rBMAVWelN	Modified pTvT7-BMAVS in which the N and C terminal amino acids of the N protein have been modified to mimic the termini of Weldona virus N protein.	This study
rBMAVWelUN	Modified pTvT7-BMAVS in which the N and C terminal amino acids of the N protein have been modified to mimic the termini of Weldona virus N protein and UTR sequences have been switched with those of Weldona virus.	This study
rBMAVn6A rBMAVn12A rBMAVn18A rBMAVn24A rBMAVn2aA rBMAVn2bA rBMAVn2cA rBMAVn2dA rBMAVn2eA rBMAVn2fA rBMAVn3aA rBMAVn3bA rBMAVn3cA rBMAVn3dA rBMAVn4xA	Modified pTvT7-BMAV S plasmid containing alanine substitutions at N protein amino acid residues 6, 12, 18 and/or 24. Detailed descriptions in Table 7-1.	This study
rBMAVn11Δ rBMAVn13Δ rBMAVn19Δ rBMAVn24Δ rBMAVn2aΔ rBMAVn2bΔ rBMAVn2cΔ rBMAVn2dΔ rBMAVn2eΔ rBMAVn2fΔ rBMAVn3aΔ rBMAVn3bΔ rBMAVn3cΔ rBMAVn3dΔ rBMAVn4xΔ	Modified pTvT7-BMAV S plasmid in which N protein amino acid residues 11, 13, 19 and/or 24 have been deleted. Detailed descriptions in Table 7-1.	This study

A description of transcription plasmids used in this thesis is given. rBMAV transcription plasmids are described in detail in Chapter 7, Table 7-1.

2.6 Antibodies

Table 2-5: Primary Antibodies

Target	Antibody	Dilutions	
		WB	IF
BUNV N	Rabbit anti-BUNV (non-commercial, bleed 593)	1:1000	1:2000
BUNV NSs	Rabbit anti-BUNV (non-commercial)	1:500	-
FLAG epitope	Mouse anti-FLAG M2 (Sigma-Aldrich)	1:2000	1:100
IFI44	Mouse anti-IFI44 (Ab-cam)	1:500	1:50
IFITM1	Rabbit anti-IFITM1 (Abgent)	1:1000	-
IFITM2	Rabbit anti-IFITM2 (Abgent)	1:1000	-
IFITM3	Rabbit anti-IFITM3 (Abgent)	1:1000	-
MxA	Rabbit anti-MxA (Santa-Cruz)	1:500	-
Tubulin	Mouse anti-tubulin (Cell Signaling)	1:3000	1:100

A description of primary antibodies used in western blotting (WB) and immunofluorescence (IF).

Table 2-6: Secondary Antibodies

Target	Antibody	Dilutions	
		WB	IF
Mouse IgG	Anti-mouse HRP (New England BioLabs)	1:2000	-
Rabbit IgG	Anti-rabbit HRP (New England BioLabs)	1:3000	-
Mouse IgG	Anti-mouse IgG Cy5 (New England BioLabs)	-	1:200
Rabbit IgG	Anti-rabbit IgG FITC (New England BioLabs)	-	1:200

A description of secondary antibodies used in western blotting (WB) and immunofluorescence (IF).

2.8 Chemicals and Buffers

2.8.1 Multipurpose Chemicals

- Ethanol (Various, molecular grade)
- Isopropanol (Various)
- Formaldehyde (Fisher Scientific, F/1501/PB17)
- Glycerol (BDH, 101186M)
- Igepal CA-630: Gima I3021-500ML
- HEPES: HEPES, free acid (Melford, B2001)
- Methanol (Fisher Scientific, M/4000/17)

- Phosphate buffered saline (PBS) (Various)
- Sodium azide (Sigma Aldrich, S2002-500G)
- Sodium dodecyl sulphate (Sigma Aldrich L4509-500G)
- Sucrose (Sigma Aldrich, S7903-1KG)
- Tris-HCl (Sigma Aldrich, T5941-500G)

2.8.2 Mammalian Cell Culture

- Avicel RC/CL: Microcrystalline cellulose and sodium carboxymethylcellulose (CAS# 51395-75-6, FMC BioPolymer)
- Avicel overlay 0.6% (w/v): 1.2% (w/v) Avicel RC/CL diluted at a ratio of 1:2 at time of use with 2X MEM (20% (v/v) 10X MEM, 2% (v/v) L-glutamine, 0.435% (v/v) sodium bicarbonate, 4% (v/v) FCS, diluted in sterile water
- Blasticidin S (Life Technologies, A1113903)
- Crystal violet solution: 1 g methyl violet (Fisher Scientific) dissolved in 200 ml ethanol and 10 ml methanol, and made up to a final volume of 1 L with distilled water. Stored at room temperature shielded from light.
- Dulbecco's modified Eagle media: DMEM (1X) (Life Technologies, 41966-029)
- DMEM (Met-): DMEM without L-methionine, L-cysteine and L-glutamine (Sigma-Aldrich, D0422-100ML)
- DMSO (Sigma Aldrich, D8418-100ML)
- Fetal calf serum (FCS) (Gibco, various)
- Formaldehyde fixing buffer: 8% (v/v) formaldehyde in PBS
- G418: Geneticin™ (Life Technologies, 10131035)
- Glasgow minimum essential medium: G-MEM BHK-21 (1X) (Life Technologies, 21710-025)
- Hygromycin B (Calbiochem, Merck Millipore International, 400052-20ML)
- Interferon: Universal type I IFN alpha (PBL, 11200-2)
- L-glutamine (Life Technologies, 25030-024)
- Minimum essential medium: MEM (10X) (Life Technologies, 21430-020)
- Non-essential amino acids: MEM NEAA (100X) (Life technologies, 11140-035)
- Newborn calf serum (NCS) (Gibco, various)

- Opti-MEM: Opti-MEM® I with GlutaMax-I (Life Technologies, 51985-026)
- Pen/Strep (Life Technologies, 15140-122)
- Plasticware: CELLSTAR® (VWR, various), Nunc® (Sigma-Aldrich, various) and Corning® (Sigma-Aldrich, various)
- Puromycin (Life Technologies, A1113803)
- Sodium bicarbonate 7.5% (Life Technologies, 25080-60)
- Tet System Approved FCS (Clontech, 631101)
- Tetracycline hydrochloride (Sigma-Aldrich, T-3383)
- Trypsin EDTA 0.05% (Life Technologies, 25300-054)
- Tryptose phosphate broth (TPB) (Life Technologies, 18050-039)

2.8.3 Transfection of Cultured Cells

- Dual luciferase® Reporter Assay System (Promega, E1960)
- Lipofectamine 2000 (Life Technologies, 11668027)
- *Trans*IT®-LT1 (Mirus Bio, MIR 2300)
- Polystyrene tubes: 5 ml polystyrene tubes, 12 x 75 mm style (SLS, 352054)

2.8.4 Immunofluorescence

- Mowiol® 4-88 (Calbiochem, 475904)
- Permeabilisation buffer: PBS with 5% (v/v) FCS and 0.3% (v/v) Triton X-100 or PBS with 1% (w/v) BSA and 0.1% (w/v) saponin
- Saponin (Sigma Life science, 84541-100G)
- Triton X-100 (Sigma Aldrich, X100-100ML)

2.8.5 Protein Analysis

- Benzonase: Benzonase, purity >90% (Novagen®, 70746)
- Bromophenol Blue: PlusOne bromophenol blue (Amersham Bioscience, 17-1329-01)

- BSA: Bovine serum albumin (Sigma Aldrich, A2153-50G)
- DTT: Dithiothreitol (Melford, MB1015)
- Horseradish peroxidase (HRP) substrate: SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080)
- MES SDS running buffer: NuPAGE® MES SDS Running Buffer (20X) (Life Technologies, NP0002-02) diluted to 1X in distilled water
- Nitrocellulose membrane: Hybond-C Extra 0.45 μm (GE Healthcare, RPN303E)
- Protein disruption buffer (PDB): 100 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 0.02% (w/v) bromophenol blue, 200 mM DTT and 125 U/ml benzonase
- Protein gels: NuPAGE® SDS gel 4-12% Bis Tris (Life Technologies, various)
- Protein ladder: PageRuler^{TM} Plus Prestained Protein Ladder (Life Technologies, 26619)
- Transfer buffer: NuPAGE® Transfer Buffer (20X) (Life Technologies, NP0006-1)
- Western blot blocking buffer: 5% (w/v) skimmed milk powder diluted in PBS, 0.1% (v/v) Tween-20
- Western blot washing buffer: PBS, 0.1% (v/v) Tween-20

2.8.6 Nucleic Acid Isolation and Analysis

- Agarose: Molecular grade agarose (Various)
- DNA ladders: 100 and 1000 bp with loading dye (6X) (Promega, G2101 and G5711)
- DNA polymerase: GoTaq® Hot Start polymerase (Promega, M5006) and KOD hot start DNA polymerase (Merck Millipore, 71086-4)
- DNase: RQ1 RNase-Free DNase (Promega, M6101)
- dNTP Mix: 10 mM dNTP Mix (Promega, U1511)
- DpnI (Promega, R6231)
- E-PAP: *E. coli* Poly(A) polymerase I (Life Technologies, AM1350)
- Ethidium bromide solution (Various)
- Klenow Fragment (3'→5' exo-) (New England BioLabs, M0212S)
- Magnetic bead DNA purification: Agencourt AMPre XP system (Beckman Coulter, A63880)

- PEG-8000, nuclease free (Sigma-Aldrich, P5413)
- Poly(A) Tailing Kit (Life Technologies, AM1350)
- QIAamp® Viral RNA Mini kit (Qiagen, 52904)
- Random primers (Promega, C1181)
- Reverse Transcriptase (RT): GoScript™ RT (Promega, A5003), M-MLV (H-) Point mutant RT (Promega, M3681), and Transcriptor RT (Roche, 03531295001).
- RNasin: Recombinant RNasin® Ribonuclease Inhibitor (Promega, N2511)
- RNeasy Mini Kit (Life Technologies, 74104)
- RNase (H) (Life Technologies, 18021014)
- Sequencing library preparation: KAPA Library Preparation and Adapter Kits for Ion Torrent™ Platforms (Kapa Biosystems, KK8301, KK8331, KK8332, KK8333)
- Series II RNA 6000 Nano Reagents (Agilent Technologies, 5067-1511)
- SuperScript® III reverse transcriptase (Life Technologies, 18080044)
- T4 DNA ligase (Promega, M1801)
- T4 RNA ligase 1 (ssRNA Ligase) (New England BioLabs, M0204L)
- TAE (Tris-acetic acid-EDTA) buffer: 40 mM Tris base (w/v), 12%(v/v) acetic acid, 0.1mM (w/v) EDTA diluted in sterile water
- TNE buffer: 10mM Tris pH 6.5, 1 mM EDTA, 100mM NaCl
- TRIzol reagent (Life Technologies, 15596026)
- Wizard® SV Gel and PCR Clean-Up System (Promega, A9282)

Chapter 3: Methods

3.1 Cell Culture

3.1.1 Cell Maintenance

Cell cultures were maintained in vented 75 cm^2 flasks and split every 3 - 4 days as required. Cell cultures were split by removing the cell culture medium and washing the cell monolayer with 5 ml sterile PBS. Cells were then washed in 5 ml of 0.05% trypsin-EDTA and placed at 37°C with ≤ 0.5 ml residual trypsin remaining to aid cell detachment. Cells were resuspended in 10 ml cell culture media, seeded to new flasks at a final ratio of 1:10 with additional fresh cell culture medium and incubated at 37°C in a 5% CO_2 humidified atmosphere.

3.1.2 Liquid Nitrogen – Cell Storage and Resuscitation

To maintain low passage stocks cell lines were stored in liquid nitrogen. Cell monolayers at low passage number in vented 75 cm^2 flasks were trypsinised as per cell maintenance. The resuspended cells were then pelleted by centrifugation at 1700 x g for 5 minutes (min) and the supernatant discarded. The cells were then resuspended in 5 ml cell culture medium containing 10% (v/v) DMSO and 20% (v/v) serum and stored overnight at -80°C, after which they were transferred for long term storage in liquid nitrogen.

Cells from liquid nitrogen were rapidly thawed in 37°C water bath. To remove DMSO cells were washed twice in 12 ml ice cold medium by centrifugation at 200 x g for 5 min. The cell pellet was then gently resuspended and cells seeded into vented 75 cm^2 flasks with the appropriate cell culture medium, selective antibiotic and 20% (v/v) serum. Cells were incubated overnight at 37°C in a 5% CO_2 humidified atmosphere to allow cell attachment, and the cell culture media was then changed to remove dead cells and cell debris. Cells were then maintained with normal levels of serum as described in section 3.1.1.

3.1.3 Determination of Selective Antibiotic Concentration

The optimal concentration of puromycin for the selection of transduced A549 cells was determined by kill curve experiment. A549 cells were seeded at 2 x 10^5 cells ml^{-1}, 500 μl per well, in 24-well cell culture plates. Six hours after seeding cells were checked for attachment and puromycin added at final concentrations of 0, 1, 2, 4, 8 and 10 μg ml^{-1} to cell culture media. Concentrations were tested in triplicate and cells checked every 24 hours (h) for cell death. At 3 days post antibiotic addition cell media was changed, with fresh antibiotic added. The optimal concentration was determined as the concentration needed to kill 90% of cells in 3 – 5 days. At day 4, 90% of cells in all wells with 2 μg ml^{-1} puromycin had died, and 2 μg ml^{-1} was chosen as the optimal concentration for antibiotic selection of A549-derived cell lines.

3.2 Virus Propagation

3.2.1 Preparation of Lentivirus Pseudoparticles

SCRPSY plasmids encoding puromycin resistance and either TagRFP alone, or TagRFP and IFITM protein were kindly provided by Dr Sam Wilson, University of Glasgow. The SCRPSY-based plasmids were co-transfected into subconfluent 293FT cells in vented 75 cm^2 flasks with pVSV-G and pCMVR8.91 plasmids at a ratio of 5:3:3, respectively to create lentivirus pseudoparticles. Transfections were carried out with Lipofectamine 2000 as per manufacturer's instructions in a total volume of 6 ml Opti-MEM for 5 h at 37 °C. Following incubation, the transfection mix was removed and the cell monolayer washed once with 5 ml DMEM 10% (v/v) FCS. Cells were then incubated at 37°C with 10 ml DMEM 10% (v/v) FCS and cell supernatant harvested 24 h p.i. and stored at 4°C. Fresh medium was then added to the cell monolayer, and the cells incubated for a further 24 h; at this point the supernatant was harvested and pooled with that collected 24 h earlier. The pooled supernatant was then clarified by centrifuging at 1700 x g for 5 min, passed through a 0.45 μm Truffryn or PVDF filter and stored in 1.5 ml aliquots at -80°C.

3.2.2 Lentivirus Transduction

Subconfluent A549 cells in vented 25 cm^2 flasks were transduced with lentivirus pseudoparticles. Briefly, cells were washed with 2 ml serum free DMEM prior to the addition of 1 ml DMEM supplemented with 8 μg ml^{-1} polybrene and 500 μl lentivirus pseudoparticles or 500 μl DMEM for the mock-transduced control. Following incubation for 2 h at 37°C, 2 ml DMEM 10% (v/v) FCS was added directly to each flask, without removing the lentivirus inoculum. At 48 h p.i. cell culture medium was removed and replaced with DMEM 10% (v/v) FCS supplemented with 2 μg ml^{-1} puromycin. Cells were then monitored for TagRFP expression and cultures split as required.

3.2.3 Virus Rescue

Recombinant viruses were created using a 3 plasmid rescue system. Five microlitres *Trans*IT-LT1 was mixed with 175 μl Opti-MEM per reaction in a polystyrene tube, and incubated at room temperature for 5 min. Five hundred nanograms of 3 pTvT7 plasmids encoding the complete S, M and L virus genome segments in the antigenome orientation were then mixed with 175 μl Opti-MEM and slowly added to the *Trans*IT-LT1 Opti-MEM mix. This solution was gently mixed by pipetting and incubated at room temperature for 30 min. Subconfluent BSR-T7/5 cells in 6-well cell culture plates were then washed with 1 ml Opti-MEM and 350 μl transfection mix/well pipetted directly onto the cell monolayer. Cells were then incubated at 37°C for 1 h, prior to the addition of 2 ml cell culture medium, and then moved to 33°C. Twenty-four hours post-transfection the cells were washed and fresh medium added to minimise transfection reagent toxicity. Supernatant was harvested 6 days post-transfection, clarified by centrifuging at 1700 x g for 5 min and stored at -80°C. Longer incubations of up to 14 days were tested for mutant viruses, although this did not increase the success of virus resuce.

3.2.4 Preparation of Virus Stocks

Freeze-dried and lyophilised virus isolates were resuspended in 0.5 to 1 ml sterile PBS, as specified by isolate labels. Elite virus stocks were created by infecting subconfluent Vero E6 cells in 25 cm^2 vented flasks with 100 µl inoculum (rescue supernatant or freeze-dried virus suspension) and 400 µl OptiMem for 1 h at 37°C, 5% CO_2 in a humidified atmosphere. Four point five millilitres cell culture media was then added and cells incubated at 33°C, 5% CO_2 in a humid atmosphere until cytopathic effect (CPE) reached 90% or for 7 days. When generating elite virus stocks from freeze-dried virus isolates, cell culture media and the first inoculum were supplemented with 100 U ml^{-1} Pen/Strep; all further passages of freeze-dried virus isolates were carried out in antibiotic-free media.

Working stocks were then created by infecting subconfluent Vero E6 cells in vented 150 cm^2 flasks with 100 µl elite stock and 4 ml serum free medium for 1 h at 37°C 5% CO_2 in a humidified atmosphere. Twenty-one millilitres cell culture medium was then added and cells incubated at 33°C, 5% CO_2 in a humid atmosphere until CPE reached 90% or for 7 days.

3.2.5 Blind Passage of Virus Isolates

Elite stocks of freeze-dried virus isolates were blind passaged in vented 25 cm^2 flasks of subconfluent Vero E6 cells until the production of CPE or passage 8. Briefly, 7 days d p.i. elite stock supernatants were collected and centrifuged for 5 min at 1700 x g. One hundred microlitres clarified culture supernatant was then used to infect subconfluent Vero E6 cells as per the protocol for generating elite virus stocks. Additionally, 1 ml aliquots of each passage were stored at -80°C.

3.2.6 Virus Titration by Plaque Assay

Virus titre was determined by plaque assay on Vero E6 cells. Cells were seeded at $5x10^5$ cells per well in 6-well cell culture plates 18 - 22 h prior to infection. Cell culture

medium was then removed and replaced with 200 µl of virus supernatant that had been serially diluted in sterile PBS, 2% (v/v) FCS. Cells were then incubated for 1 h at 37°C in 5% CO_2, and rocked every 15 min to prevent drying. Two millilitres 0.6% (w/v) Avicel overlay was then added and cells were incubated at 33°C for 6 to 14 days. The cell monolayers were then fixed for 1 h in 4% (v/v) formaldehyde, and monolayers stained in crystal violet for 15 min at room temperature. The stain was removed by rinsing the plates in water and virus titre calculated as number of plaque forming units (p.f.u) per ml using the formula: t = (p/d) x 5, where t = titre in p.f.u ml^{-1}, p = number of plaques, d = dilution factor of counted well.

3.2.7 Virus Yield Assays

Virus yield assays were carried out in 6-well plates. For experiments with Flp-In™ T-REx™ 293 cell lines, cells were induced with 1 µg ml^{-1} tetracycline or mock-induced with PBS for 48 h prior to infection. For all virus yield assays subconfluent cells were infected at the specified multiplicity of infection (MOI) for 1 h at 37°C in 5% CO_2. Virus dilutions were carried out in Opti-MEM, which also acted as the negative control in mock infections. Following infection cell monolayers were washed three times with 1 ml Opti-MEM prior to the addition of fresh medium and incubation at 37°C in 5% CO_2. Cell monolayers and supernatants were harvested at specified time points. Supernatants were clarified by centrifuging at 1700 x g for 10 min and stored at -80°C for viral titre determination by plaque assay. Pellets were resuspended in 100 µl protein disruption buffer and pooled with the corresponding cell monolayer to which another 200 µl protein disruption buffer was added. Cell lysates were stored at -20°C for analysis by Western Blot.

3.3 Interferon Experiments

3.3.1 Induction of Interferon-Stimulated Genes

Cells were stimulated with IFN by the addition of universal IFN at a final concentration of 1000 U ml^{-1} for 4 h – 24 h. To test for ISG stimulation cell monolayers were

harvested at specified time points in 300 μl PDB and stored at -20°C for analysis by Western Blot.

3.3.2 Resistance to Interferon

Virus resistance to IFN was measured by treating Vero E6 cells seeded at 1.5 x 10^5 cells ml^{-1} with 100, 1 000 and 10 000 U ml^{-1} IFN 24 hours prior to infection at MOI 0.01. Supernatants were then harvested at 48 h p.i. and virus titre determined by plaque assay.

3.3.3 Interferon-β Promoter Reporter Assays

Two reporter assays measuring activation and antagonism of the IFN-β promoter were carried out using the Dual-Luciferase Reporter System. Luciferase activity was measured on the Glomax® 96 Microplate Luminometer (Promega) with a 2 s delay and 1 s integration time.

Induction of the IFN-β promoter during virus infection was assessed by transfecting subconfluent A549 cell monolayers in 6-well cell culture plates with 750 ng pIFΔ(-125)lucter and 5 ng phRL-CMV for 5 h at 37°C with rocking every 20 min to prevent drying of the cell monolayer. The transfection mix was then removed, cells washed 3 times with 1 ml Opti-MEM and either infected at an MOI of 1 with the specified viruses or mock infected with Opti-MEM. Following infection cell monolayers were washed 3 times with 1 ml Opti-MEM prior to the addition of cell culture medium and incubation at 37°C in 5% CO_2. Cell monolayers were harvested at 18 h p.i. in 1X passive lysis buffer as per manufacturer's instructions and assayed for luciferase activity.

A second reporter assay measuring antagonism of the IFN-β promoter by viral proteins was also performed using the Dual-Luciferase Reporter System. Subconfluent 293T cells in 12-well cell culture plates were transfected with 25 ng p2CARD-CAGGS to stimulate the IFN-β promoter, 750 ng p(-125)IFNlucter to measure activity of the IFN-

β promoter, 5 ng phRL-CMV to allow normalisation to Renilla, 10 ng - 500 ng viral proteins under control of the CMV promoter, and empty pCMV plasmid to equalise the concentration of DNA across all reactions. Cells were harvested 18 h p.i. in passive lysis buffer as per manufacturer's instructions.

For reporter assays transfection reagent, Lipofectamine 2000 or *Trans*IT-LT1 was mixed with Opti-MEM in polystyrene tubes, and incubated at room temperature for 5 min. Plasmid DNA was mixed with Opti-MEM, added to the transfection/Opti-MEM solution, gently mixed by pipetting and incubated at room temperature for 30 min. The cell monolayer was then washed with 1 ml Opti-MEM and transfection mix added directly to cells. Cells were then incubated at 37°C in 5% CO_2 for 1 h for all cell types except the A549 cell lines, which were incubated for 5 h. Following incubation transfection mix was removed and the appropriate cell culture medium added.

3.3.5 Biological Interferon Assay

Subconfluent A549 cells were infected at an MOI of 1 with the specified viruses, or mock infected with Opti-MEM, for 1 h at 37°C in 5% CO_2. Following infection cell monolayers were washed 3 times with 1 ml Opti-MEM prior to the addition of cell culture medium and incubation at 37°C in 5% CO_2. Forty-eight hours post infection supernatant was harvested and clarified by centrifugation at 1700 x g for 5 min. Clarified culture supernatant was then UV-inactivated by placing in a fresh 6-well cell culture plate and placing a UV light tube directly over the plate with no lid on for 2 min. The plate was then agitated to mix the supernatant before exposure to UV light for a further 2 min. One hundred microlitres of 2-fold dilutions of the UV-irradiated supernatant were then applied to A549-NPro cells in 96-well cell culture plates and incubated for 24 h at 37°C. Without removing the UV-irradiated supernatant encephalomyocarditis virus (EMCV), which is sensitive to IFN, was then added to the A549-Npro cells at a final dilution of 1:10 000. Cell monolayers were then fixed in 4% (v/v) formaldehyde 72 h post EMCV infection and stained with crystal violet to measure cell death. Intact cell monolayers correspond with the death of EMCV and thereby the presence of IFN in the UV-inactivated supernatants.

3.4 Nucleic Acid Manipulation and Cloning

3.4.1 Extraction of Total Cellular and Virion RNA

Total cellular RNA for IFN-β mRNA PCR was extracted using TRIzol as per manufacturer's instructions from A549 cells infected at MOI of 1, 18 h p.i. Virion RNA was extracted using the QIAamp® Viral RNA Mini kit (Qiagen), with carrier RNA as per manufacturer's instructions.

3.4.2 RNA Extraction for High-throughput Sequencing

20 ml working virus stock was harvested and clarified by centrifugation at 1000 x g for 10 min. The resulting supernatant was then further clarified by centrifugation at 3000 x g for 5 min in a fresh Falcon tube. The supernatant was then passed through a 0.2 μm filter and incubated at 4°C for 30 min with 10.8 ml sterile PEG-8000 in TNE buffer and 1.56 ml sterile 5M NaCl. Virions were then precipitated by centrifugation at 4000 x g for 1 h 45 min at 4°C. The pellet was resuspended in 500 μl PBS, 25 U benzonase and incubated at 37°C for 30 min. RNA was then extracted with 2 ml TRIzol according to the manufacturer's instructions.

3.4.3 DNase Treatment

Approximately 2 μg RNA was incubated with 2 μl (2 U) DNase in a total volume of 20 μl at 37°C for 30 min, followed by heat inactivation at 65°C for 15 min.

3.4.4 RNA Ligation

Twelve microlitres virion RNA was denatured by heating at 90°C for 3 min and immediately cooled on ice. Twelve microlitres denatured RNA, 10 U rRNasin, 1X T4 RNA ligase 1 buffer, 20 U T4 RNA ligase 1 (ssRNA ligase), and 20 mM ATP were

heated at 37°C for 2 h, followed by heat inactivation at 65°C for 15 min. Ligated RNA was then amplified by RT-PCR and sequenced as per section 3.4.5, 3.4.7 and 3.4.10.

3.4.5 Standard Reverse-transcription

For IFN-β mRNA RT-PCR with γ-actin controls, 10 μl DNase treated total cellular RNA was heated at 70°C for 5 min with 1 μl (500 ng) random primers. For all other RT reactions 12 μl virion RNA and 1 μl of 100 μM target-specific RT primer were heated at 70°C (90°C for ligated RNA) for 5 min and immediately cooled on ice. Standard RT reactions were carried out using M-MLV based systems: M-MLV (H-) Point mutant RT or GoScript™ RT. The change from M-MLV (H-) Point mutant RT to GoScript™ RT was made following relocation from The University of St Andrews to The University of Glasgow and the differing availability of kits at onsite stores. GoScript™ reactions were carried out in a final volume of 25 μl with 13 μl heat-denatured RNA/Primer mix, 5 μl 5X GoScript™ buffer, 3 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTPs, 40 U rRNasin, 1 μl GoScript™ RT. The RT reaction was incubated for 1 h at 42°C. M-MLV (H-) Point mutant reactions were carried out in a final reaction volume of 20 μl with 13 μl heat denatured RNA/Primer mix, 4 μl 5X M-MLV buffer, 1 μl 10 mM dNTPs, 40 U rRNasin®, 200 U M-MLV (H-) Point mutant RT. The RT reaction was incubated for 1 h at 37°C.

3.4.6 Rapid Amplification of cDNA Ends

Virion RNA was poly-A tailed using the Poly(A) Tailing Kit (Ambion). Each 25 μl reaction contained 14 μl virion RNA, 5 μl 5X buffer, 2.5 μl 25 mM $MnCl_2$ 2.5 μl 10 mM ATP and 1 μl EPAP. Reactions were incubated at 37 °C for 1 h and poly-A tailed RNA was then purified using the RNeasy minikit (Qiagen) and eluted in 30 μl nuclease free water as per manufacturer's instructions. Twelve microlitres poly-A tailed RNA and 1 μl 100 μM oligo d(T) primer were then heat-denatured at 70°C for 5 min and immediately cooled on ice. Thirteen microlitres heat-denatured RNA/primer mix was then reverse transcribed in a 20 μl reaction containing 4 μl 5X Transcriptor RT reaction

buffer, 2 µl 10 mM dNTPs and 1 µl (20 U) Transcriptor RT for 40 min at 55°C. Five microlitres cDNA was then amplified by PCR using KOD polymerase, a virus specific primer and a primer targeting the oligo-d(T) anchor sequence. This technique was used to obtain sequences for both the 5' and 3' cRNA UTRs, as the 5' cRNA UTR corresponds to the 3' vRNA.

3.4.6 Standard PCR

PCRs, excluding colony PCR, were performed using KOD polymerase in a final reaction volume of 50 µl and contained 5 µl cDNA or 0.5 µl plasmid DNA, 5 µl 10X buffer, 5 µl dNTP mix (2 mM each), 3 µl 25 mM $MgSO_4$, 2.5 µl 10 µM forward primer, 2.5 µl 10 µM reverse primer and 1 µl (1 U) KOD polymerase. PCR cycling conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of denaturing at 95°C for 30 s, primer annealing at 50-60°C for 30 s, and extension at 70°C for 30 s, and a final extension step at 70°C for 10 min. The primer annealing temperature was determined by primer characteristics, whilst the cycling extension time was determined by amplicon size (30 s per 1 kb amplicon).

3.4.7 QuickChange® PCR

QuickChange® site-directed mutagenesis (Stratagene) was performed according to the manufacturer's instructions, with the exception that KOD polymerase and primers of standard salt purification were used. Following amplification template DNA was degraded by the addition of 20 U DpnI per reaction and incubation at 37°C for 1 h. treatment. Bacterial cells were then transformed with 2 µl of the QuickChange® reaction.

3.4.8 Agarose Gel Electrophoresis

Gel electrophoresis was carried out in horizontal slab 1% (w/v) agarose gels in 1X TAE buffer supplemented with 0.004% (v/v) ethidium bromide. The volume of samples loaded ranged from 5 µl – 50 µl and loading dye was added to samples at a final concentration of 1X. Gels were run at 100 V for 30 – 45 min in 1X TAE running buffer. DNA fragments were visualised using a UV transilluminator. If required, gel bands were excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as per manufacturer's instructions.

3.4.9 High-throughput Sequencing

RNA samples from ANAV, CAPV, BORV, BMAV and TETEV virions were submitted to Glasgow Polyomics for sequencing on the illumina MiSeq platform. Insufficient reads were generated for ANAV and CAPV and these viruses, and all other viruses with HTS data were sequenced at the Center for Infection and Immunity, NY. Briefly, first strand cDNA synthesis was carried out on 11 µl virion RNA using random hexamer primers and superscript III reverse transcriptase enzyme. Samples were then RNAse(H) treated by 1 µl 37°C 20 min, 75°C 15 min. Second strand cDNA synthesis was performed using 600 ng random hexamer primers, 1X NEB buffer 2, 50 mM deoxynucleotides and 20 U Klenow Fragment, exo- in a total volume of 100 µl, 37°C 45 min. The KAPA Library Preparation Kit for Ion Torrent Platforms was then followed as per manufacturer's instructions, with sample clean-up using Agencourt AMPure XP reagent to prepare cDNAs for sequencing on the Ion Proton sequencer. Samples were quantified using the Qubit® 2.0 Fluorometer (Life Technologies) and 2100 Bioanalyser (Agilent).

3.4.10 Sanger Sequencing

RACE-PCR products and purified plasmids were sent to GATC or Source BioScience for sequence confirmation by Sanger sequencing. The sample requirements recommended by the companies were followed.

3.4.11 Transformation of Bacterial Cells

Competent JM109 *E.coli* bacteria were prepared using the Z-Competent *E. coli* Transformation kit and buffer set (Zymo Research) according to the manufacturer's instructions and stored in 100 µl aliquots at -80°C. Prior to use cells were defrosted on ice for 5 min, plasmid DNA was then added and cells incubated on ice for 3-5 min. Transformed cells were then plated on pre-warmed LB agar with selective antibiotic and incubated overnight at 37°C. Colonies were then selected for confirmation by colony PCR and Sanger sequencing as required.

3.4.12 Plasmid Propagation

Transformed bacterial cells were grown on solid LB agar supplemented with ampicillin overnight at 37°C. Single colonies were then picked, inoculated into 5 ml LB broth containing ampicillin and incubated at 37°C with shaking at 225 rpm for 10 - 12 h. Plasmid purification was performed using the PureYield^{TM} Plasmid Miniprep System (Promega), as per the manufacturer's instructions.

3.4.13 Restriction-Free Cloning

Plasmids were linearised by excision PCR with KOD polymerase and primers which flank the insertion site, followed by DpnI treatment. Inserts were prepared by KOD PCR with primers that contain a 5' overhang of 15 bp, which is complementary to the desired insert site, followed by agarose gel electrophoresis and purification. Inserts and linearised vectors were then combined using the InFusion HD Cloning Kit (Clontech Laboratories Inc.). Reactions included 100 ng insert, 100 ng linearised vector, 1 µl 5X In Fusion enzyme pre-mix and nuclease free water in a total reaction volume of 10 µl, and were incubated at 50°C for 15 min prior to cooling on ice. The entire 10 µl reaction was then used to transform 100 µl competent JM109 cells as detailed in section 3.4.11.

3.4.14 pGEM-T Easy

The pGEM-T easy vector is supplied as linearised plasmid with single thymidine overhangs at both ends of the insertion site. Therefore, adenine overhangs were added to agarose gel-purified PCR products to allow ligation between the vector and insert. Adenine overhangs were created using the following 'Taq reaction': 30 µl gel-purified PCR product, 5 µl 5X Flexi buffer, 1 µl 10 mM dNTPs, 5 µl 25 mM $MgCl_2$, 0.5 µl GoTaq G2, 8.5 µl water, incubated at 72°C for 15 min and cooled on ice. Reaction products were then purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and eluted in 35 µl nuclease free water as per manufacturer's instructions. Overnight ligation reactions with T4 DNA ligase were carried out at 4°C with preliminary insert:vector ratios of 3:1 and 1:3, according to the manufacturer's instructions.

3.4.15 Colony PCR

Colony PCR was used to screen transformants for the presence of a desired insert. Single colonies were resuspended in 14.5 µl sterile H_2O and 2 µl used to dot inoculate an LB agar plate supplemented with ampicillin. The remaining 12.5 µl of the colony suspension was then used as the template in a 50 µl PCR reaction with final concentrations of 1X GoTaq PCR buffer, 200 nM dNTPs, 200 nM forward primer, 200 nM reverse primer and 0.3 U GoTaq polymerase. PCR cycling conditions were 95°C for 5 min denaturation, followed by 30 cycles of 95°C for 30 s, primer annealing for 30 s, extension at 72°C, and a final extension step at 72°C for 10 min. The primer annealing temperature and extension time were determined by primer characteristics and amplicon size (30 s per 1 kb amplicon), respectively. Colonies with PCR products of the correct size were then selected for plasmid propagation and confirmation by Sanger sequencing.

3.5 Protein Analysis

3.5.1 Western Blotting

Proteins were prepared for western blot analysis by removing cell culture media, rinsing cells in PBS, 2% (v/v) FCS and lysing cells in 30 µl PDB per cm^2 cell monolayer. Lysates were stored at -20°C and then, prior to analysis, boiled at 100°C for 3 min and immediately placed on ice. Samples (10 µl – 15 µl per well) were then size separated on NuPAGE 4-12% Bis-Tris gels, with 5 µl PageRule Plus pre-stained protein ladder, for approximately 50 min at 180 V in 1 X MES SDS running buffer. Proteins were transferred to nitrocellulose membrane (GE Healthcare) using the Bio-Rad Trans-Blot Turbo Transfer System and 1x NuPAGE Transfer Buffer. Once transfer was complete membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk in PBS, 0.1% (v/v) Tween-20. Membranes were then reacted with primary and secondary antibodies for 1 h using the specified dilutions (Table 2-4, Table 2-5). Visualisation of proteins was achieved by the addition of SuperSignal West Pico Chemiluminescent Substrate as per manufacturer's instructions. Blots were then exposed to x-ray films which were subsequently developed using KODOmat and Kograph Compact x4 automatic processors.

3.5.2 Metabolic Labelling

Metabolic labelling was carried out using subconfluent Vero E6 cells seeded in 6-well cell culture plates. Two hours prior to labelling cell culture medium was replaced with 2 ml DMEM (Met-) to starve cells of methionine. Following methionine starvation DMEM (Met-) was replaced with 350 µl DMEM (Met-) supplemented with 15 µCi [^{35}S]-methionine, and cells were incubated for a further 2 h at 37ºC in 5% CO_2. Radiolabelled medium was then removed, cell monolayers lysed in PDB and stored at -20°C prior to analysis. For analysis [^{35}S]-methionine labelled proteins were size separated and transferred to nitrocellulose membrane as in 3.5.1. The protein gels were then dried and labelled products visualised by autoradiography.

3.6 Software Packages

3.6.1 Bioinformatics

- *De novo* assembly of high-throughput sequencing data was carried out in CLC genomics workbench v7.5.1 (Qiagen) [http://www.clcbio.com/products/clc-genomics-workbench].

- Mega 6.0 (for Mac) [http://www.megasoftware.net/] and NetNGlyc 1.0 [http://www.cbs.dtu.dk/services/NetNGlyc] were used for sequence alignments, phylogeny and *N*-Glycosylation prediction respectively (Tamura *et al.*, 2013; Gupta *et al.,* 2004).

- Disorder predictions and domain searches were carried out by Dismeta [http://www-nmr.cabm.rutgers.edu/bioinformatics/disorder] and InterPro Scan [http://www.ebi.ac.uk/interpro] (Huang *et al.,* 2014; Mitchell *et al.*, 2015).

- Prediction of transmembrane topology and signal peptides were carried out by TMHMM server v2.0 [http://www.cbs.dtu.dk/services/TMHMM] and Phobius [http://phobius.sbc.su.se/].

- Structural models were generated by Phyre2 [http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index] and manipulated in PyMol v1.7.6 (for Mac) [https://www.pymol.org/] (Kelley and Sternberg, 2009).

3.6.2 Graph Generation and Statistical Analysis

Statistical analysis was carried out and graphs prepared using Prism v5.0a (for Mac OS X), GraphPad Software, La Jolla California, USA [http://www.graphpad.com/]

3.6.3 Imaging Programmes

- IF images were prepared in ImageJ 1.45s 64-bit [http://imagej.nih.gov/ij].

- Agarose gel electrophoresis and western blot images were prepared in Image Lab v5.0, Bio-Rad Laboratories [http://www.bio-rad.com/en-uk/product/image-lab-software]

- Graphical schematics were prepared in Autodesk® Graphic for Mac® v3.0.1 [http://www.graphic.com/]

Chapter 4: Orthobunyavirus Growth Characteristics and Protein Profiles

4.1 Introduction

The World Health Organisation's Collaborating Centre for Arbovirus Reference and Research has determined antigenic relationships between viruses collected by the Rockefeller Institute and collaborating centres since the late 1950s (Berge 1975; Elliott and Blakqori 2011). These data have shaped virus classification and our understanding of bunyavirus ecology and evolution, with Casals and Whitman proposing the formation of the Bunyamwera 'group' in 1960 (Casals and Whitman 1960). As complex interrelated antigenic relationships were discovered this was amended to the Bunyamwera 'Supergroup' in 1963 (Okuno 1961; Whitman and Casals 1961; Whitman and Shope 1962; Casals 1963). This proposal would go on to form the foundation of the *Bunyaviridae* family and orthobunyavirus genus, which now contains over 170 named viruses, classified into 18 ICTV-recognised serogroups (Fenner 1976; King 2012). However, for many of these viruses research stalled after their original isolation and antigenic classification, with detailed molecular studies focusing only on viruses in the Simbu, California, Bunyamwera and Group C serogroups (Dunn et al., 1994; Bowen *et al.,* 1995; Nunes *et al.*, 2005; Chowdhary *et al*., 2012; Ladner *et al.*, 2014; Tilston-Lunel *et al.*, 2015). Obtaining virus isolates from the less studied serogroups would allow further characterisation and improve genus wide knowledge. For example, the NSs protein has been identified as the primary virulence factor of orthobunyaviruses and was thought to be key in overcoming the vertebrate host immune response (Bridgen *et al.*, 2001; Weber *et al*., 2002; Léonard *et al.*, 2006; Barry *et al*., 2014). However, in 2009 Mohammed *et al.,* utilised consensus primers to obtain S segment sequence data of viruses in the Anopheles A, Anopheles B and Tete serogroups, and in doing so identified viruses associated with human infection that contained severely truncated and non-functional NSs ORFs. Despite this discovery no further work has been carried out on viruses within these serogroups with no published information on growth kinetics, or plaque morphology. Furthermore, there are currently 12 named virus isolates in the Anopheles A serogroup and only 2 viruses, ANAV and TCMV, were included in Mohamed *et al.,* (2009) studies.

In addition to documenting plaque morphology and growth kinetics determining the biochemical profile of virus isolates can act as a screen, confirming the presence of orthobunyavirus-like infectious agents. This is possible as orthobunyaviruses have a

unique biochemical profile that is distinct from other arboviruses and genera within the *Bunyaviridae* family (Table 1-3). Orthobunyaviruses typically encode an NSs protein of 12 ± 1 kDa, N protein of 22 ± 3 kDa, NSm protein of 16 ± 2 kDa, Gn and Gc proteins of 37 ± 8 kDa and 115 ± 7 kDa, respectively, and a 259 kDa L protein. However, this orthobunyavirus protein profile is based on viruses from only 10 serogroups, namely the Anopheles A, Anopheles B, Bunyamwera, California, Capim, Group C, Guama, Patois, Simbu, and Turlock serogroups (White, 1975; Gentsch *et al.*, 1977; Pennington *et al.,* 1977; Gentsch *et al.*, 1979; Klimas *et al.*, 1981; McPhee and Westaway 1981; Ushijima *et al.*, 1981; Short *et al.*, 1982; Elliott, 1985; McPhee and Della-Porta, 1988). Furthermore, the unclassified orthobunyavirus Brazoran virus has an N protein of 49.8 kDa and NSs protein of 19.6 kDa demonstrating that there is greater diversity in the orthobunyavirus genus than previously thought (Lanciotti *et al.*, 2013). It would therefore be of interest to determine the protein profiles of viruses in the remaining 8 recognised serogroups, which are the Bakau, Bwamba, Gamboa, Koongol, Minatitlan, Nyando, Olifantsvlei and Tete serogroups.

4.2 Aims

The aims of this part of my studies were to create a panel of orthobunyaviruses capable of replication in mammalian cell culture with information on plaque morphology, virus protein profiles and virus growth kinetics in IFN-competent and incompetent cell lines. In addition to the virus panel, I would create a spreadsheet detailing geographical distribution, known vectors, and host species for all ICTV-recognised orthobunyavirus species and all named orthobunyaviruses with complete genome sequences.

4.3 Results

4.3.1 Virus Propagation and Plaque Morphology

Fifty orthobunyavirus isolates, supplied as freeze-dried infected mouse brain homogenates or freeze-dried low passage tissue culture samples were resuspended and blind passaged on Vero E6 cells until the production of CPE, or passage 10 (Table 4-1).

In total 25 viruses developed CPE during virus passage, 2 on passage 1, 13 on passage 2, 7 on passage 3, 1 on passage 4, 1 on passage 5 and 1 on passage 6 (Table 4-1).

Of the 26 viruses that developed CPE 7 belonged to the Anopheles A serogroup. ANAV, ColAn57389, CPEV and LMV developed CPE at passage 2, whilst TRMV and TUCV required further passaging, developing CPE at passage 4 and passage 6, respectively (Table 4-1). ColAn57389, LMV and TRMV formed plaques 2 mm - 3 mm in diameter that remained slightly opaque, with incomplete clearing of the cell monolayer (Figure 4-1). The plaques of CPEV, TUCV and TCMV were smaller at only 1 mm - 2 mm in diameter at 6 d p.i. (Figure 4-1). Viruses in the Anopheles B serogroup, ANBV and BORV, both developed CPE at passage 2 and formed semi opaque/turbid plaques 2 mm - 3 mm in diameter at 6 d p.i. (Table 4-1, Figure 4-1).

Nine viruses in the Capim serogroup were passaged, however, only 4 developed CPE, GJAV and JDV at passage 2 and CAPV and MORV at passage 3 (Table 4-1). CAPV, GJAV and JDV formed sharp clear pin-prick plaques of 1 mm - 2 mm in diameter, whilst MORV formed slightly larger plaques of ≤ 3 mm at 6 d p.i. (Table 4-1, Figure 4-1). GMAV, the sole member of the Guama serogroup to show evidence of infection, developed CPE on passage 2 and formed large clear plaques of 4 mm - 5 mm in diameter which were comparable to those of BUNV (Figure 4-2). The only other virus to form large plaques comparable to BUNV was MPOV, a member of the Turlock serogroup, which produced CPE on passage 1 and formed large clear plaques 2 mm – 5 mm in size (Table 4-1, Figure 4-2).

Table 4-1: Virus Propagation in Vero E6 Cells

Virus Classification		Isolates In This Study			In the Literature
Virus Name	**ABBV**	**Passage Number**	**CPE**	**Virus Titre** (p.f.u ml^{-1})	**Plaque Morphology** (d p.i., size [mm])
Anopheles A Serogroup					
Anopheles A	ANAV	p3	+	1.80×10^6	(4, 2)
ColAn 57389	ColAn57389	p2	+	2.20×10^5	N/A
CoAr 1071	CoAr1071	p10	-	N/A	N/A
CoAr 3624	CoAr3624	p10	-	N/A	N/A
Arumateua	ARTV	p10	-	N/A	N/A
Caraipe	CPEV	p2	++	3.65×10^6	N/A
Las Maloyas	LMV	p2	++	1.00×10^7	(5, <1)
Lukuni	LUKV	p10	-	N/A	(13, 0.5-1)
Tacaiuma	TCMV	p10	-	N/A	(N/A,1)
Tacaiuma BeAn73	BeAn73	p5	+	9.00×10^5	(N/A,1)
Trombetas	TRMV	p4	++	4.50×10^6	N/A
Tucurui	TUCV	p6	+	6.50×10^5	N/A
Virgin River	VRV	p10	-	N/A	(12, 3)
Anopheles B Serogroup					
Anopheles B	ANBV	p2	+	1.00×10^6	(9,3)
Boraceia	BORV	p2	+	4.50×10^7	(2,3)
Bakau Serogroup					
Bakau	BAKV	p10	-	N/A	(4,2)
Ketapang	KETV	p10	-	N/A	(4,2)
Bwamba Serogroup					
Bwamba	BWAV	p2	++	8.50×10^6	(5,1)
Pongola	PGAV	p10	-	N/A	(3,1)
Capim Serogroup					
Acara	ACAV	p10	-	N/A	(6,1)
Acara	ACAV	p10	-	N/A	(6,1)
Benevides	BVSV	p10	-	N/A	CPE
Benfica	BENV	p10	-	N/A	CPE
Bushbush	BSBV	p10	-	N/A	(6,1)
Capim	CAPV	p3	+	3.65×10^6	(3,1)
Guajara	GJAV	p2	+	8.50×10^5	N/A
Juan Diaz	JDV	p2	++	5.50×10^4	(5, N/A)
Moriche	MORV	p3	++	1.65×10^6	(4,1)
Gamboa Serogroup					
Gamboa	GAMV	p2	++	1.75×10^5	(4-6, N/A)
San Juan	SJV	p3	++	3.55×10^6	(5, N/A)
Guama Serogroup					
Bertioga	BERV	p10	-	N/A	(2,1)
Guama	GMAV	p2	+	2.50×10^5	(5, 1-1.5)
Koongol Serogroup					
Koongol	KOOV	p10	-	N/A	(15,1)
Wongal	WONV	p10	-	N/A	(10,2)
Minatitlan Serogroup					
Minatitlan	MNTV	p10	-	N/A	N/A
Palestina	PLSV	p2	+++	2.60×10^7	(7, N/A)
Nyando Serogroup					
Eretmapodites	EREV	p2	+++	2.60×10^7	N/A
Nyando	NDV	p1	+	1.80×10^7	(5,2)
Olifantsvlei Serogroup					
Botambi	BOTV	p10	-	N/A	N/A
Olifantsvlei	OLIV	p10	-	N/A	N/A

Patois Serogroup					
Pahayokee	PAHV	p10	-	N/A	N/A
Patois	PATV	p10	-	N/A	No plaques
Tete Serogroup					
Bahig	BAHV	p10	-	N/A	(3,3)
Batama	BMAV	p2	++	3.10×10^8	N/A
Matruh	MTRV	p3	++	1.70×10^7	(3,7)
Tete	TETEV	p3	++	3.95×10^7	N/A
Tsuruse-like	TSUV	p3	++	3.25×10^7	N/A
Weldona	WELV	p2	+	N/A	N/A
Turlock Serogroup					
M'Poko	MPOV	p1	++	5.50×10^6	N/A
Turlock	TURV	p10	-	N/A	(6,3)

Virus names and abbreviations (ABBV) of isolates passaged in this study are given. Viruses were passaged on Vero E6 cells until the development of cytopathic effect (CPE) or passage (p) 10. Level of CPE signified by + or -; +++, 90% disruption of cell monolayer by 4 days post infection (d p.i.); ++, 90% disruption of cell monolayer by 7 d p.i.; +, evidence of cell death but <90% at 7 d p.i.; -, no evidence of CPE at 7 d p.i., cells comparable to mock. The virus titre of working stocks determined by plaque assay on Vero E6 cells is also listed. Lastly, a summary of evidence of replication in Vero cells as listed in the International Catalog of Arboviruses (ArboCAT) section VI – Biologic Characteristics is given (Berge 1975); plaque morphology is reported as incubation time (days), followed by plaque size (mm). N/A no information available, CPE information on cytopathic effect only.

GAMV and SJV, members of the Gamboa serogroup, developed CPE at passage 2 and passage 3, respectively. GAMV formed clear plaques 2 mm - 4 mm in diameter whilst SJV formed smaller plaques at 1 mm - 2 mm at 6 d p.i. (Table 4-1, Figure 4-2). Similarly, 2 viruses in the Nyando serogroup, NDV and EREV, developed CPE at passage 1 and passage 2, respectively, and formed plaques on Vero E6 cells (Table 4-1). However, NDV caused notably less CPE than EREV, and further passaging of NDV did not increase CPE formation to that of EREV. NDV plaques were also difficult to distinguish at 6 d p.i. and required incubation for 9 days (Figure 4-2). PLSV, a member of the Minatitlan serogroup produced strong CPE at passage 2 and formed clear plaques of 2 mm - 3 mm (Figure 4-2).

In total 6 named virus isolates from the Tete serogroup were passaged in cell culture with 5 viruses, BMAV, MTRV, TETEV, TSUV and WELV, producing CPE (Table 4-1). All of which, except WELV, formed distinct plaques in Vero E6 cells at 6 d p.i. (Figure 4-2). WELV failed to form clear plaques on Vero E6 cells with indistinct hazing of the cell monolayer only (Figure 4-2, Figure 4-3). Further passaging of WELV did not improve plaque formation or increase the level of CPE beyond that observed at passage 2, with cell rounding and cell death at 5 d p.i. (Figure 4-3).

Viruses in the Bakau (Bakau, Ketapang), Koongol (Koongol, Wongol), Olifantsvlei (Olifantsvlei, Botambi) and Patois (Pahayokee, Patois) serogroups failed to produce CPE by passage 10 (Table 4-1). All passage 10 isolates were also negative by plaque assay at 6 d p.i. (Table 4-1) and 14 d p.i. (data not shown).

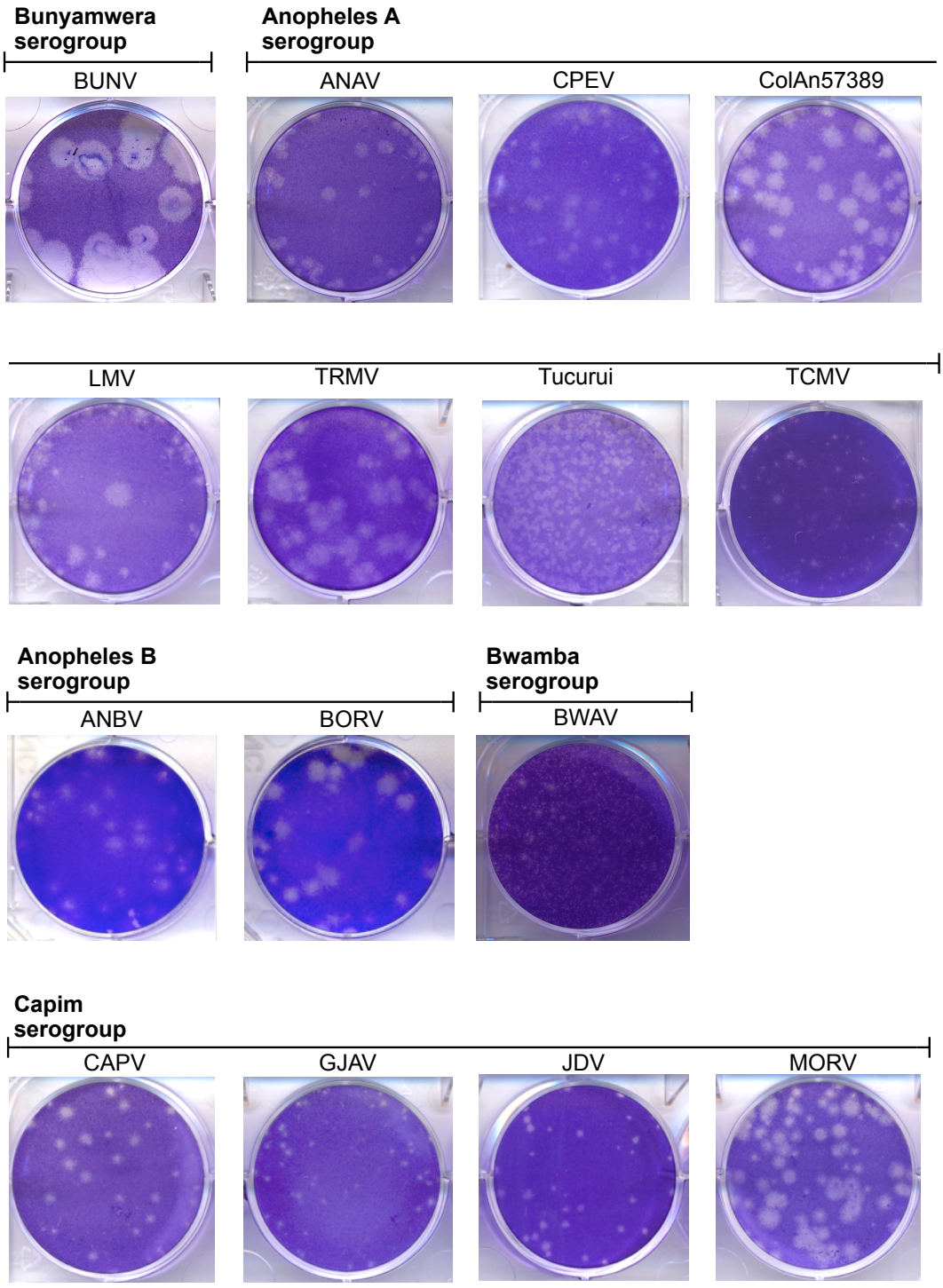


Figure 4-1: Plaque Morphology of Viruses in the Anopheles A, Anopheles B and Capim Serogroups

Plaque morphology at 6 days post infection on Vero E6 cells with a 0.6% (w/v) Avicel overlay.

Virus abbreviations: Anopheles A virus (ANAV), Caraipe virus (CPEV), ANAV strain ColAn57389 (ColAn57389), Las Maloyas (LMV), Trombetas virus (TRMV), Tucurui virus (TUCV), Tacaiuma virus (TCMV), Anopheles B virus (ANBV), Boraceia virus (BORV), Bunyamwera virus (BUNV), Bwamba virus (BWAV), Capim virus (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV) and Moriche virus (MORV).

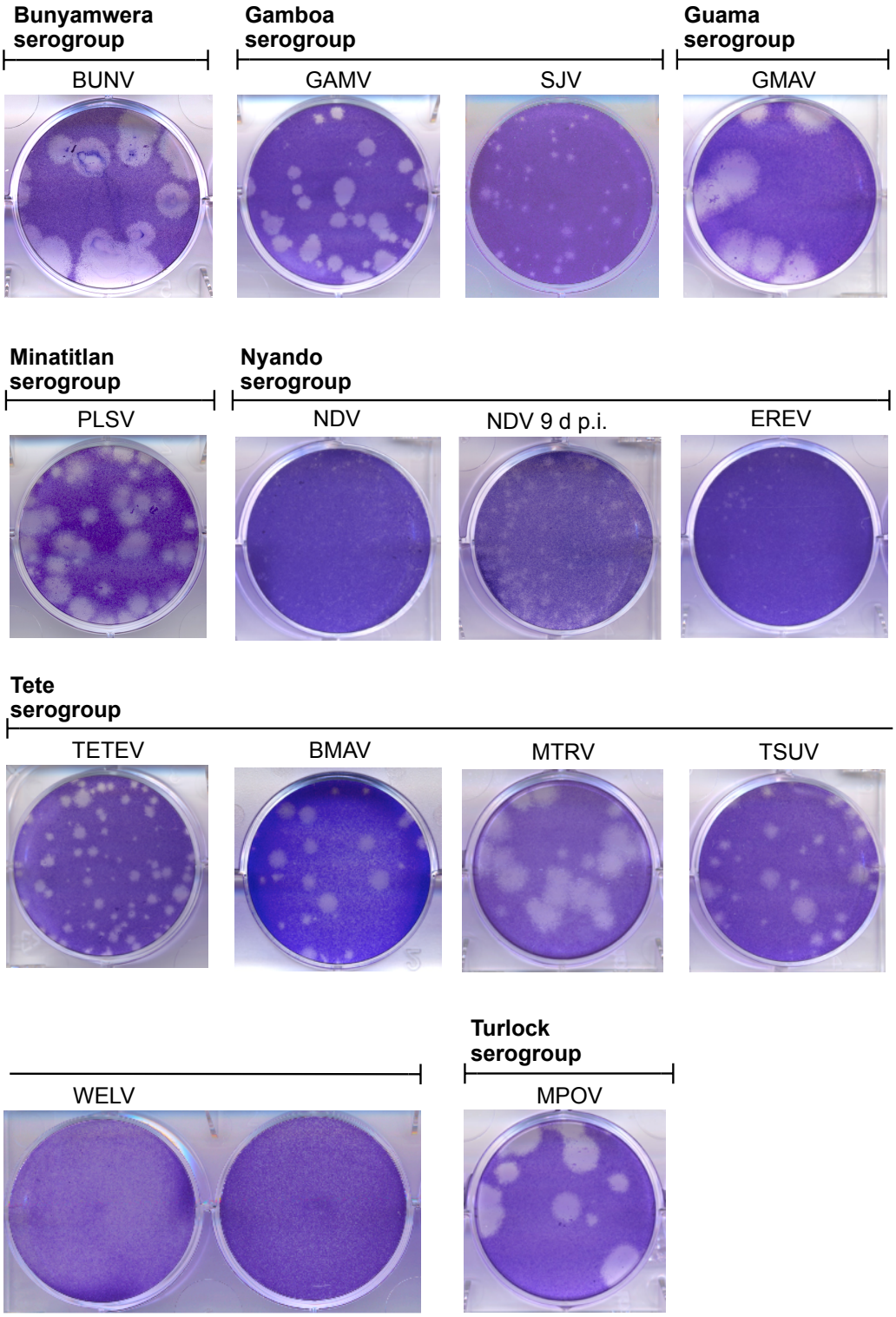


Figure 4-2: Plaque Morphology of Gamboa, Guama, Minatitlan, Nyando, Tete and Turlock Serogroup Viruses

Plaque morphology at 6 days post infection (d p.i.) unless stated, on Vero E6 cells with a 0.6% (w/v) Avicel overlay.

Virus abbreviations: Bunyamwera virus (BUNV), Gamboa virus (GAMV), San Juan virus (SJV), Guama virus (GMAV), Palestina virus (PLSV), Nyando virus (NDV), Eretmapodites virus (EREV), Tete virus (TETEV), Batama virus (BMAV), Matruh virus (MTRV), Tsuruse-like virus (TSUV), Weldona virus (WELV) and M'Poko virus (MPOV).

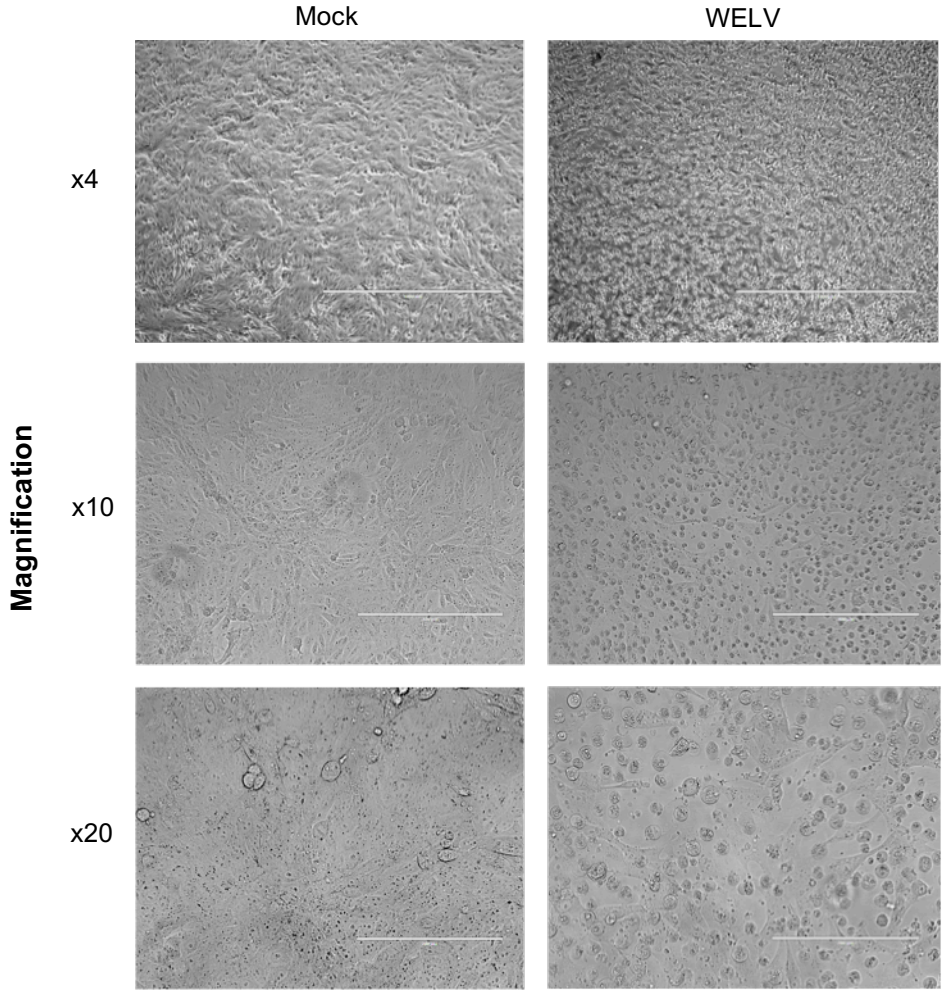


Figure 4-3: Weldona Virus Cytopathic Effect in Vero E6 Cells

Weldona virus (WELV) cytopathic effect 5 days post infection in Vero E6 cells. Cells were inoculated with 100 µl clarified culture supernatant from passage 1 virus stocks or mock infected with 100 µl PBS 2% (v/v) FCS. Widespread cell rounding and cell death is present only in WELV infected cells. Scale bars represent 1000 µm, 400 µm and 200 µm at x4, x10 and x20 magnification, respectively.

4.3.2 Virus Growth Kinetics

Growth kinetics in A549 and Vero E6 cell lines were determined for the 25 viruses titrated by plaque assay in section 4.3.1., the type species of the genus, BUNV, and rBUNdelNSs2. BUNV grew to similar titres in both cell lines at all time points, with titres of 4.0 x 10^6 p.f.u ml^{-1} and 5.0 x 10^6 p.f.u ml^{-1} in A549 and Vero E6 cells respectively at 72 h p.i. (Figure 4-4). However, growth of rBUNdelNSs2 was suppressed in A549 cells with > 1 log drop in virus titre at 24 h p.i., 48 h p.i. and 72 h p.i. compared with Vero E6 cells (Figure 4-4).

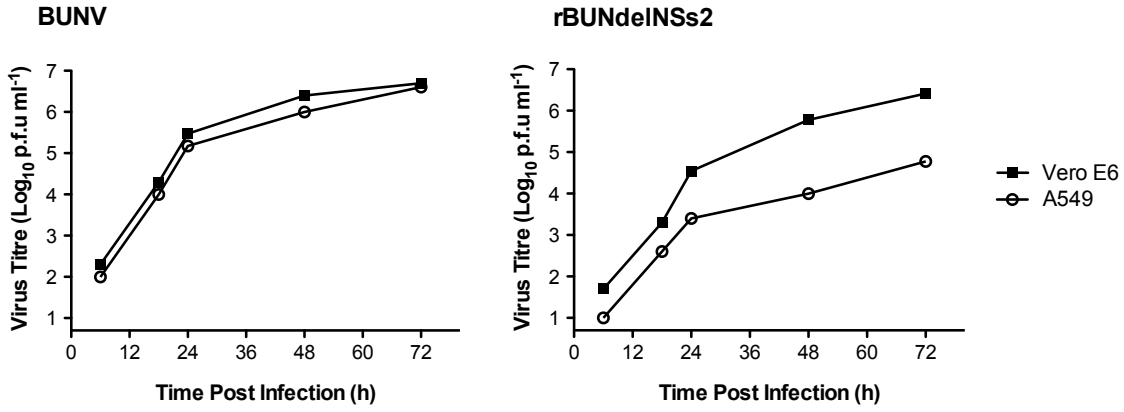


Figure 4-4: Growth Kinetics of Wild Type and Recombinant Bunyamwera Virus
Growth kinetics of wild type Bunyamwera virus (BUNV) and recombinant virus rBUNdelNSs2, which lacks a functional NSs protein. A549 and Vero E6 cells were infected at multiplicity of infection 0.01 and virus titre determined by plaque assay on clarified culture supernatant harvested at specified time points post infection.

With the exception of TCMV, all viruses in the Anopheles A serogroup showed reduced titres in A549 cells in comparison to Vero E6 cells, with a > 1 log drop in virus titre at 48 h p.i. and 72 h p.i. (Figure 4-5). In contrast the virus titre of TCMV remained within < 1 log at all time points when the 2 cell lines were compared, with final titres of 8.5 x10^4 p.f.u ml^{-1} and 2.25 x10^5 p.f.u ml^{-1} in A549 and Vero E6 cells at 72 h p.i. (Figure 4-5). BWAV, a member of the Bwamba serogroup, also displayed similar growth kinetics in both cell lines, with no notable difference in virus titre at any time point tested (Figure 4-6).

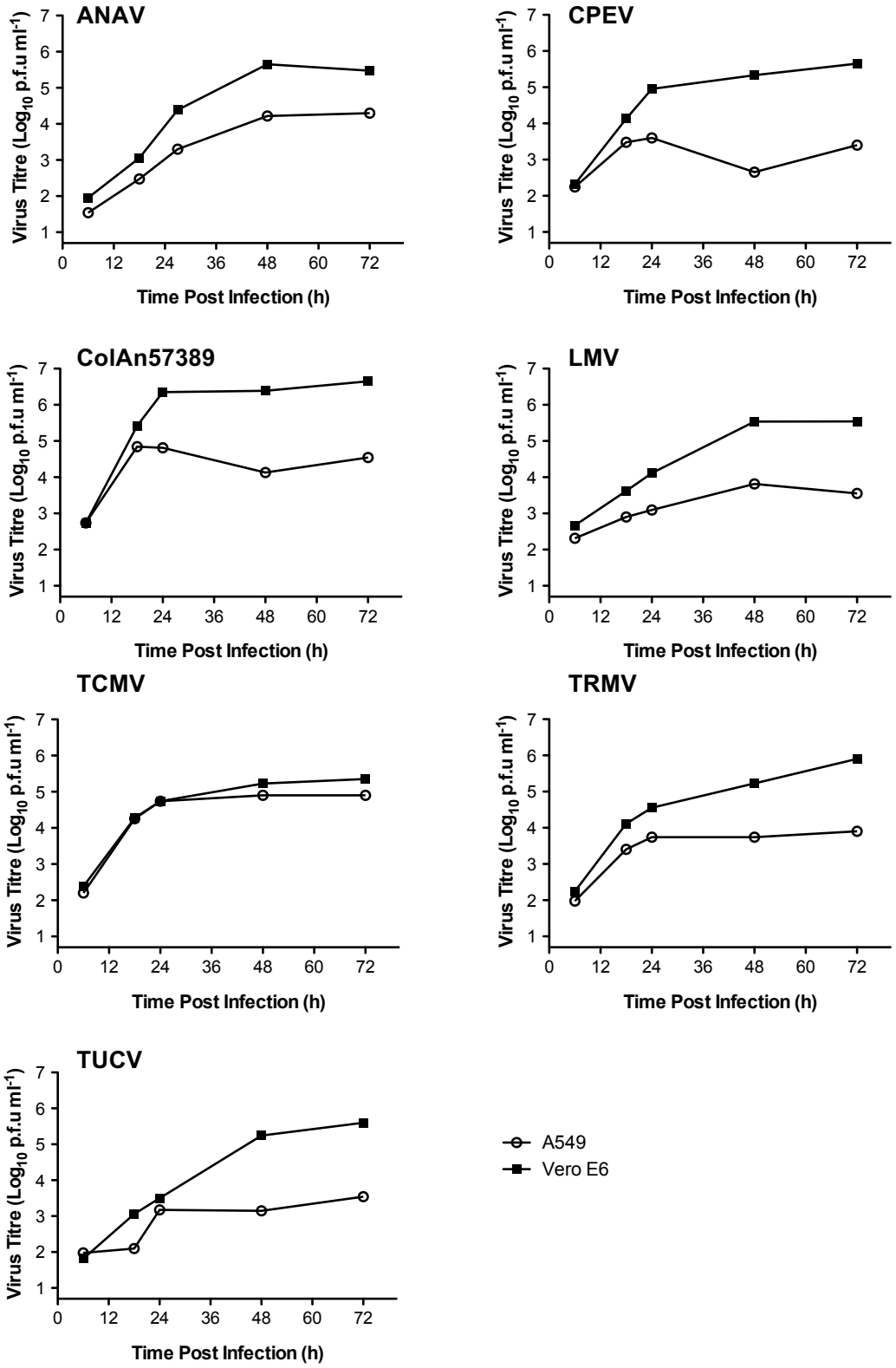


Figure 4-5: Growth Kinetics of Viruses in the Anopheles A Serogroup

Growth kinetics in A549 and Vero E6 cell lines. Cells were infected at multiplicity of infection 0.01 and clarified culture supernatant harvested at specified hours (h) post infection. Virus titre was then determined by plaque assay on Vero E6 cells.

Virus abbreviations: Anopheles A virus (ANAV), Caraipe virus (CPEV), ANAV strain ColAn57389 (ColAn57389), Las Maloyas (LMV), Tacaiuma virus (TCMV), Trombetas virus (TRMV) and Tucurui virus (TUCV).

Titres of the Anopheles B serogroup viruses ANBV and BORV were > 1 log lower in A549 cells at 48 h p.i. and 72 h p.i. (Figure 4-6). Reduced growth in A549 cells was also detected in Capim serogroup viruses although the degree of growth suppression varied (Figure 4-6). CAPV only showed > 1 log difference at 72 h p.i. with final titres of 2.65 $x10^5$ p.f.u ml^{-1} and 6.85 $x10^6$ p.f.u ml^{-1} in A549 and Vero E6 cells (Figure 4-6). The titre of GJAV in A549 cells was >1 log lower at 48 h p.i. and 72 h p.i. compared with Vero E6 cells (Figure 4-6). JDV titres were lower at all time points, although this difference was > 1 log only at 72 h p.i. (Figure 4-6). The titre of MORV in A549 cells was >1 log lower at 24 h p.i., 48 h p.i. and 72 h p.i. with a final titre of only 3.50 $x10^3$ p.f.u ml^{-1} at 72 h p.i. in A549 cells (Figure 4-6).

Viruses in the Gamboa serogroup, GAMV and SJV, replicated in Vero E6 cells with titres of 2.50 x 10^5 p.f.u ml^{-1} and 2.60 x $x10^6$ p.f.u ml^{-1}, respectively, at 72 h p.i. (Figure 4-7). The titre of SJV in A549 cells at 72 h p.i. was only 500 p.f.u ml^{-1} and no plaques of GAMV were detected at 48 h p.i. or 72 h p.i. (Figure 4-7).

GMAV, a member of the Guama serogroup, had ≥ 1 log reduction in A549 cells at 48 and 72 h p.i. with final titres of only 1.00 x 10^3 p.f.u ml^{-1} and 5.80 x 10^4 p.f.u ml^{-1} in A549 and Vero E6 cell lines at 72 h p.i., respectively (Figure 4-7). Virus titres of PLSV, a member of the Minatitlan serogroup, were also reduced across all time points in A549 cells with ≥ 1 log drop at 18 h p.i., 24 h p.i. and 72 h p.i.. Final virus titres of PLSV were 1.00 x 10^5 p.f.u ml^{-1} and 2.60 x 10^6 p.f.u ml^{-1} in A549 and Vero E6 cell lines, respectively (Figure 4-7). Virus titres of the Nyando serogroup viruses NDV and EREV were reduced across all time points in A549 cells, although titres remained within 1 log of those in Vero E6 cells (Figure 4-7).

Virus titres of MPOV were severely suppressed with > 2 log drop at 48 h p.i. and 72 h p.i. in A549 cells compared with Vero E6 cells (Figure 4-8). There was also a > 2 log reduction in the titre of BMAV, a member of the Tete serogroup, at 72 h p.i. in A549 cells compared with Vero E6 cells. Other members of the Tete serogroup, TETEV, MTRV and TSUV, also showed lower levels of virus titre in A549 cells, however there was < 2 log reduction at all time points tested (Figure 4-8).

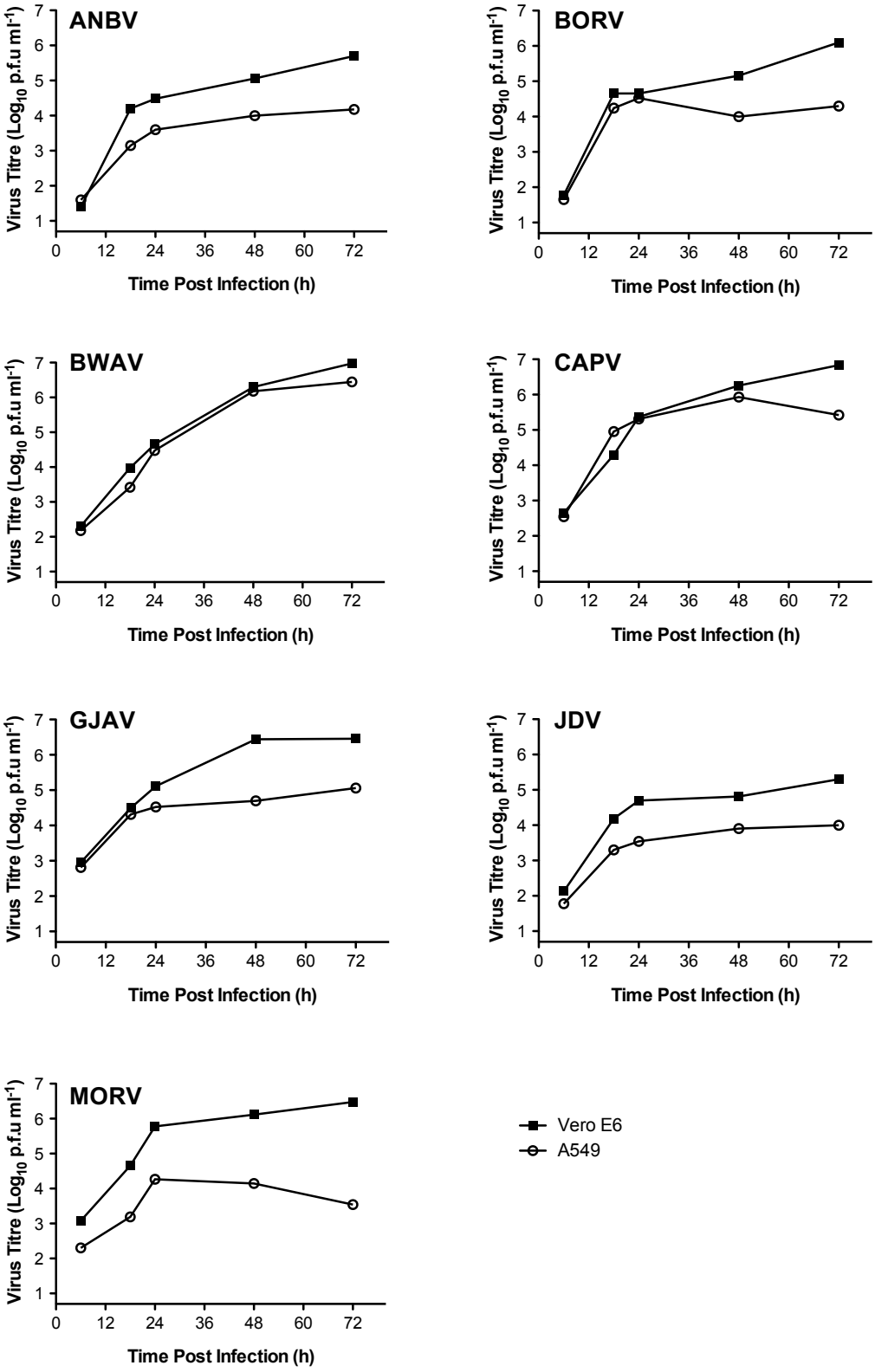


Figure 4-6: Growth Kinetics of Viruses in the Anopheles B, Bwamba and Capim Serogroups

Growth kinetics in A549 and Vero E6 cell lines. Cells were infected at multiplicity of infection 0.01 and clarified culture supernatant harvested at specified hours (h) post infection. Virus titre was then determined by plaque assay on Vero E6 cells.

Virus abbreviations: Anopheles B virus (ANBV), Boraceia virus (BORV), Bwamba virus (BWAV), Capim virus (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV) and Moriche virus (MORV).

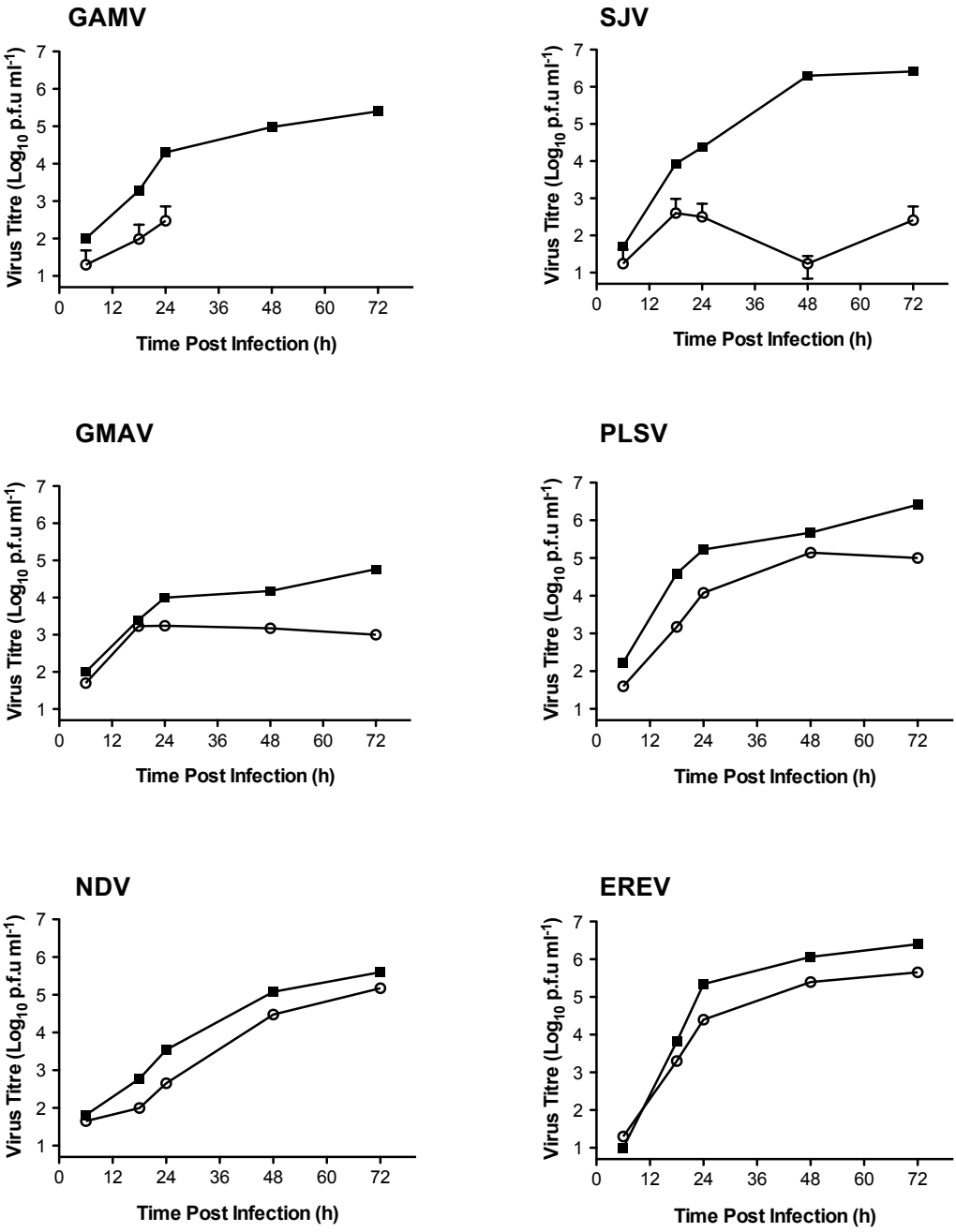


Figure 4-7: Growth Kinetics of Viruses in the Gamboa, Guama, Minatitlan and Nyando Serogroups.

Growth kinetics in A549 and Vero E6 cell lines. Cells were infected at multiplicity of infection 0.01 and clarified culture supernatant harvested at specified hours (h) post infection. Virus titre was then determined by plaque assay on Vero E6 cells.

Virus Abbreviations: Gamboa virus (GAMV), San Juan virus (SJV), Guama virus (GMAV), Palestina virus (PLSV), Nyando virus (NDV), and Eretmapodites virus (EREV).

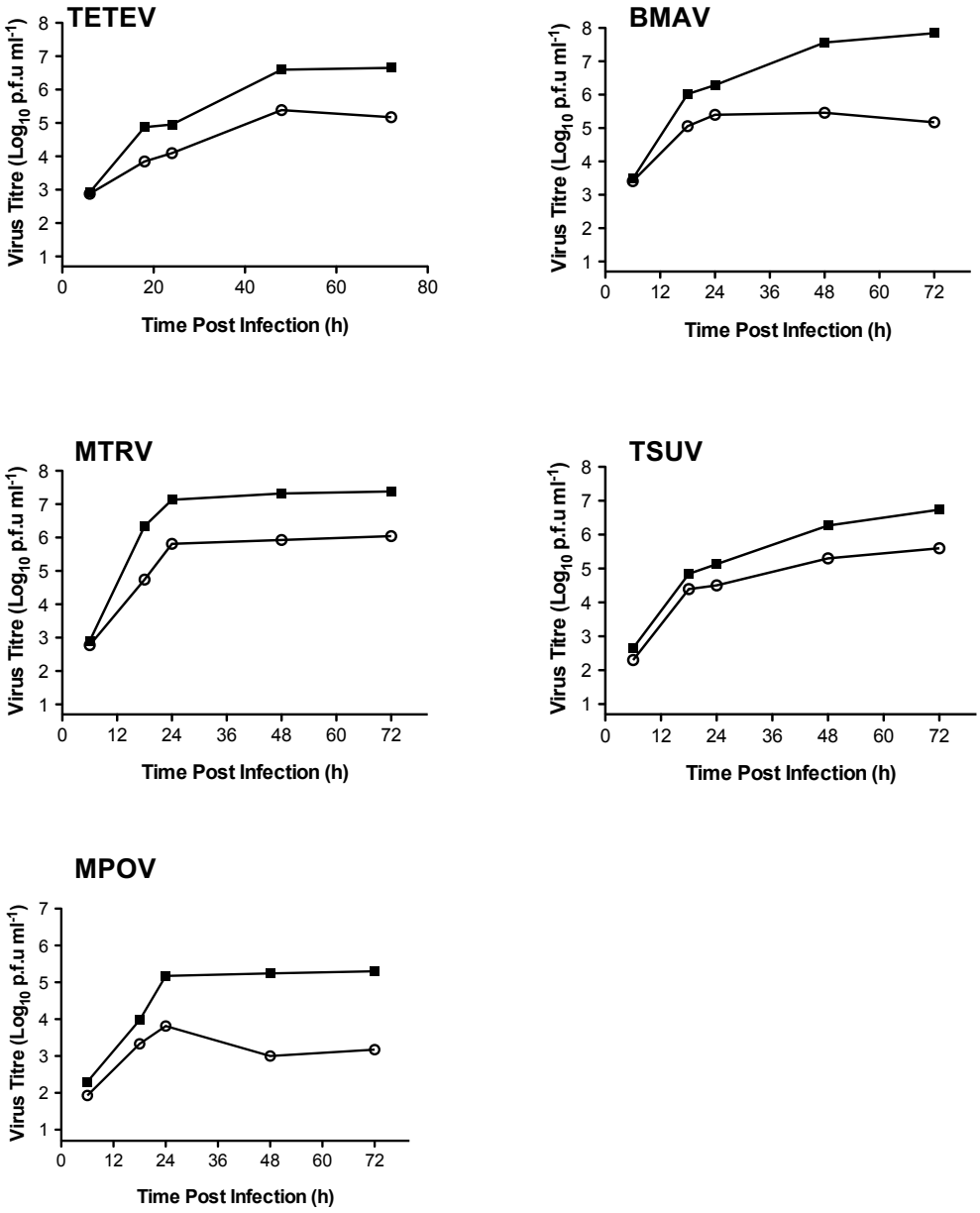


Figure 4-8: Growth Kinetics of Viruses in the Tete and Turlock Serogroups
Growth kinetics in A549 and Vero E6 cell lines. Cells were infected at multiplicity of infection 0.01 and clarified culture supernatant harvested at specified hours (h) post infection. Virus titre was then determined by plaque assay on Vero E6 cells.

Virus abbreviations: Tete virus (TETEV), Batama virus (BMAV), Matruh virus (MTRV), Tsuruse-like virus (TSUV) and M'Poko virus (MPOV).

4.3.3 Metabolic Labelling

Metabolic labelling of proteins during virus infection in Vero E6 cells was carried out for the 26 viruses described in section 4.3.1, with BUNV and rBUNdelNSs2 as controls. In BUNV infected cells clear bands at approximately 11 kDa, 15 kDa, 25 kDa and 115 kDa, that were not present in mock infected cells, were detected (Figure 4-9). These bands correspond in molecular weight to the virus proteins NSs, NSm, N and Gc proteins. A high molecular weight band (>180 kDa) was also detected and may represent the virus L protein, which has a predicted molecular weight of 259 kDa (Figure 4-9). An additional band at approximately 32 kDa, the predicted molecular weight of the virus Gn protein, was also present in infected cells at 48 h p.i. (Figure 4-9). At 48 h p.i. and 72 h p.i. there is a dramatic reduction in the number and intensity of bands as virus induced protein shut-off and cell death occurs. rBUNdelNSs2 gave a similar protein profile to BUNV, although bands corresponding to the molecular weight of virus Gn or NSs proteins were not visualised (Figure 4-9).

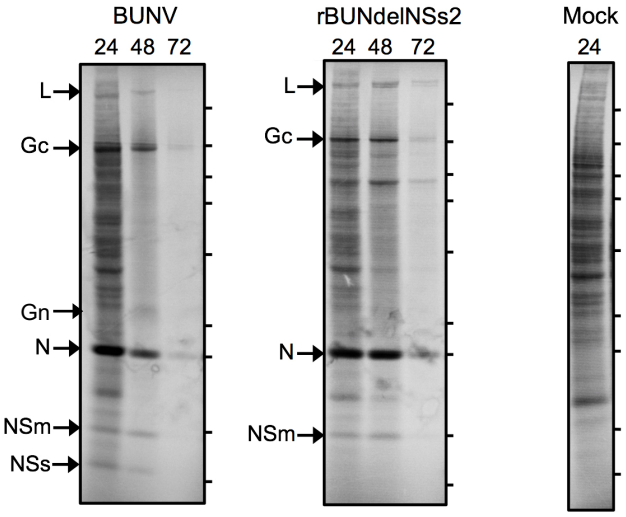


Figure 4-9: Metabolic Labelling of BUNV and rBUNdelNSs2 Viruses
Vero E6 cells were infected at multiplicity of infection 1 with Bunyamwera virus (BUNV), recombinant BUNV rBUNdelNSs2, or mock infected with PBS 2% (v/v) FCS. Cells were radiolabelled with 35 S methionine cysteine for 2 hours prior to harvesting at 24, 48 and 72 hours post infection Black arrows indicate bands not present in mock infected cells, and where possible are labelled with the virus protein of corresponding molecular weight. BUNV and rBUNdelNSs2 were included as controls. Black lines on the right hand side of the protein gels indicate the migration of the non-labelled protein ladder with sizes of approximately 10, 15, 25, 30, 50, 65, 80, 115 and 180 kDa in 4-12% Bis-Tris gels with 1xMES running buffer.

In cells infected with Anopheles A serogroup viruses, excluding TCMV, clear strong bands at approximately 26 kDa, the size of orthobunyavirus N proteins, were present at 24 h p.i. and 48 h p.i. and, at reduced intensity, 72 h p.i. (Figure 4-10). In TCMV infected cells a band at approximately 26 kDa was detected, although it was very faint at 24 h p.i., increasing in intensity at 48 h p.i. and 72 h p.i. (Figure 4-10). Bands at approximately 117 kDa, which is analogous to the size of the virus Gc protein, were detected in ANAV, CPEV, ColAn57389, LMV and TUCV infected cells (Figure 4-10). In TRMV and TCMV only very faint bands, which were difficult to differentiate from bands in mock infected cells, were present within the 115 kDa - 125 kDa molecular weight range (Figure 4-10). Bands corresponding to the size of Gn could not be detected in any cells infected with Anopheles A serogroup viruses. High molecular weight bands, >180 kDa, that were distinct from those in mock infected cells were present only in TCMV infected cells at 24 h p.i., 48 h p.i. and 72 h p.i. (Figure 4-10). Low molecular weight bands corresponding to the molecular weight of NSm proteins, 16 ± 2 kDa, were only present in LMV infected cells at 24 h p.i. and 48 h p.i. (Figure 4-10). Bands conforming to the NSs protein size 11 kDa – 13 kDa were not detected at any time point in cells infected with Anopheles A serogroup viruses (Figure 4-10).

In cells infected with Anopheles B serogroup viruses, ANBV and BORV, only bands at approximately 26 kDa and 117 kDa were detected, corresponding to virus N and Gc proteins (Figure 4-11). In Capim serogroup viruses CAPV, GJAV and JDV the band presumed to represent virus N protein migrated to approximately 24 kDa. However, in cells infected with MORV, which also belongs to the Capim serogroup, 2 bands within the molecular weight range for orthobunyavirus N proteins were detected (Figure 4-11). Bands at approximately 115 kDa, thought to be virus Gc protein, were detected in CAPV, GJAV, JDV and MORV infected cells (Figure 4-11). High molecular weight bands not present in mock infected cells and possibly representing virus L protein were only present in MORV infected cells (Figure 4-11). Low molecular weight bands within the range of orthobunyavirus NSm or NSs proteins were not present in any Capim or Anopheles B serogroup viruses (Figure 4-11).

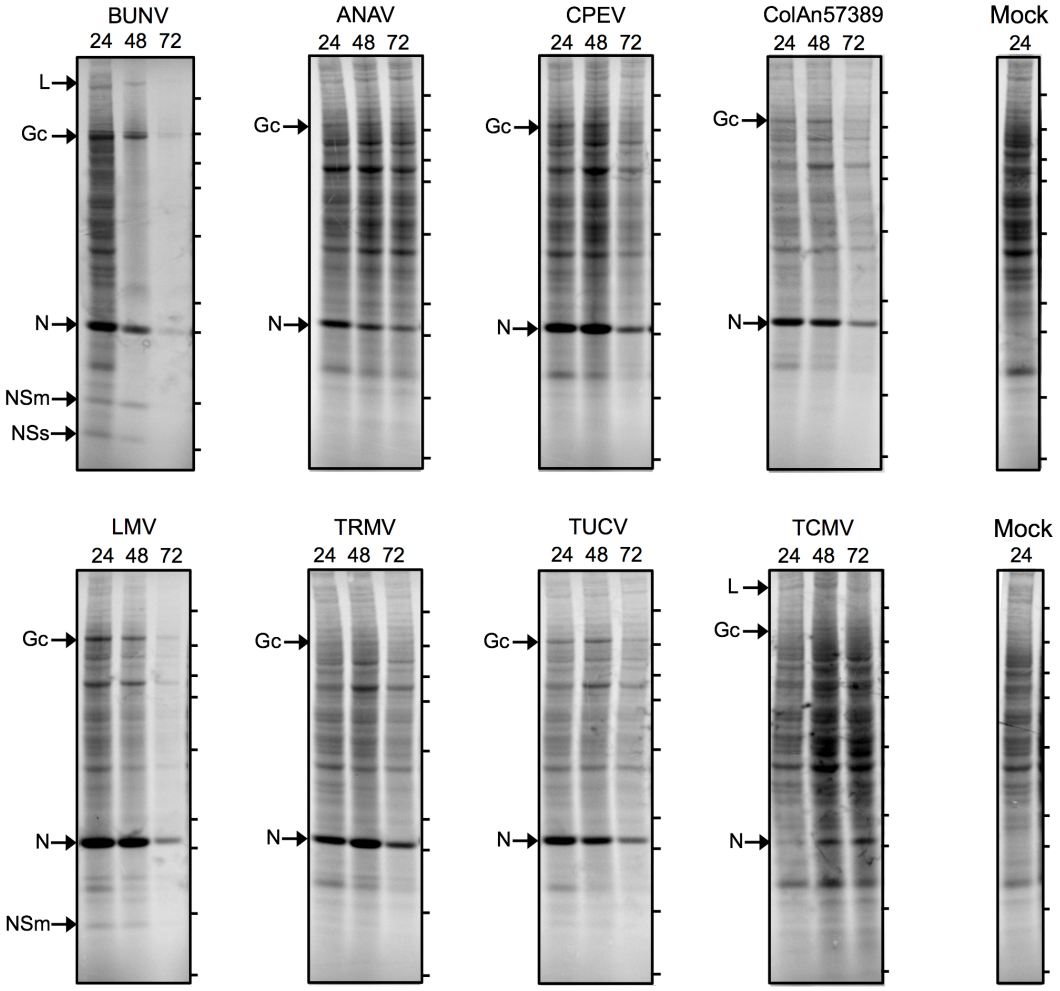


Figure 4-10: Metabolic Labelling of Viruses in the Anopheles A Serogroup

Vero E6 cells were infected at multiplicity of infection 1 or mock infected with PBS 2% (v/v) FCS. Cells were radiolabelled with 35 S methionine cysteine for 2 hours prior to harvesting at 24, 48 and 72 hours post infection Black arrows indicate bands not present in mock infected cells, and where possible are labelled with the virus protein of corresponding molecular weight. BUNV was included as a control. Black lines on the right hand side of the protein gels indicate the migration of the non-labelled protein ladder with sizes of approximately 10, 15, 25, 30, 50, 65, 80, 115 and 180 kDa in a 4-12% Bis-Tris gel with 1xMES running buffer.

Virus abbreviations: Bunyamwera virus (BUNV), Anopheles A virus (ANAV), Caraipe virus (CPEV), Anopheles A virus strain ColAn57389 (ColAn57389), Las Maloyas virus (LMV), Trombetas virus (TRMV), Tucurui virus (TUCV) and Tacaiuma virus (TCMV).

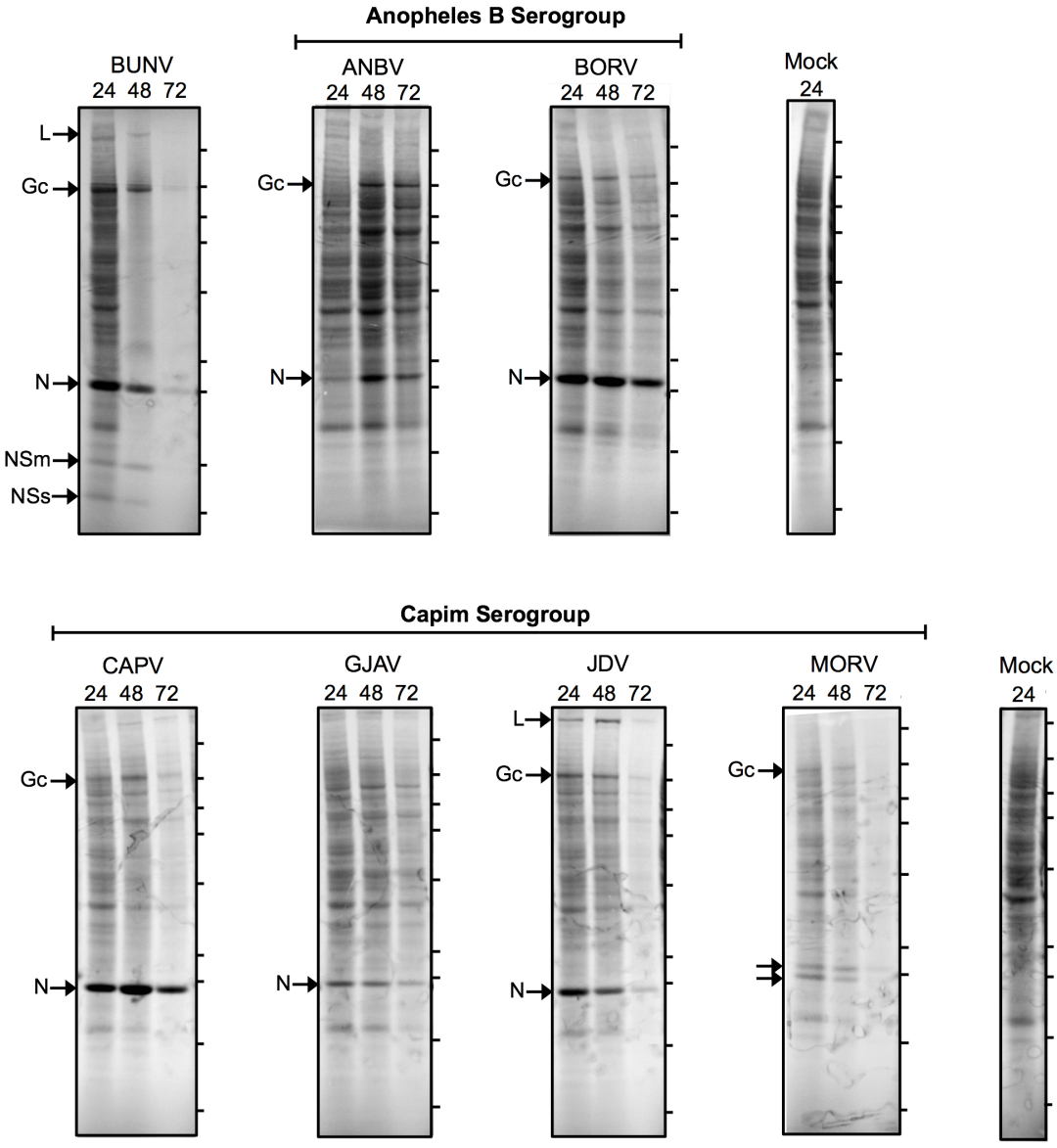


Figure 4-11: Metabolic Labelling of Viruses in the Anopheles B and Capim Serogroups

Vero E6 cells were infected at multiplicity of infection 1 or mock infected with PBS 2% (v/v) FCS. Cells were radiolabelled with 35 S methionine cysteine for 2 hours prior to harvesting at 24, 48 and 72 hours post infection Black arrows indicate bands not present in mock infected cells, and where possible are labelled with the virus protein of corresponding molecular weight. BUNV was included as a control. Black lines on the right hand side of the protein gels indicate the migration of the non-labelled protein ladder with sizes of approximately 10, 15, 25, 30, 50, 65, 80, 115 and 180 kDa in a 4-12% Bis-Tris gel with 1xMES running buffer.

Virus abbreviations: Bunyamwera virus (BUNV), Anopheles B virus (ANBV), Boraceia virus (BORV), Capim (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV), and Moriche virus (MORV).

In cells infected with the Gamboa serogroup viruses, GAMV and SJV, bands corresponding to the size of orthobunyavirus N (25 kDa) and Gc (120 kDa) proteins were detected (Figure 4-12). In GMAV and PLSV infected cells the only band distinct from those in mock infected cells migrated at approximately 25 kDa, analogous with the size of orthobunyavirus N proteins (Figure 4-12). Bands of 25 kDa were also present in NDV and EREV infected cells. In addition, bands corresponding in size to virus NSs, Gn, Gc and L proteins were also detected in EREV infected cells, and NSs protein in NDV infected cells (Figure 4-12).

Bands corresponding in size to orthobunyavirus N and Gc protein could be detected in cells infected with Tete and Turlock serogroup viruses (Figure 4-13). In BMAV, MTRV, TETEV and TSUV infected cells the band posited as N protein migrated at approximately 27/28 kDa, whilst in WELV infected cells it migrated slightly faster at approximately 25 kDa and was only visible at 24 h p.i. (Figure 4-13). A band predicted to be virus Gc protein migrated at 115 kDa - 120 kDa in all Tete serogroup viruses, although it was only present in WELV infected cells at 24 h p.i. (Figure 4-13). In MPOV infected cells bands corresponding in size to virus N and Gc proteins at 25 kDa and 115 kDa were clearly visible only at 24 h p.i., with very faint bands at 48 h p.i. (Figure 4-13).

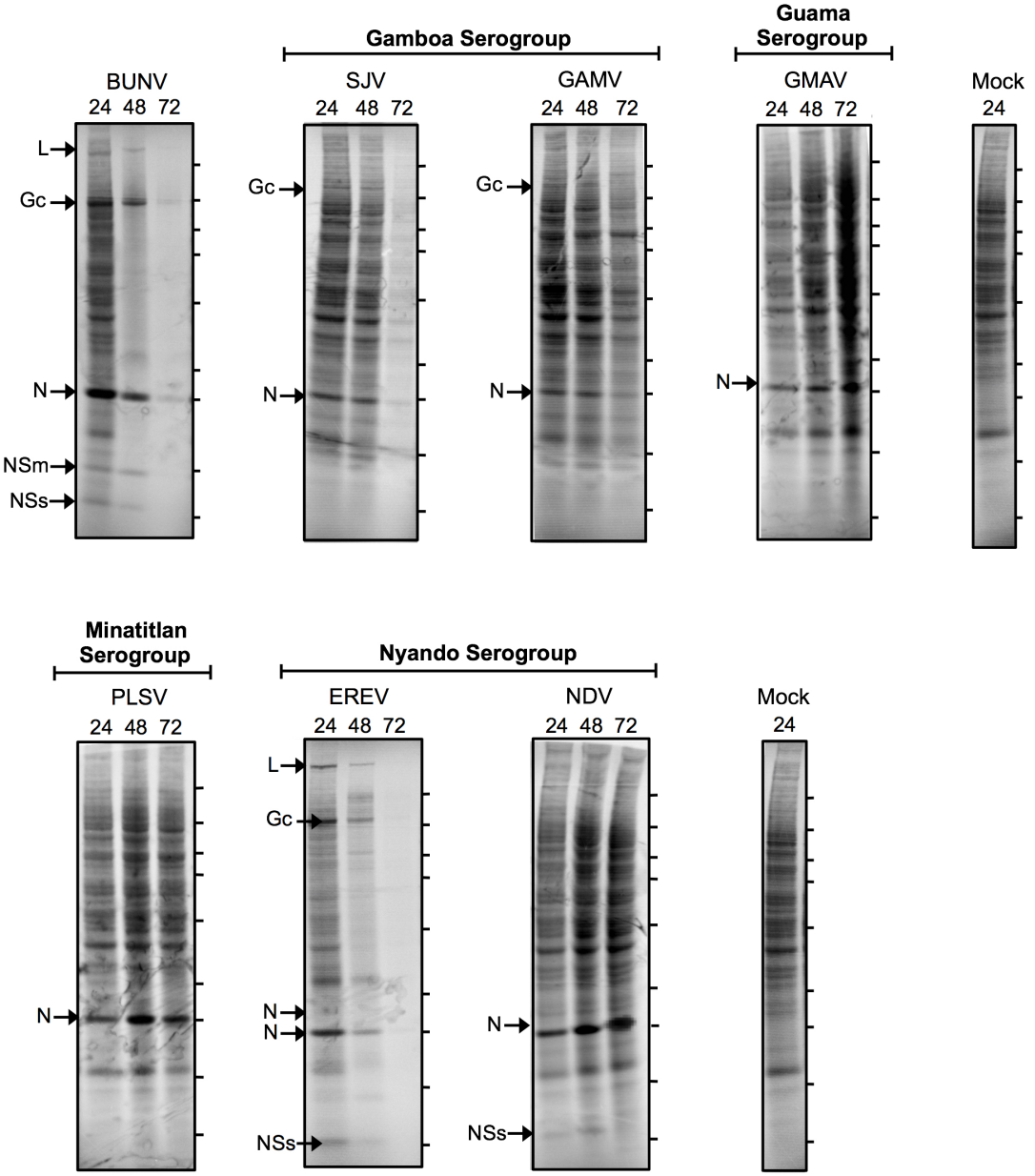


Figure 4-12: Metabolic Labelling of Viruses in the Gamboa, Guama, Minatitlan and Nyando Serogroups

Vero E6 cells were infected at multiplicity of infection 1 or mock infected with PBS 2% (v/v) FCS. Cells were radiolabelled with 35 S methionine cysteine for 2 hours prior to harvesting at 24, 48 and 72 hours post infection Black arrows indicate bands not present in mock infected cells, and where possible are labelled with the virus protein of corresponding molecular weight. BUNV was included as a control. Black lines on the right hand side of the protein gels indicate the migration of the non-labelled protein ladder with sizes of approximately 10, 15, 25, 30, 50, 65, 80, 115 and 180 kDa in a 4-12% Bis-Tris gel with 1xMES running buffer.

Virus abbreviations: Bunyamwera virus (BUNV), San Juan virus (SJV), Gamboa virus (GAMV), Guama virus (GMAV), Palestina virus (PLSV), Eretmapodites virus (EREV) and Nyando virus (NDV).

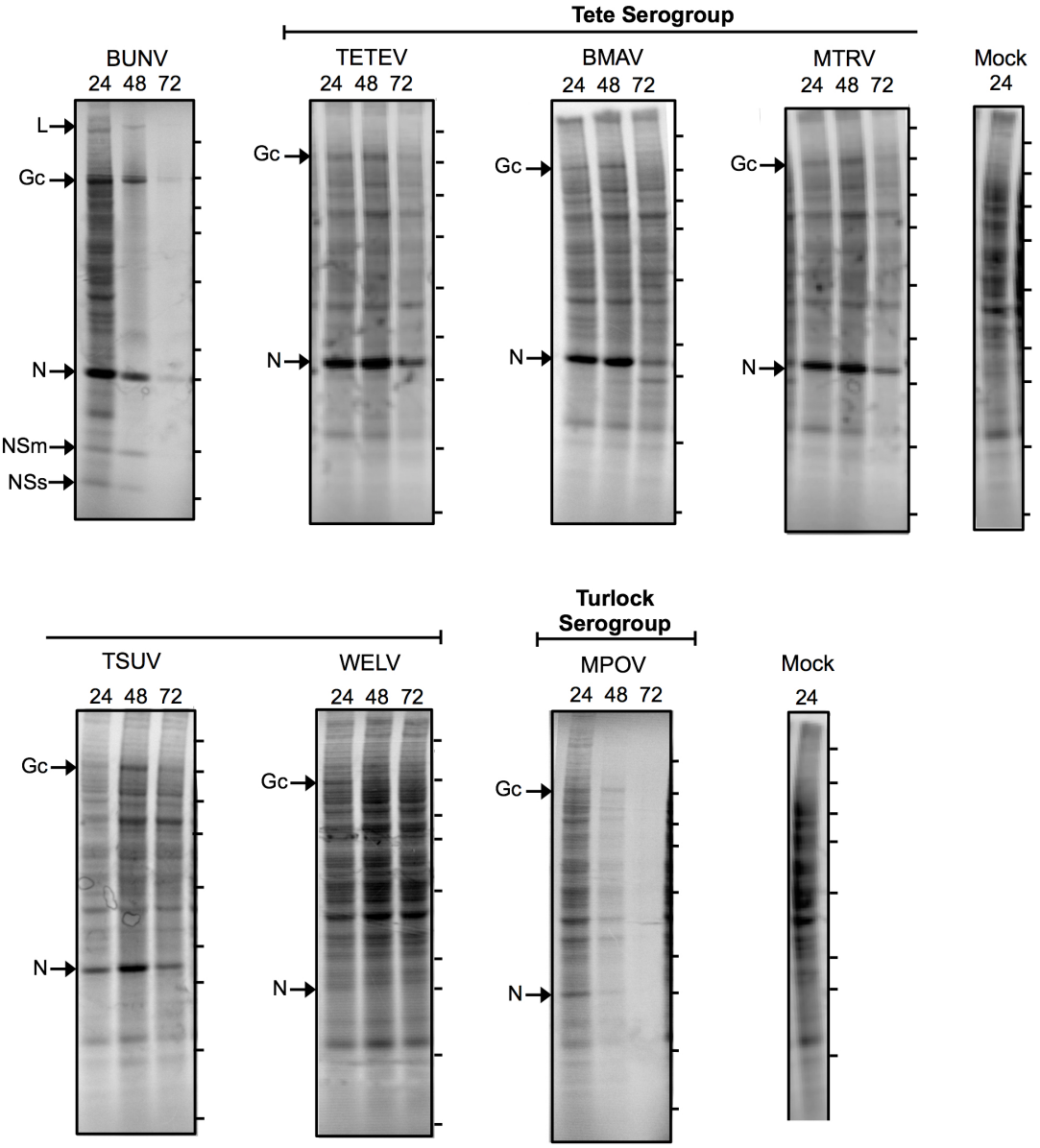


Figure 4-13: Metabolic Labelling of Viruses in the Tete and Turlock Serogroups
Vero E6 cells were infected at multiplicity of infection 1 or mock infected with PBS 2% (v/v) FCS. Cells were radiolabelled with 35 S methionine cysteine for 2 hours prior to harvesting at 24, 48 and 72 hours post infection Black arrows indicate bands not present in mock infected cells, and where possible are labelled with the virus protein of corresponding molecular weight. Black arrows indicate bands not present in mock infected cells and where possible labelled with the virus protein of corresponding molecular weight. BUNV was included as a control. Black lines on the right hand side of the protein gels indicate the migration of the non-labelled protein ladder with sizes of approximately 10, 15, 25, 30, 50, 65, 80, 115 and 180 kDa in a 4-12% Bis-Tris gel with 1xMES running buffer.

Virus abbreviations: Bunyamwera virus (BUNV), Tete virus (TETEV), Batama virus (BMAV), Matruh virus (MTRV), Tsuruse-like virus (TSUV), and M'Poko virus (MPOV).

4.4 Discussion

4.4.1 Virus Propagation and Plaque Morphology

In this study only 26 of 50 freeze-dried orthobunyaviruses were successfully propagated in Vero E6 cells giving a recovery rate of 52%. Passaging virus isolates on only 1 cell line introduced a clear restriction factor and possible barrier to virus growth as Vero E6 cells may not be permissive to all of the viruses that were screened. The Vero E6 cell line was selected as it was the most frequently documented permissive cell line in the ArboCAT for the viruses included in this study (Table 4-1) (Berge, 1975). However, ArboCAT contained no information on cell line permissiveness for 7 of the 24 viruses that failed to show CPE or form plaques at 6 d p.i. or 14 d p.i.. These were Anopheles A ColAr1071 and CoAr3624, Arumateua, Botambi, Olifantsvlei, Minatitlan and Pahayokee viruses (Berge, 1975). In 1968 T.B. Stim carried out a detailed investigation into arbovirus plaque morphology on 2 simian kidney-derived cell lines, Vero cells and LLC-MK_2 cells. In disagreement with ArboCAT records, Stim (1968) reported that LUKV, a virus that failed to form plaques or CPE by passage 10 in this study, did not form plaques on Vero cells. Furthermore, it was reported that although mouse passage 5 Olifantsvlei virus SAAr 5133 did not form plaques the virus preparation was fatal to infant mice (Stim 1968). Therefore, the failure of viruses to show CPE or form plaques on Vero cells does not rule out the presence of infectious particles. For all isolates the residual freeze-dried resuspension, passage 1 cell monolayers harvested in Trizol, and clarified culture supernatant from each blind passage were stored at -80°C. This will facilitate future studies by providing RNA for possible screening by HTS and resuspended freeze-dried isolates for passaging on alternative cell lines.

In addition to the choice of cell line, sample viability will also have impacted on virus recovery. Information on the date isolates were freeze-dried was available for all (n=50) of the isolates passaged in this study. Freeze-dried dates ranged from 1959 for TURV to 2011 for BVSV (Table 2-1). Comparing the year in which samples were freeze-dried revealed a significant difference between viruses that were successfully propagated (n=27, median 1984, range 1963 - 2009) and those which were not (n=23 median 1970, range 1959 - 2011) (t-test, $p<0.05$). However, it should be noted that

sample age was not an absolute predictor of recovery success as viruses freeze-dried in 1963 (ANBV), 1966 (EREV, MORV) and 1968 (CAPV) were successfully propagated, whilst BVSV, which is documented as causing CPE in Vero cells and freeze-dried in 2011, failed to produce CPE or plaques in this study. Pastorino *et al.,* (2015) investigated the thermostability and infectivity of freeze-dried viruses, observing < 2 log drop in $TCID_{50}$ values for freeze-dried Chikungunya virus (CHIKV), an enveloped ssRNA virus, when it was stored at +20°C for 7 days. The viruses included in this study were shipped from the United States of America at room temperature and may have undergone loss in infectivity due to thermal instability. Furthermore, 27 of the viruses included in this study were originally received in the laboratory in 2003. The samples were resuspended and stored at -20°C, however, prior to this study they have been subject to at least 3 freeze-thaw cycles due to power failures and freezer breakdown, possibly further impacting negatively on sample viability. Fourteen of the viruses that failed to form CPE or plaques belonged to this group.

In total 26 viruses, belonging to 10 serogroups, developed CPE on virus passage, however only 25 could be titrated by plaque assay. Despite forming clear CPE WELV, a member of the Tete serogroup, could not be titrated by plaque assay (Figure 4-2, Figure 4-3). Altering the length of incubation (3 days - 12 days), temperature (32°C, 33°C and 37°C), cell type (BHK-21, Vero E6 or CPT-Tert cells) or overlay concentration (0.6% (w/v) - 1.2% (w/v)) did not improve plaque formation. Interestingly, 4 other viruses in the Tete serogroup, formed clear plaques and grew to a titre of 1.0×10^7 p.f.u ml^{-1} - 3.10×10^8 p.f.u ml^{-1} (Figure 4-2, Table 4-1). WELV was isolated from Culicoides flies whilst other Tete serogroup viruses (BAHV and MTRV) have been found in Hyalomma marginatum ticks (Table 4-2) (Converse *et al*., 1974; Moussa *et al.*, 1974). Divergent vectors and plaque phenotype suggest that WELV may be distant from other viruses in the Tete serogroup.

Table 4-2: Orthobunyavirus Ecology

Virus ABBV	Geographical	Vector Subfamily	Hosts	Human Infection
Anopheles A Serogroup				
ANAV	South America	N/A	N/A	N/A
ColAn57389	N/A	N/A	N/A	N/A
CPEV	N/A	N/A	N/A	N/A
LMV	South America	Anophelinae	N/A	N/A
TCMV	South America	Anophelinae and Culicinae	Bats, birds, horses, primates and rodents	Y
TRMV	South America	N/A	N/A	N/A
TUCV	N/A	N/A	N/A	N/A
Anopheles B Serogroup				
ANBV	South America	Anophelinae	N/A	N/A
BORV	South America	Anophelinae and Culicinae	N/A	NtAb
Bwamba Serogroup				
BWAV	Africa	Anophelinae	N/A	Y
Capim Serogroup				
CAPV	South America	Culicinae	Rodent	N/A
GJAV	N/A	N/A	N/A	N/A
JDV	South America	N/A	Sentinal mice	N/A
MORV	South America	Culicinae	N/A	N/A
Gamboa Serogroup				
GAMV	South America	Culicinae	N/A	N/A
SJV	N/A	N/A	N/A	N/A
Guama Serogroup				
GMAV	South America	Anophelinae and Culicinae	Bats, marsupials and rodents	Y
Minatitlan Serogroup				
PLSV	South America	Culicinae	Sentinal hamster	N/A
Nyando Serogroup				
EREV	Africa	Anophelinae	N/A	N/A
NDV	Africa	Anophelinae	N/A	Y
Tete Serogroup				
BMAV	Africa	N/A	Birds	N/A
MTRV	Egypt, Italy	Hyalomminae	Birds	N/A
TETEV	Africa	N/A	Birds	N/A
TSUV	Japan	N/A	Birds	N/A
WELV	USA	Ceratopogonidae	N/A	N/A
Turlock Serogroup				
MPOV	Africa	Culex	N/A	NtAb

Exert from Orthobunyavirus spreadsheet. Published information on the geographical location, virus vector, host and association with human infection is summarised. Unless stated information was sourced from the International Catalog of Arboviruses (ArboCAT). Vector information is given at the subfamily level; Anophelinae or Culicinae (mosquitoes), Ceratopogonidae (*Culicoides* flies) and Hyalomminae (hard bodies Hyalomma ticks). Association with human infection is indicated by "Y" symbolising yes, for clinical cases and isolation of virus from man, where no clinical cases have been detected but neutralising antibodies are present "NtAb".

Plaque morphology has been linked with virus attenuation in orthobunyaviruses as rBUNdelNSs2, a recombinant BUNV that lacks the NSs protein, forms smaller plaques than the parental wt virus BUNV (Bridgen *et al.*, 2001; Hart *et al.,* 2009; van Knippenberg *et al.,* 2010*)*. In this study the only viruses to form plaques of a comparable size to that of BUNV were MPOV and GMAV, both of which have been linked with human infection (Figure 4-2, Table 4-2). The other known human pathogens, BWAV, TCMV, NDV and EREV formed small plaques of only ≤ 1 mm in diameter at 6 d p.i. (Figure 4-1, Figure 4-2, Table 4-2). Moreover, the plaque size of Guaroa virus, an orthobunyavirus in the Bunyamwera serogroup, does not correlate with pathogenicity as small and large plaque variants had similar pathogenicity in suckling mice inoculated intracranially (Tauraso 1969). Consequently, plaque size of orthobunyaviruses on Vero E6 cells cannot be correlated with *in vivo* pathogenicity or virulence.

4.4.2 Virus Growth Kinetics

BUNV, BWAV, TCMV, NDV and EREV were the only viruses with < 1 log reduction in virus titre in A549 cells compared with Vero E6 cells. The A549 cell line, originally derived from human alveolar cells, is IFN-competent whilst the simian kidney derived cell line Vero E6 is IFN-deficient having lost the *IFNA* and *IFNB1* genes (Mosca and Pitha, 1986; Diaz *et al.,* 1988). BUNV, BWAV, TCMV, and Nyando serogroup viruses have all been documented as causing clinical disease in humans (Smithburn *et al.*, 1941 Digoutte *et al.*, 1972; Moore *et al.*, 1975; Johnson *et al.*, 1978; Calisher *et al.,* 1980; Lutwama *et al*., 2002). It is therefore perhaps to be expected that virus replication would not be severely attenuated in an IFN-competent cell line. However, the virus titres of BORV, GMAV and MPOV were suppressed by > 1 log in A549 cells despite their association with human infection (Figure 4-5, Figure 4-7, Figure 4-8) (Whitman and Casals, 1961; de Souza Lopes and de Abreu Sacchetta, 1974; Berge, 1975). Therefore, virus pathogenicity cannot be extrapolated from comparative growth kinetics in IFN-competent A549 cells and IFN-deficient Vero E6 cells.

Viruses in the Anopheles A, Anopheles B and Tete serogroup (excluding TCMV) also showed >1 log drop in virus titres in A549 cells at 72 h p.i.. Six viruses in these

serogroups, ANAV, TCMV, ANBV, BORV, BMAV and TETEV, have previously been shown to lack an NSs protein (Mohamed *et al.*, 2009). The NSs protein is the primary virulence factor of orthobunyaviruses, blocking RNA polymerase II and antagonising the type I IFN response (Léonard *et al.*, 2006; Thomas 2004; van Knippenberg *et al.*, 2010). This leads to the hypothesis that non-NSs viruses would be vulnerable to the IFN response as this action of antagonism is not present. This hypothesis is supported by the growth kinetics of rBUNdelNSs2, a recombinant virus in which the expression of NSs has been abolished through the introduction of 6 mutations that are synonymous for the BUNV N protein (Figure 4-6) (van Knippenberg *et al*., 2010; Bridgen *et al*., 2001). However, TCMV opposes this theory, showing similar growth kinetics in both IFN-competent and IFN-deficient cell lines, despite lacking an NSs protein (Figure 4-4). Furthermore, Gamboa serogroup viruses, GAMV and SJV, showed > 2 log drop in virus titre in A549 cells, yet Nunes *et al.* (2014) published complete coding sequence data for 4 Gamboa serogroup viruses and all were predicted to contain full length NSs ORFs. Therefore, the presence or absence of an NSs ORF can no longer act as a predictor of virus growth in IFN-competent cell lines.

4.4.3 Protein Labelling

The titrated viruses had protein profiles corresponding to the characteristic biochemical footprint of orthobunyaviruses with N and Gc protein bands at approximately 24 ± 3 kDa and 117 ± 3 kDa respectively. The higher molecular weight of Tete serogroup N proteins corroborates S segment sequencing data published by Mohamed *et al.,* (2009) that reported BMAV and TETEV encode N proteins of 258 aa, in comparison to the 233 aa N protein of BUNV. However, the N protein of WELV was smaller than that of the other Tete serogroup viruses at 25 kDa, and only visible at 24 h p.i. (Figure 4-12). WELV failed to form clear plaques on virus titration and protein labelling was carried out at an unknown MOI with 200 µl neat working stock, whilst all other viruses were tested at an MOI of 1. If Vero E6 cells were not permissive for WELV infection the titre of the working stock preparation used in this experiment would be approximately a 1 in 6.25 x 10^5 dilution of the original freeze-dried suspension, or 160 p.f.u ml^{-1} if the freeze-dried suspension contained 1 x 10^8 p.f.u ml^{-1}. Non-replicating residual cell bound virus at this titre would be insufficient to form visible bands on radiolabelling,

therefore some level of virus propagation in Vero E6 cells must have occurred. Vero E6 cells are also IFN-deficient and apparent virus clearance at 48 h p.i. or 72 h p.i. would be highly unusual. In contrast to WELV, the intensity of all other virus protein bands increased over time and only reduced in intensity in conjunction with a reduction of host protein bands, most probably due to host protein shut-off and/or cell death.

There was very low detection of bands corresponding to the Gn and L proteins in infected cells. This was partly due to masking by host cellular protein bands of a similar molecular weight. Radiolabelling concentrated virions instead of infected cell supernatants may therefore improve detection of these proteins, especially Gn, which frequently gives rise to much fainter bands than the other structural proteins (Gentsch *et al*., 1979; Ushijima *et al*., 1980; Short *et al.*, 1982). In concordance with previous studies, the detection of non-structural proteins was also poor with both NSm and NSs proteins identified only in BUNV infected cells (Figure 4-9) (Gentsch *et al.*, 1977; Gentsch *et al.*, 1979; Ushijima *et al.,* 1980; Klimas *et al.,* 1981; Stuckly and Wright 1983). The NSs protein of BUNV undergoes lysine-dependent degradation by the proteasome and the addition of proteasome inhibitors such as MG132 leads to improved detection of NSs by western blot analysis (van Knippenberg *et al.*, 2013). The addition of proteasome inhibitors may also increase the detection of NSs by radiolabelling. However, not all orthobunyaviruses encode an NSs protein as viruses in the Anopheles A, Anopheles B, Mapputta, Simbu and Bunyamwera serogroup have been shown to contain severely truncated and presumably non-functional NSs ORFs (Mohamed *et al*., 2009; Chowdhary *et al*., 2012; Ladner *et al.,* 2014; Gauci *et al.*, 2015). It will therefore be of interest to determine if the viruses in the Capim, Guama, Gamboa, Minatitlan and Turlock serogroups, which had no detectable NSs protein band on radiolabelling, are in fact lacking a full length NSs ORF.

4.4.4 Concluding Remarks

In conclusion, a panel of 25 orthobunyaviruses representing 10 serogroups of the orthobunyavirus genus was created. The presence of replicating virus was confirmed by virus titration on Vero E6 cells and metabolic labelling of proteins during virus infection. Growth kinetics in IFN-competent A549 cells and IFN-deficient Vero E6

cells at low MOI were also determined. A spreadsheet collating information on vector, geographical location and vertebrate hosts was also created to facilitate result interpretation and complement future sequencing studies (Table 4-2, Appendix 2).

Chapter 5: Orthobunyavirus Genome Sequencing

5.1 Introduction

Orthobunyaviruses, the largest of the 5 bunyavirus genera, are transmitted by arthropods, a feature that contributes to their continual evolution and geographical spread. This was exemplified by the emergence of SBV, a novel orthobunyavirus causing abortion and foetal abnormalities in cattle and sheep, that spread rapidly throughout Europe in 2011 (van den Brom *et al.*, 2012; Gibbens, 2012; Rasmussen *et al.*, 2012). There are currently 48 ICTV-registered orthobunyavirus species but over 170 named viruses in the literature, with 'new' viruses increasingly described by HTS screens (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012; Lanciotti *et al.*, 2013; Bakonyi *et al.*, 2013; Li *et al.*, 2015). In addition to HTS, consensus primers targeting the conserved termini of orthobunyavirus genome segments have been used for PCR amplification and Sanger sequencing (Yandoko *et al.*, 2007; Mohamed *et al.,* 2009) .

Utilising consensus primers targeting the orthobunyavirus S segment Mohammed *et al.,* (2009) identified viruses within the Anopheles A, Anopheles B and Tete serogroups that naturally lack a functional NSs ORF. Subsequently, viruses within the Mapputta, Simbu and Wyeomyia groups have also been found to contain severely truncated and presumably non-functional NSs ORFs (Mores *et al.*, 2009; Chowdhary *et al.*, 2012; Ladner *et al.*, 2014; Gauci *et al.*, 2015). The NSs protein has been identified as the major virulence factor of orthobunyaviruses, blocking RNAPII and inhibiting the transcription of cellular mRNAs (Bridgen *et al.*, 2001; Thomas, 2004). The transcriptional blockade has a profound effect on the IFN-β promoter and viruses genetically engineered to lack a functional NSs show increased sensitivity to type I IFNs (Bridgen *et al.*, 2001; Blakqori *et al.*, 2007). Studying naturally occurring "non-NSs" bunyaviruses will further our understanding of bunyavirus evolution and replication, and may reveal previously unidentified mechanisms by which orthobunyaviruses can overcome or subvert the host's innate immune system. As yet, no complete genome sequences are available for viruses within the Anopheles A, Anopheles B or Tete serogroups. Furthermore, complete genome sequencing of orthobunyaviruses as a whole is extremely limited with 12 out of the 18 recognised orthobunyavirus serogroups lacking a single, published, complete genome sequence. Full genome sequence information will enable the relationships between orthobunyaviruses to be established and their evolutionary history tracked. Moreover,

at a practical level, it will provide essential information for the rational classification of bunyaviruses as well contributing to our understanding of bunyavirus emergence and facilitating in depth molecular research on virus replication and interactions with the host cell.

5.2 Aims

The aim of the research presented in this chapter was to carry out complete genome sequencing of orthobunyaviruses belonging to serogroups with no published complete genome sequences[1]. Viruses in serogroups identified by S segment sequencing to contain isolates lacking a full length NSs ORF, namely the Anopheles A, Anopheles B, Capim and Tete serogroups, were prioritised (Mohamed, 2007).

5.3 Results

In total 21 viruses were selected for HTS with viruses belonging to serogroups known to contain non-NSs isolates preferentially picked (n=15) (Table 5-1). The remaining viruses belonged to serogroups containing isolates with full length NSs ORFs (n=4) or no sequence information (n=2) (Table 5-1). Twenty-one viruses were selected as this made optimal use of sequencing reagents. To confirm the presence of replicating virus in each sample, an aliquot of the clarified and filtered cell culture supernatant processed for HTS was titrated by plaque assay on Vero E6 cells (Table 5-1).

HTS on virion RNA was carried out on the illumina® MiSeq and Ion Proton™ platforms followed by *de novo* genome assembly in CLC Genomics Workbench v7.5.1

[1] Complete genome and coding sequences were published for Nyando and Gamboa serogroup viruses during data analysis and have been included in data analysis (Groseth *et al.*, 2014; Nunes *et al.*, 2014). Schetinin *et al*., (2015) have also determined complete coding sequence for viruses in the Anopheles A, Capim, Guama, Tete and Turlock serogroups, although sequences were not available on GenBank® on 3rd December 2015, and are therefore not included in data analysis.

Table 5-1: Virus Isolates for Genome Sequencing

Virus Name	Serogroup	Date of isolate (DD.MM.YYYY)	Strain Information	NSs ORF	Sample Titre (p.f.u. ml^{-1})
ANAV	Anopheles A	23.12.1984	Isolated 1940	N	1.4×10^5
57389	Anopheles A	13.10.1981	ColAn 57389	N/A	2.1×10^5
LMV	Anopheles A	10.09.1981	AG 80-14	N/A	7.0×10^5
TCMV	Anopheles A	21.07.2001	BeAn73	N	4.1×10^5
TRMV	Anopheles A	21.09.1981	BeAn 306771	N/A	2.6×10^6
ANBV	Anopheles B	15.02.1963	DMC 1537	N	1.0×10^6
BORV	Anopheles B	21.06.1965	SP Ar395	N	8.6×10^5
CAPV	Capim	08.02.1994	N/A	N	1.5×10^6
JDV	Capim	12.06.2001	Maru 8563	N/A	1.5×10^5
MORV	Capim	17.11.1966	N/A	N/A	3.0×10^5
GAMV	Gamboa	17.12.1990	GML 438524	Y	4.5×10^5
GMAV	Guama	13.01.1992	BeAn 277	N/A	3.5×10^4
PLSV	Minatitlan	31.08.1982	76V 1565	N/A	3.0×10^5
EREV	Nyando	10.07.1966	E147V	Y	4.1×10^5
NDV	Nyando	11.10.2003	Mp401	Y	2.7×10^5
BMAV	Tete	15.12.1984	DAK AnB1292	N	2.2×10^7
MTRV	Tete	12.07.1985	An 1047-61	N/A	1.1×10^7
TETEV	Tete	12.07.1984	SA An3518	N	3.5×10^6
TSUV	Tete	03.11.1999	Magpie 271580	N/A	1.2×10^7
WELV	Tete	30.04.2009	76V 21935	N/A	N/A
MPOV	Turlock	10.03.2003	BA365	Y	4.2×10^6

Details of the 21 viruses selected for genome sequencing. Isolates were obtained from the Emerging Viruses and Arboviruses Reference Collection, University of Texas Medical Branch and propagated as detailed in Chapter 4. The presence of an NSs ORF as known in December 2013 is also given; (N) none or severely truncated, (Y) putative complete NSs ORF, (N/A) not available (Mohamed, McLees and Elliott, 2009; Mohamed, no date). The virus titre of RNA cultures after filtration was determined by plaque assay on Vero E6 cells with a 0.6% (w/v) Avicel overlay.

Virus abbreviations: ANAV (Anopheles A virus), 57389 (ColAn 57389 virus), LMV (Las Maloyas virus), TCMV (Tacaiuma virus), TRMV (Trombetas virus), ANBV (Anopheles B virus), BORV (Boracéia virus), CAPV (Capim virus), JDV (Juan Diaz virus), MORV (Moriche virus), GAMV (Gamboa virus), GMAV (Guama virus), PLSV (Palestina virus), EREV (Eretmapodites virus), NDV (Nyando virus), BMAV (Batama virus), MTRV (Matruh virus), TETEV (Tete virus), TSUV (Tsuruse-like virus), WELV (Weldona virus), MPOV (M'Poko virus).

(Qiagen). HTS data were trimmed by quality score and adapter sequences removed prior to contig assembly with word and bubble size optimised for average HTS read length. Contigs were then assembled into genome scaffolds through BLASTn and BLASTx comparisons with cyclical rounds of read mapping. Regions of low coverage (< 100 reads) and terminal UTR sequences were confirmed by Sanger sequencing and RACE PCR (Table 5-2).

5.3.1 N and NSs Coding Sequences

All S segment sequences contained bunyavirus nucleocapsid domain pfam00952. Capim, Guama, Gamboa, Minatitlan, Nyando and Turlock serogroup viruses are predicted to encode N proteins of 233 - 238 aa, whereas viruses belonging to the Anopheles A, Anopheles B and Tete serogroups encode larger N proteins at 244 – 245 aa, 247 aa, and 248 - 258 aa, respectively (Table 5-2). In the Anopheles A and Anopheles B serogroup the additional amino acids align at the C terminus of the N protein. In Tete serogroup N proteins the additional amino acids form a 25 - 26 aa insertion at the N terminus, with only 3 - 4 aa aligning to the C terminus of the N protein (Figure 5-1). The BUNV N protein E128A change associated with a small-plaque, high-titre phenotype in BUNV was only present in MPOV. All other viruses in this study retained the E residue at this position. N protein residues that remain fully conserved within the orthobunyaviruses are at alignment positions K77, T119 and G180 (BUNV N protein K50, T91, 147G) (Figure 5-1).

In addition to the N protein ORF a second ORF, that identifies as bunyavirus NSs pfam01104 by BLASTx and is in a plus 1 reading frame relative to N, was identified in 5 viruses. The NSs encoding viruses are Nyando serogroup viruses NDV and EREV, Gamboa serogroup virus GAMV, Turlock serogroup virus MPOV and Tete serogroup virus WELV (Table 5-2). All sequenced viruses in the Anopheles A, Anopheles B, Capim, Guama and Minatitlan serogroups lacked an NSs ORF, and contained no additional or severely truncated (< 40 aa) ORFs (Table 5-2).

Table 5-2: Virus Genome Size

Virus	Nucleotide sequence (nt)									Amino acid sequence (aa)			
	S segment			M segment			L segment						
	5' UTR	ORF	3' UTR	5' UTR	ORF	3' UTR	5' UTR	ORF	3' UTR	N	NSs	M	L
ANAV	53†	738	155¥	52†	4224	339¥	33†	6729	119†	244	N/D	1405	2242
57389	53†	738	208¥	43†	4218	124†	32†	6723	94†	245	37*	1405	2240
LMV	54†	738	210¥	51†	4236	109¥	32†	6723	100†	245	37*	1411	2240
TCMV	42†	738	134¥	35†	4278	124†	33†	6723	93†	245	27*	1425	2240
TRMV	44†	738	203¥	34†	4248	118†	32†	6723	94†	245	37*	1415	2240
ANBV	35†	744	212¥	38†	4296	280†	48†	6729	180†	247	24*	1431	2242
BORV	35†	744	203†	32†	4293	224†	39†	6729	91†	247	38*	1430	2242
CAPV	49†	708	307¥	51†	4293	454¥	58†	6759	162†	235	11*	1430	2252
JDV	44†	705	323¥	42†	4296	478¥	61†	6752	188†	234	10*	1431	2253
MORV	44†	705	379¥	42†	4329	434¥	61†	6792	154†	234	10*	1442	2253
GAMV	86†	717	438	42†	4779	236†	47†	6822	79†	238	130	1592	2273
GMAV	70†	714	355¥	139†	4320	240	57	6753	178	237	18*	1439	2250
PLSV	89†	711	>167	62†	4335	>145†	51†	6726	181†	236	21*	1444	2241
EREV	70¥	702	169¥	34¥	4338	135¥	30¥	6798	111¥	233	92	1445	2265
NDV	45†	702	155¥	48†	4299	102†	29†	6807	121†	233	92	1432	2268
BMAV	73†	777	171†	39†	4302	110†	57†	6843	93†	258	33*	1433	2280
MTRV	58†	777	228¥	39†	4299	115†	58†	6843	125†	258	36*	1432	2280
TETEV	73†	777	172¥	39†	4299	116†	57†	6846	92†	258	20*	1432	2281
TSUV	58¥	777	183¥	39†	4302	111¥	57†	6843	124†	258	23*	1433	2280
WELV	60†	747	185†	N/A	N/A	N/A	N/A	N/A	N/A	248	114	N/A	N/A
MPOV	117†	711	247	77†	4386	266†	73†	6828	118†	236	79	1461	2275
BUNV	85	702	174	56	4302	100	50	6717	108	233	101	1433	2238

Size of virus genomes and predicted proteins are listed. Viruses sequenced in this study are highlighted in bold. The genome details of Bunyamwera virus (BUNV) NC001927 are given for comparison. UTR; untranslated region, ORF; open reading frame, nt; nucleotide, aa; amino acid. N/D; not detected, † Confirmed by RACE PCR. ¥ Confirmed by cloning into pGEM®-T Easy or pTVT7 vectors, terminal 15 nucleotides primer/HTS sequence data only. * truncated and presumably non-functional ORF.

Virus abbreviations: ANAV (Anopheles A virus), 57389 (ColAn 57389 virus), LMV (Las Maloyas virus), TCMV (Tacaiuma virus), TRMV (Trombetas virus), ANBV (Anopheles B virus), BORV (Boracéia virus), CAPV (Capim virus), JDV (Juan Diaz virus), MORV (Moriche virus), GAMV (Gamboa virus), GMAV (Guama virus), PLSV (Palestina virus), EREV (Eretmapodites virus), NDV (Nyando virus), BMAV (Batama virus), MTRV (Matruh virus), TETEV (Tete virus), TSUV (Tsuruse-like virus), WELV (Weldona virus), MPOV (M'Poko virus).

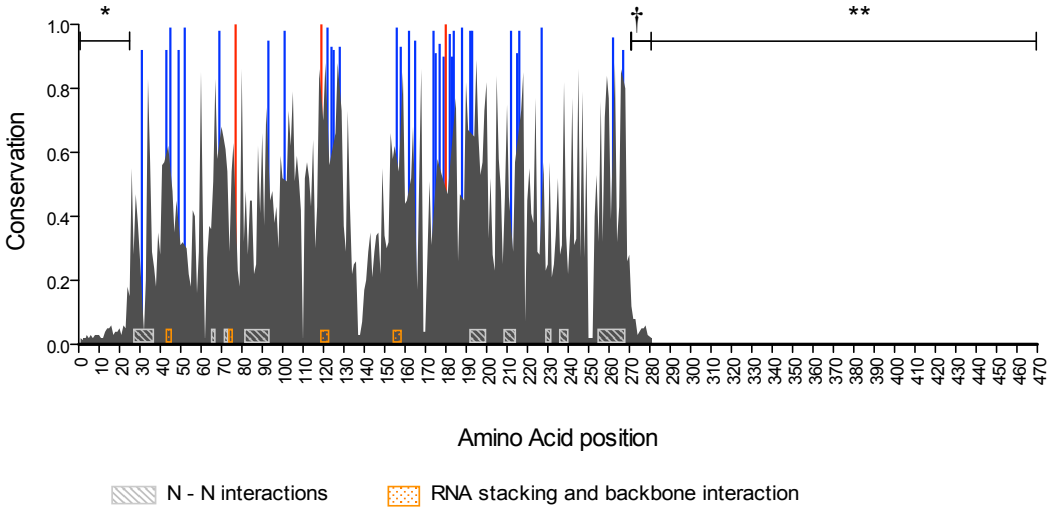


Figure 5-1: N Protein Conservation

N protein sequences of viruses sequenced in this study were aligned with previously published orthobunyavirus sequences; when more than 1 sequence for a named virus was available 1 was selected. Sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. Fully conserved residues are highlighted in red. Residues present in > 90% of named virus are marked in blue. Functional annotations as per Reguera *et al*., (2013). (*) Oyo, I612045 and Tete serogroup viruses only. (†) Mermet, M'poko, Brazoran viruses and Anopheles A, Anopheles B, Tete serogroup only. (**) Brazoran virus only.

The Nyando serogroup viruses EREV and NDV were predicted to encode a 93 aa NSs protein, which contains conserved SS, LxL and IxI sequence motifs (Figure 5-2). MPOV was predicted to encode an NSs protein of only 79 aa, which lacks the conserved LxL motif, containing FxL at this position. GAMV was predicted to encode a much larger NSs protein at 130 aa (Table 5-2). The additional aa in GAMV, and published Gamboa serogroup NSs proteins, align to the C terminus and form an incredibly serine-rich region (Figure 5-2) (Nunes *et al.*, 2014). WELV was predicted to encode a 114 aa NSs protein and although it contained the conserved LxL and IxI it lacked the conserved serine residues and had a 10 aa insertion in this region. WELV was the only member of the Tete serogroup predicted to contain an NSs ORF, with BMAV, TETEV, MTRV and TSUV containing severely truncated and presumed non-functional second ORFs. To confirm correct assembly of the WELV S segment, the complete S segment was amplified from virion RNA in a single RT-PCR reaction with primers targeting RACE-PCR confirmed terminal UTR sequences. The amplified segment was then cloned into a plasmid backbone for Sanger sequencing, confirming HTS data and the presence of an NSs ORF.

5.3.2 M Coding Sequences

The M segments of the viruses sequenced in this study are predicted to encode a single polyprotein that will be post-translationally cleaved to form the 2 virus glycoproteins, Gn (pfam03563) and Gc (pfam03557), and the non-structural protein NSm (TIGR04210). M polyproteins of Anopheles B, Capim, Guama, Nyando, Minatitlan, Tete and Turlock serogroups were 1430–1454 aa in length. The Anopheles A serogroup virus M polyproteins, excluding TCMV, were slightly smaller at 1405 - 1415 aa (Table 5-2). In agreement with complete coding sequences published for Gamboa serogroup viruses, GAMV encodes a larger M polyprotein at 1592 aa with a 149 aa insertion at the start of the NSm domain, which manifests as a clear drop in conservation in the M polyprotein alignment (Figure 5-3A) (Nunes *et al.*, 2014).

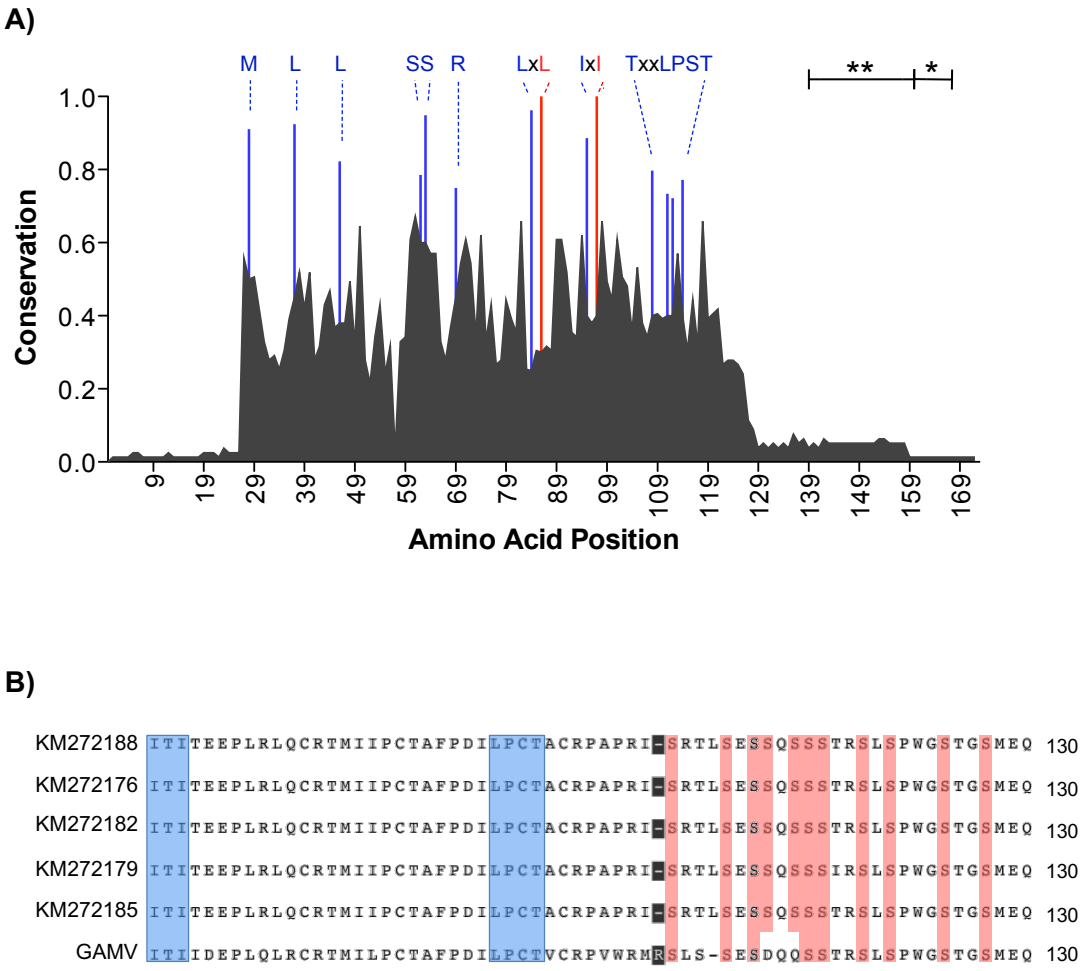


Figure 5-2: NSs Protein Conservation

A) NSs protein sequences of viruses sequenced in this study were aligned with previously published orthobunyavirus sequences; when more than 1 sequence for a named virus was available 1 was selected. Sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. Fully conserved residues are highlighted in red. Residues present in > 75% of named virus isolates are marked in blue. (*) Brazoran virus only, (**) Brazoran virus and Gamboa serogroup NSs proteins only. **B)** C terminal alignment of Gamboa serogroup NSs proteins, published sequences are named by their accession number, (GAMV) Gamboa virus sequenced in this study. Orthobunyavirus conserved motifs are highlighted in blue, serine residues in red.

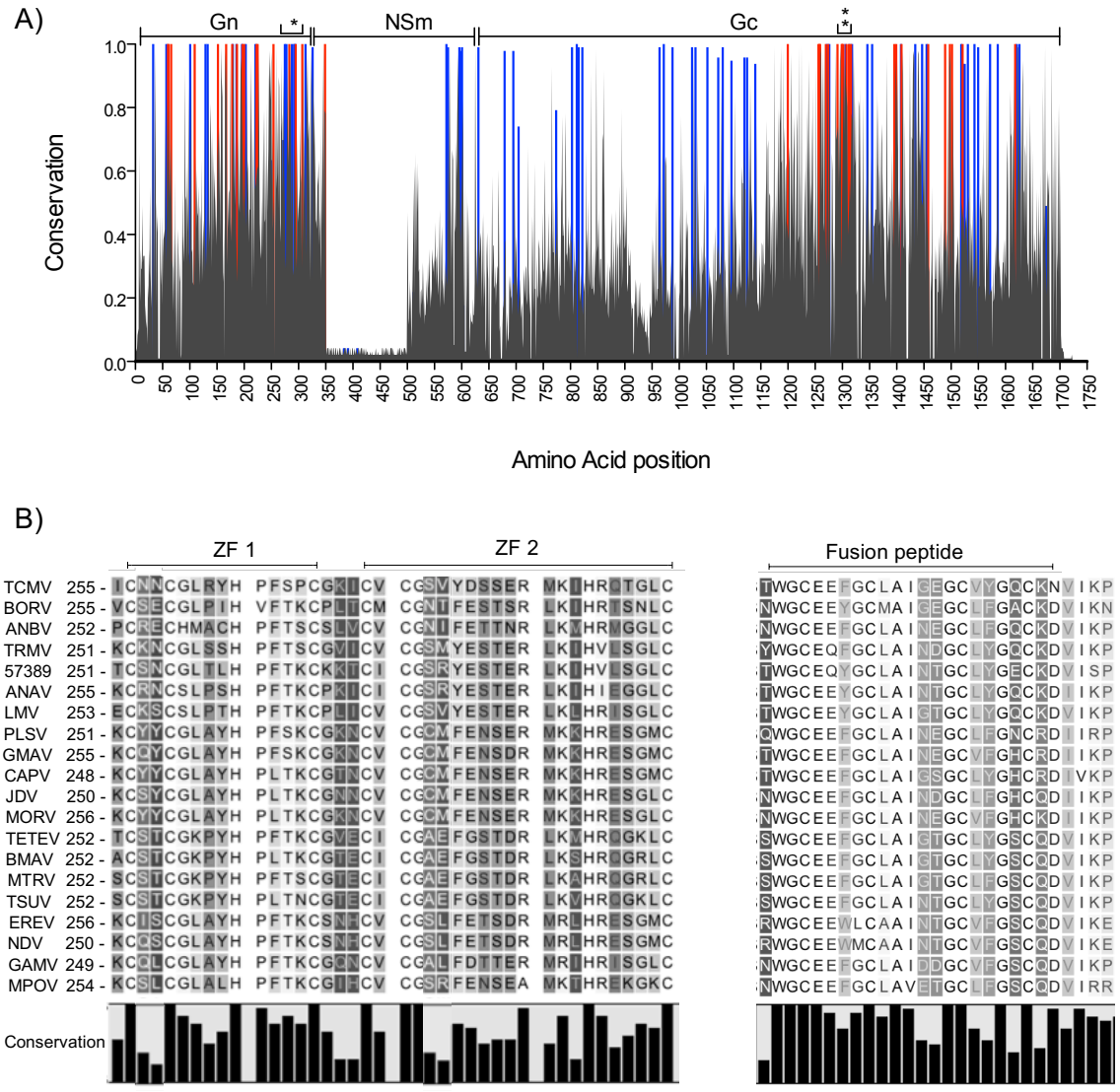


Figure 5-3: M Polyprotein Conservation

A) M polyprotein sequences of viruses sequenced in this study were aligned with previously published orthobunyavirus sequences; when more than 1 sequence for a named virus was available 1 was selected. Sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. Cysteine residues are highlighted in blue, fully conserved residues, excluding cysteine, are highlighted in red. (*) Gn zinc finger motif, (**) Gc fusion peptide. **B)** Gn zinc finger (ZF1 and ZF2) and Gc fusion peptide motifs of the viruses sequenced in this study. Sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. Black bars represent percentage conservation at each alignment position.

Virus abbreviations: ANAV (Anopheles A virus), 57389 (ColAn 57389 virus), LMV (Las Maloyas virus), TCMV (Tacaiuma virus), TRMV (Trombetas virus), ANBV (Anopheles B virus), BORV (Boracéia virus), CAPV (Capim virus), JDV (Juan Diaz virus), MORV (Moriche virus), GAMV (Gamboa virus), GMAV (Guama virus), PLSV (Palestina virus), EREV (Eretmapodites virus), NDV (Nyando virus), BMAV (Batama virus), MTRV (Matruh virus), TETEV (Tete virus), TSUV (Tsuruse-like virus), WELV (Weldona virus), MPOV (M'Poko virus).

The alignment also demonstrates striking conservation of cysteine residues, with 53 of 73 cysteine residues fully conserved across all sequences and 63 conserved in 90% of published M proteins (Figure 5-3A). Zinc finger motifs located in the Gn cytoplasmic tail and the Gc fusion peptide were readily detected in all sequences and were highly conserved (Figure 5-3B). N-glycosylation patterns remained largely conserved within serogroups (Figure 5-4). For example, all Anopheles A serogroup viruses lack a single glycosylation site in Gn, whilst GMAV and PLSV lack any glycosylation sites in the Gc C terminus (Figure 5-4).

5.3.3 L Coding Sequences

The characteristic domains of bunyavirus L proteins, namely the endonuclease domain (pfam15518) and 'polymerase module' (pfam04196) were present in all sequences and all sequences were of comparable length to those previously published for orthobunyavirus L segments (Table 5-2). Within the polymerase module premotif A, motifs A, B, C (containing the catalytic site S1633DD), D, and E were readily detected and highly conserved (Figure 5-5B). Two drops in aa conservation at alignment positions 604-607 and 2241-2253 can be attributed to an insertion of 4 aa (FxSD) by viruses in the Gamboa serogroup and 23 aa insertion by Tete serogroup viruses, respectively (Figure 5-5A).

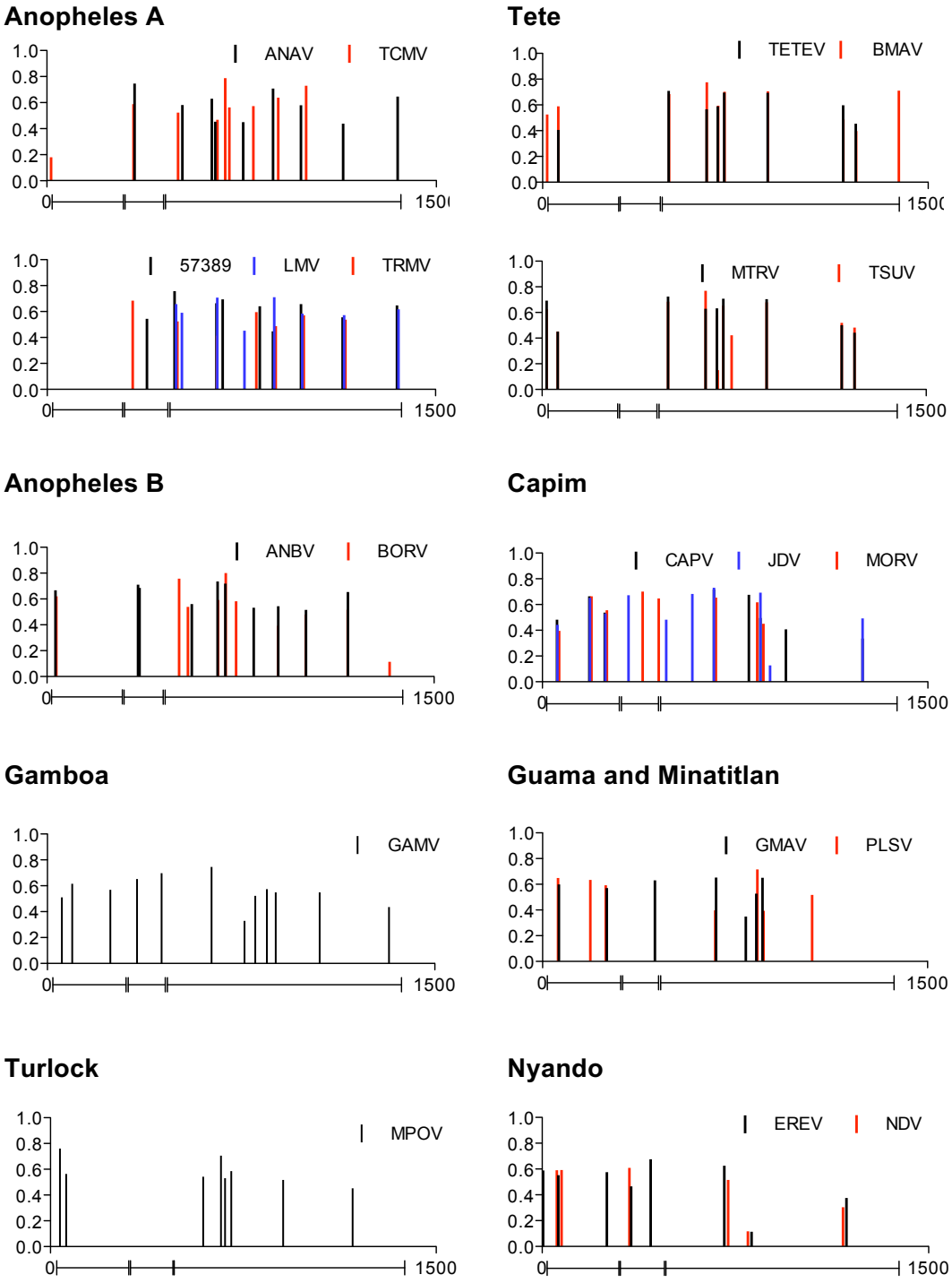


Figure 5-4: Predicted M Polyprotein N-glycosylation

N-glycosylation patterns as predicted by NetNGlyc 1.0. Viruses grouped into serogroups, with the exception of GMAV and PLSV, members of the Guama and Minatitlan serogroups, which are grouped together. Capped lines indicate Gn, NSm and Gc.

Virus abbreviations: ANAV (Anopheles A virus), 57389 (ColAn 57389 virus), LMV (Las Maloyas virus), TCMV (Tacaiuma virus), TRMV (Trombetas virus), ANBV (Anopheles B virus), BORV (Boracéia virus), CAPV (Capim virus), JDV (Juan Diaz virus), MORV (Moriche virus), GAMV (Gamboa virus), GMAV (Guama virus), PLSV (Palestina virus), EREV (Eretmapodites virus), NDV (Nyando virus), BMAV (Batama virus), MTRV (Matruh virus), TETEV (Tete virus), TSUV (Tsuruse-like virus), WELV (Weldona virus), MPOV (M'Poko virus).

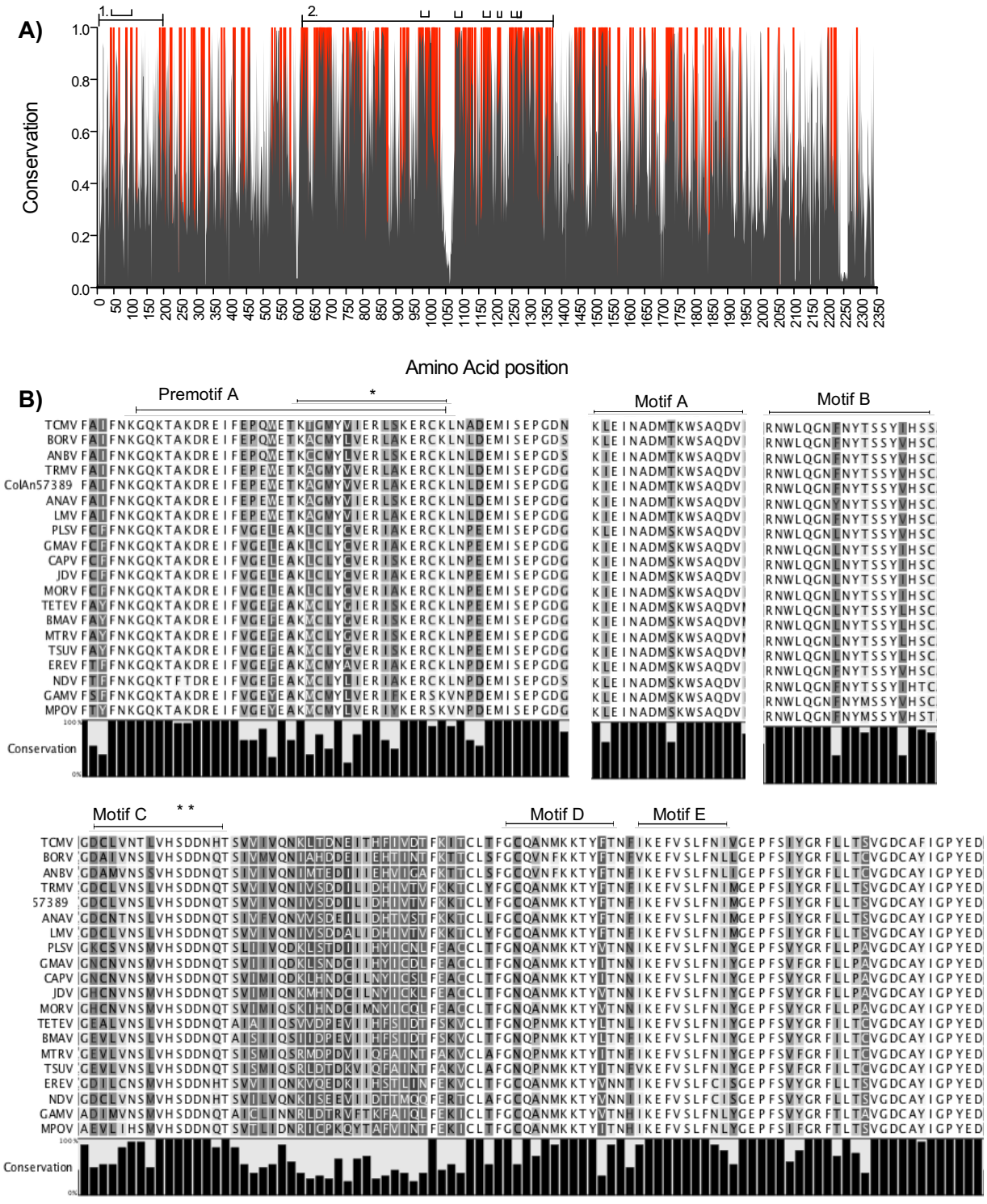


Figure 5-5: L Protein Conservation

A) L protein sequences of viruses sequenced in this study were aligned with previously published orthobunyavirus sequences; when more than 1 sequence for a virus species was available 1 was selected. Sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. Fully conserved residues are shown in red. 1. Endonuclease domain (pfam15518) with conserved nuclease superfamily motif marked. 2. Polymerase module (pfam04196) with pre-motif A and motifs A, B, C, D and E marked. B) Polymerase module of L proteins sequenced in this study. Sequence were aligned CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm. * 3' vRNA binding site ** Nucleotide addition sites.

5.3.4 Untranslated Sequences

The archetypal conserved orthobunyavirus termini (AGUAGUGUA, CACACUACU) were present in the S, M and L segments of ANAV, ANBV, BORV, GAMV, NDV, EREV, PLSV, BMAV, MTRV, TSUV and MPOV. However, members of the Capim and Guama serogroups contained changes at the 8th nucleotide position in both the S and M segments, and Anopheles A serogroup viruses, ColAn57389, LMV and TRMV contained a change in the M segment only (Table 5-3). When additional segment specific conserved residues are considered (S segment 10-13, M segment 10-12, L segment 10-13) even greater diversity is seen (Table 5-3). In addition to diversity in terminal sequences, there was a large range in the length of UTRs amongst the viruses sequenced in this study. MPOV S segment cRNA 5'UTR was 117 nt, in comparison to 35 nt for ANBV and 85 nt for BUNV, the type species of the genus (Table 5-2). Moreover, the cRNA 3'UTRs of JDV S, M segments were 323 and 478 nt long and incredibly A rich, UTRs of a similar length were also present in both MORV and CAPV (Table 5-2).

Despite the variation in UTR length and conserved termini sequences some conservation remains. The conserved motif UGGGUGGGUGGU, present in the S segment antigenomic 3'UTR of California, Bunyamwera and Wyeomyia group viruses, was detected in Anopheles A (excluding TCMV), Anopheles B, Gamboa and Nyando serogroups (Dunn *et al.,* 1994; Bowen *et al.*, 1995; Campbell and Huang, 1996; Chowdhary *et al.*, 2012). Slightly modified versions were also present in the Tete serogroup ([GW]GGUGGGUGGU), Capim and Guama serogroups ([W]GGGU[U]GGGUGGU) and TCMV (UGGG[A]GGGUGGU). The only virus found to lack even a truncated version of this motif was MPOV, a member of the Turlock serogroup.

Table 5-3: Variation in Conserved UTR Terminal Sequences

	S Segment cRNA		M Segment cRNA		L Segment cRNA	
	5'UTR	3'UTR	5'UTR	3'UTR	5'UTR	3'UTR
Virus	AGU AGU GUA C-UC C	G GAG CAC ACU ACU	AGU AGU GUA CUA C	G UAG CAC ACU ACU	AGU AGU GUA C-UC C	G GAG CAC ACU ACU
57389	... ...-.. .	. ...	... ... .A.	. ...	... ...	... ...
LMV	... ...-.. .	. ...	... ... .A.	. ...	... ...	... ...
TRMV	... ...-.. .	. ...	... ... .A.	. ...	... ...	... ...
CAPV	... ... .A. .-.U .	. A.A G..	... ... .A. .CG .	. CG. A..	... ... .C. U	A .G. ... ...
MORV	... ... .A. .-.U .	. A.A	... ... .A. .CG .	. CG. A..	... ... .C. U	A .G. ... ...
JDV	... ... .A. .-.U .	. A.A	... ... .A. .CG .	. CG. A..	... ... .C. U	A .G. ... ...
GMAV	... ... .A. U-.U .	. A.A	... ... .A. .CG .	. CG. A..	... ... .C. U	A .G. ... ...
PLSV	... ...-.. .	. ...	... ... .C. .	N/A	... ... .C. U	A .G. ... ...
TETE	... ...-.. .	. ... U..	... ...	. ...	... ... .C. C	. .G. ... ...
BMAV	... ...-C. .	. ...	... ... .C. .	. ...	... ... .C. C	. .G. ... ...
MTRV	... ...-.. .	. ...	... ...	. ...	... ... .C. C	. .G. ... ...
TSUV	... ...-C. .	. ...	... ...	. ...	... ... .C. C	. .G. ... ...
Brazoran*	... ... .A. .U.. .	. A.A	... ... .A. ..G .	. C.. A..	... ...	... ...
Nyando*	... ... .A.. .	. ...	... ...	. ...	... ...	... ...
Apeu *	... .G .-.. .	. ...	... ...	. ...	... ...	... ...
La Crosse*	... ...-C. .	. .G.	... ...	. ...	... ...	... ...
Akabane*	... ... .A. .-.. .	. ...	... ... .A.	. ... A..	... ...	... ...
SBV*	... ... .A. .-.. .	. ... A..	... ... .A.	. ... A..	... ...	... ...
Jatobal*	... ...-.. .	. ...	... ...C .	. ...	... ...	... ...
Pongola*	... ...-.. .	. ...	... ...G .	. C..	... ...	... ...
Buttonwillow*	... ...-.. .	. A..	... ...	. ...	... ...	... ...

Sequences with variations in conserved termini are shown. Non-canonical base pairing in red; -, denotes a gap. * Variation in published sequences limited to 1 example per virus species. Virus abbreviations: ANAV (Anopheles A virus), 57389 (ColAn 57389 virus), LMV (Las Maloyas virus), TCMV (Tacaiuma virus), TRMV (Trombetas virus), ANBV (Anopheles B virus), BORV (Boracéia virus), CAPV (Capim virus), JDV (Juan Diaz virus), MORV (Moriche virus), GAMV (Gamboa virus), GMAV (Guama virus), PLSV (Palestina virus), EREV (Eretmapodites virus), NDV (Nyando virus), BMAV (Batama virus), MTRV (Matruh virus), TETEV (Tete virus), TSUV (Tsuruse-like virus), WELV (Weldona virus), MPOV (M'Poko virus).

5.3.5 Sequence Identity and Phylogeny

To investigate evolutionary relationships and virus classification, phylogenetic analysis and aa identity were investigated. Phylogenetic analysis largely supports traditional virus classifications with N, M and L ORFs of all viruses, excluding TCMV, branching within serogroup designations (Figure 5-6, Figure 5-7, Figure 5-8).

Anopheles A and Anopheles B serogroups formed neighbouring clusters on N, M and L ORF phylogenetic trees. However, the placement of TCMV, an Anopheles A serogroup virus, varied, branching with the Anopheles B serogroup on N and M analysis, but branching as ancestral to the Anopheles A and Anopheles B serogroups on L analysis (Figure 5-6, Figure 5-7, Figure 5-8). This also occurred in neighbour-joining phylogenetic analyses (Figure 5-9). Furthermore, TCMV shares very low sequence identity with other Anopheles A serogroup viruses, with aa identities of ≤ 55.1%, ≤ 39.9% and ≤ 60.6% for the N, M and L proteins, respectively (Table 5-4A). Indeed, TCMV shared slightly higher aa identity with ANBV M (41.1%) and L (61.3%) proteins (Table 5-4B). Interestingly, unclassified bunyavirus Tataguine, which causes febrile disease in man, branched ancestrally to the Anopheles A and Anopheles B serogroups, with high bootstrap values on N and L analyses.

Capim, Guama and Minatitlan serogroups branched very closely on phylogenetic analysis and share high aa identity (Figure 5-6, Figure 5-7, Figure 5-8, Table 5-4C). N, M and L phylogeny shows PLSV, a member of the Minatitlan serogroup, as ancestral to both the Guama and Capim serogroups. However, the phylogenetic relationships between GMAV and the Capim serogroup viruses are less clear, and only supported by high bootstrap values in the L ORF analysis (Figure 5-6, Figure 5-7, Figure 5-8).

Tete serogroup viruses formed a single clade, supported by high bootstrap values for each ORF (Figure 5-6, Figure 5-7, Figure 5-8). Moreover, unclassified virus I612045 fell within the Tete serogroup cluster and showed high levels of identity to Tete serogroup N, M and L proteins (Table 5-4D). N ORF phylogeny also reveals that Oyo may fall within the Tete serogroup cluster, with WELV, a member of the Tete virus sp. and Tete serogroup, branching basally to the group (Figure 5-6).

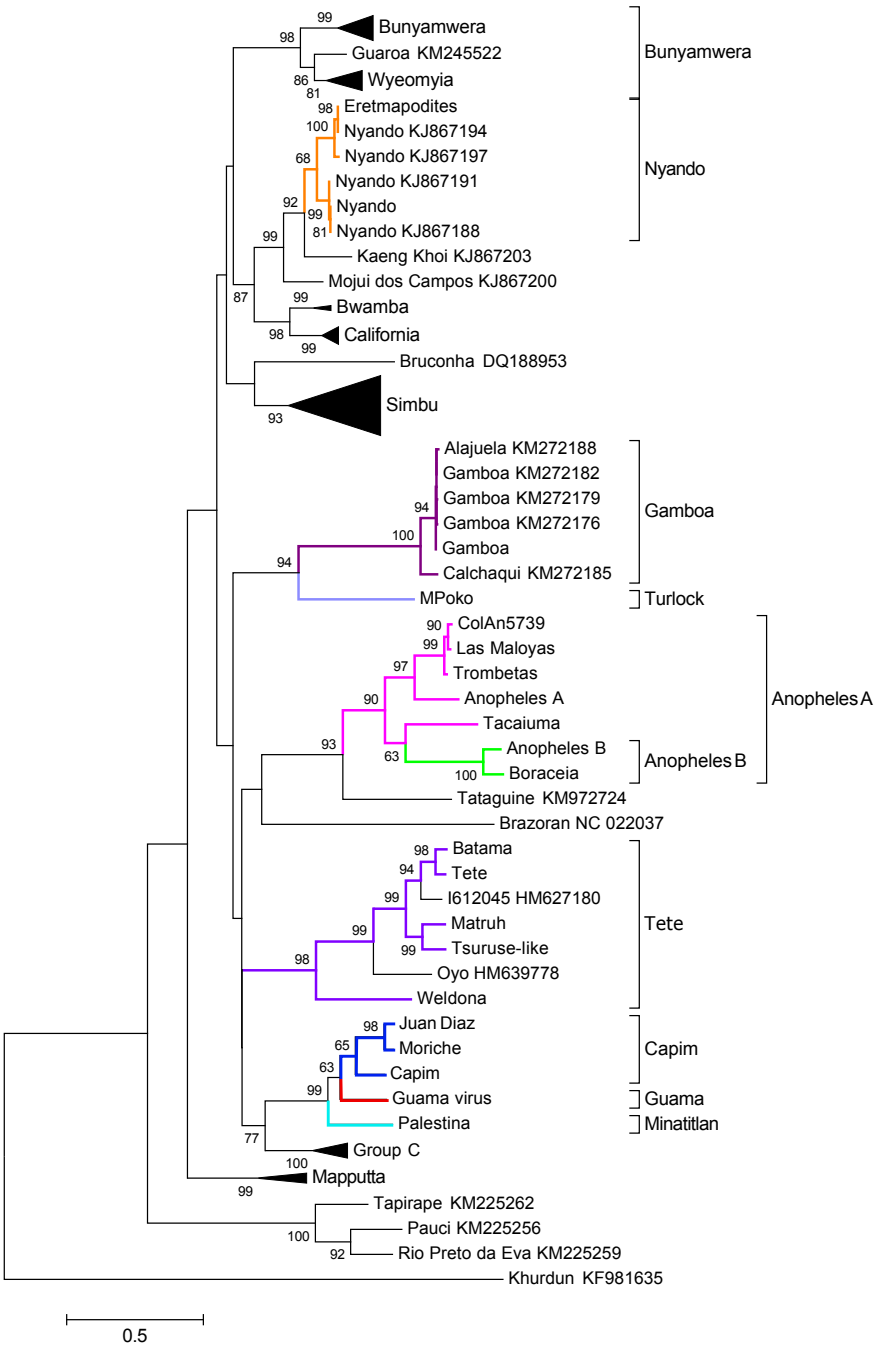


Figure 5-6: N ORF Phylogeny

Nucleotide sequences for complete N ORFs were aligned with previously published* sequences using the MUSCLE algorithm in MEGA 6.06 (Mac). Maximum likelihood phylogenies were then implemented in MEGA 6.06 (Mac) with 1000 pseudoreplicates, bootstrap values > 60% are shown. Clades containing isolates sequenced in this study are highlighted whilst large clades lacking novel sequences have been compressed. When > 1 published sequenced for a named virus isolate was available 1 was selected. A complete list of virus names and accession numbers are shown in non-compressed trees (Appendix 4).

*Published sequences available 3rd December 2015.

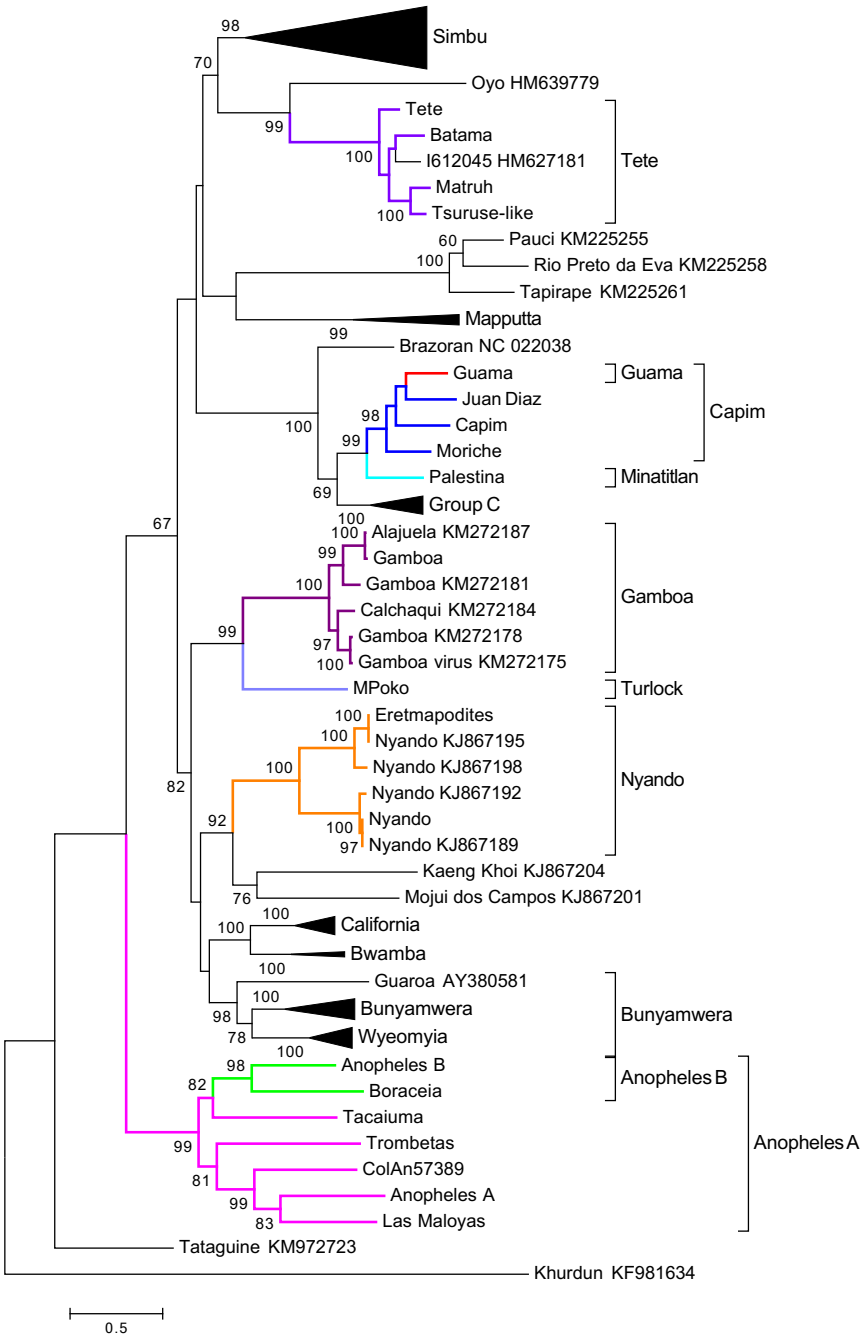


Figure 5-7: M ORF Phylogeny

Nucleotide sequences for complete M ORFs were aligned with previously published* sequences using the MUSCLE algorithm in MEGA 6.06 (Mac). Maximum likelihood phylogenies were then implemented in MEGA 6.06 (Mac) with 1000 pseudoreplicates, bootstrap values > 60% are shown. Clades containing isolates sequenced in this study are highlighted whilst large clades lacking novel sequences have been compressed. When > 1 published sequenced for a named virus isolate was available 1 was selected. A complete list of virus names and accession numbers are shown in non-compressed trees (Appendix 4).

*Published sequences available 3rd December 2015.

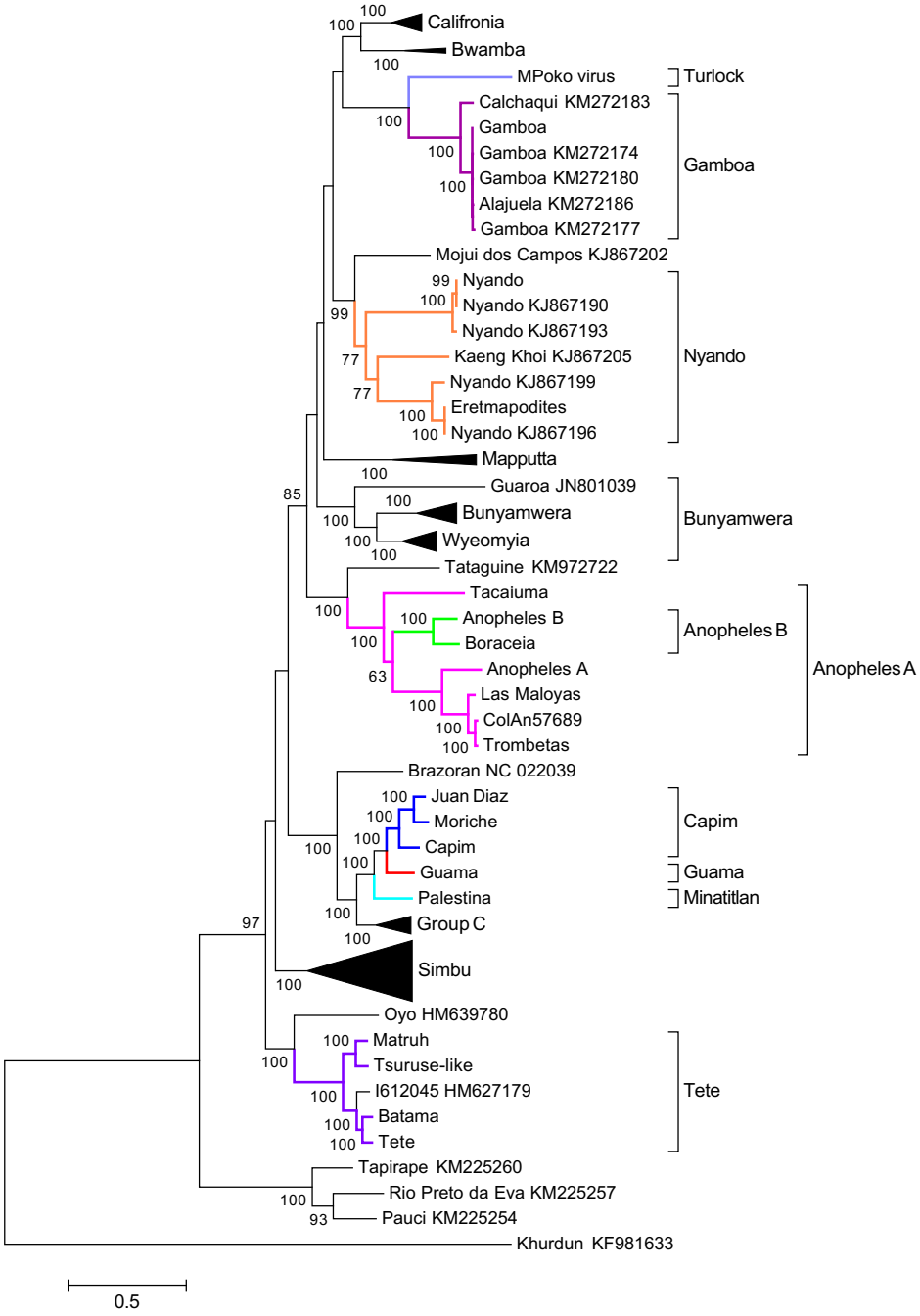


Figure 5-8: L ORF Phylogeny

Nucleotide sequences for complete L ORFs were aligned with previously published* sequences using the MUSCLE algorithm in MEGA 6.06 (Mac). Maximum likelihood phylogenies were then implemented in MEGA 6.06 (Mac) with 1000 pseudoreplicates, bootstrap values > 60% are shown. Clades containing isolates sequenced in this study are highlighted whilst large clades lacking novel sequences have been compressed. When > 1 published sequenced for a named virus isolate was available 1 was selected. A complete list of virus names and accession numbers are shown in non-compressed trees (Appendix 4).

*Published sequences available 3rd December 2015.

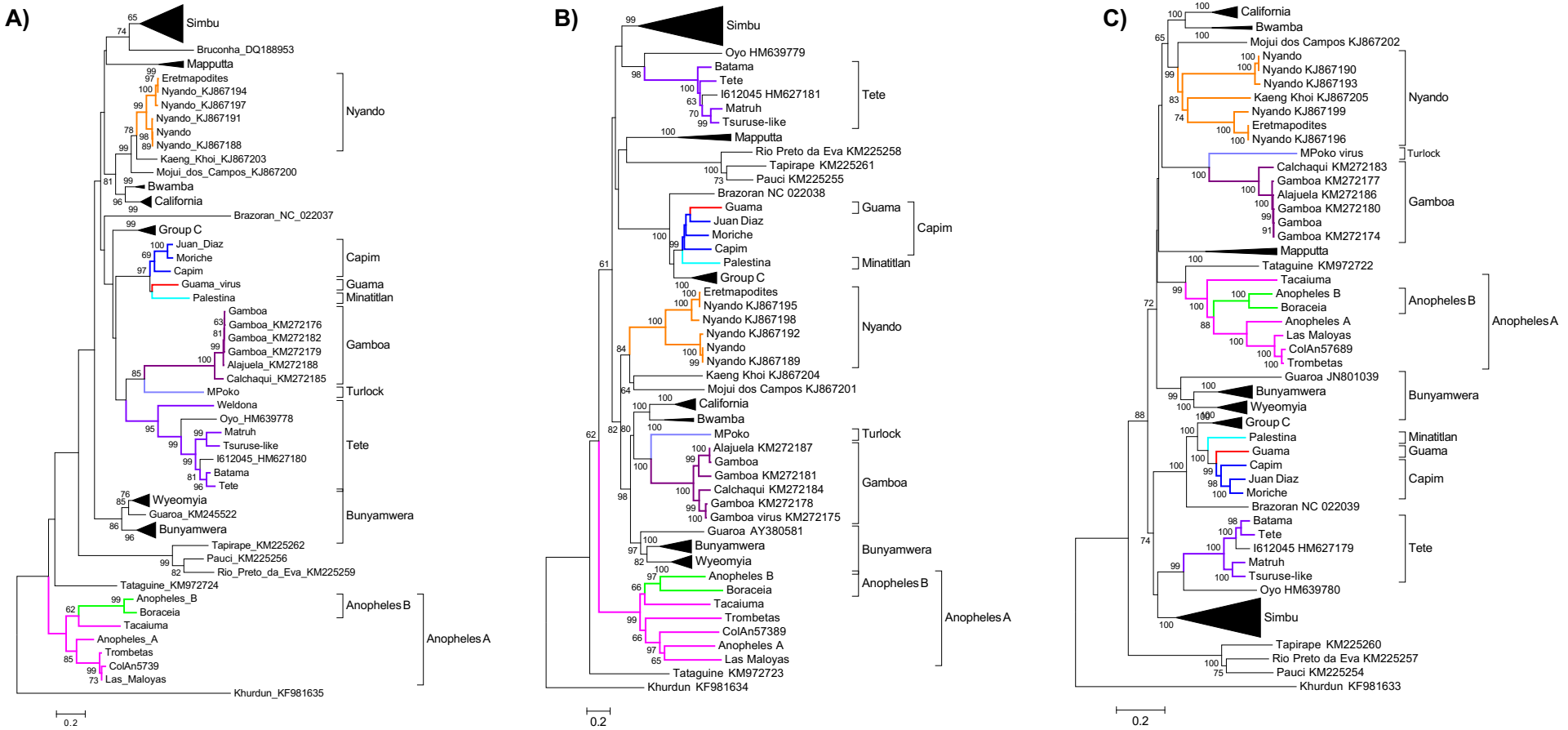


Figure 5-9: N, M and L ORF Neighbour-Joining Phylogeny

Nucleotide sequences for complete N (A), M (B) and L (C) ORFs were aligned with previously published sequences using the MUSCLE algorithm in MEGA 6.06 (Mac). Neighbour-Joining phylogenies were then implemented in MEGA 6.06 (Mac) with 1000 pseudoreplicates, bootstrap values > 60% are shown. Clades containing isolates sequenced in this study are highlighted whilst large clades lacking novel sequences have been compressed. When > 1 published sequenced for a named virus isolate was available 1 was selected.

Table 5-4: Amino Acid Identity within Serogroups

A) ANOPHELES A SEROGROUP AMINO ACID PERCENTAGE IDENTITY

	N				M				L			
	57389	LMV	TRMV	TCMV	57389	LMV	TRMV	TCMV	57389	LMV	TRMV	TCMV
ANAV	72.3	75.6	73.6	53.0	45.3	47.0	37.8	37.8	76.9	77.3	76.8	59.6
57389		98.4	97.2	54.3		46.7	41.1	35.6		94.3	98.4	60.6
LMV			97.2	55.1			39.9	38.7			94.3	60.6
TRMV				54.3				39.9				60.3

B) ANOPHELES B SEROGROUP (+ TCMV)

	N		M		L	
	BORV	TCMV	BORV	TCMV	BORV	TCMV
ANBV	82.6	51.0	48.4	41.1	83.3	61.3
BORV		48.6		39.2		62.0

C) CAPIM, GUAMA, MINATITLAN SEROGROUP AMINO ACID PERCENTAGE IDENTITY

	N				M				L			
	JDV	MORV	GMAV	PLSV	JDV	MORV	GMAV	PLSV	JDV	MORV	GMAV	PLSV
CAPV	74.9	76.2	67.5	56.8	65.0	64.3	65.1	59.0	84.0	83.7	79.1	72.4
JDV		93.2	67.5	58.5		65.4	63.6	56.5		92.1	78.1	71.8
MORV			65.4	59.3			64.0	59.9			77.5	71.8
GMAV				59.5				62.4				74.8

D) TETE SEROGROUP AMINO ACID PERCENTAGE IDENTITY

	N					M				L			
	BMAV	MTRV	TSUV	WEL	I612045	BMAV	MTRV	TSUV	I612045	BMAV	MTRV	TSUV	I612045
TETEV	88.8	68.3	68.7	45.2	83.0	75.8	74.5	75.0	76.8	94.1	83.3	82.4	91.6
BMAV		67.2	67.6	44.8	83.7		75.2	76.3	80.5		83.0	83.0	91.1
MTRV			76.4	43.6	67.9			85.6	76.8			92.1	82.4
TSUV				41.7	69.9				76.9				81.6
WEL					45.6				-				-

E) NYANDO SEROGROUP AMINO ACID PERCENTAGE IDENTITY

	N			M			L		
	EREV	ERET147	MP401	EREV	ERET147	MP401	EREV	ERET147	MP401
NDV	85.4	85.4	100.0	56.6	56.7	100.0	61.8	61.8	99.9
EREV		100.0	85.4		100.0	56.7		100.0	61.8
ERET147			85.4			56.7			61.8

F) GAMBOA SEROGROUP AMINO ACID PERCENTAGE IDENTITY

	N			M			L		
	GML	MPOV	MARU	GML	MPOV	MARU	GML	MPOV	MARU
GAMV	99.6	42.9	99.6	76.6	33.0	99.6	99.9	58.4	99.7
GML		42.9	100		40.4	76.8		58.4	99.8

Amino acid sequences were aligned using the MUSCLE algorithm in in CLC Genomics Workbench v7.5.1 (Qiagen) using MUSCLE algorithm and pairwise identity calculated. Accession numbers for previously published sequences are listed. **A)** Anopheles A serogroup. **B)** Anopheles B serogroup and TCMV. **C)** Capim, Guama and Minatitlan serogroups. **D)** Tete serogroup, I612045 virus (HM627180, HM627181, HM627179). **E)** Nyando serogroup, ERET147 (KJ867194, KJ867195, KJ867196), NDV strain MP401 (KJ867188, KJ867189, KJ867190). **F)** Turlock and Gamboa serogroup, GML; Gamboa virus (KM272176, KM272175, KM272174), MARU; Alajuela virus (KM272188, KM272187, KM272186).

Phylogenetic analysis also suggests that BMAV, I612045 and TETEV belong to 1 replicating lineage whilst MTRV and TSUV form another (Figure 5-6, Figure 5-7, Figure 5-8).

Nyando serogroup viruses branched with sequences published by Groseth *et al.,* (2014) forming 2 replicating lineages which correspond to the classical NDV and EREV classifications (Figure 5-6, Figure 5-7, Figure 5-8).

Turlock serogroup virus MPOV shares only 29.3% aa identity with the published N protein sequence of MPOV strain ArB365, accession number AM711133, and shares higher aa identity with Gamboa serogroup viruses (Table 5-4F). MPOV also branches in a distant clade from MPOV ArB365, accession AM711133, branching with the Gamboa serogroup on N, M and L ORF phylogenetic analysis (Figure 5-6, Figure 5-7, Figure 5-8, Appendix 4).

It should also be noted that there was evidence of reassortants among all serogroups with ≥ 3 sequenced isolates, namely the Anopheles A, Capim, and Tete serogroups. For example, within the Capim serogroup CAPV N and L proteins are ancestral to those of MORV and JDV, whereas M protein phylogeny shows JDV as ancestral to CAPV and MORV.

5.4 Discussion

5.4.1 Protein Conservation

In this study complete genome sequences of 20 orthobunyaviruses, representing the first complete genomes for 8 orthobunyavirus serogroups, were determined, unearthing greater genetic diversity in viral UTRs and coding regions, thus calling current classifications into question.

In 2009 Eifan *et al.*, detected 46 conserved aa in orthobunyavirus N proteins; today only 3 of these residues, alignment positions K77, T119 and G180, remain fully conserved (Figure 5-1) (Eifan and Elliott, 2009). K77 is flanked by RNA and N-N

interaction domains and T119 has been shown to directly interact with RNA in LACV crystallography studies, whilst G180 falls within a region of high conservation that currently has no assigned function (Figure 5-1) (Reguera *et al.,* 2010). Mutagenic analysis of BUNV N by Eifan and Elliott (2009) identified BUNV N protein residues 141, 144 and 158 (alignment positions 174, 177 and 191) as having potentially important roles in the virus life cycle. Mutants Y141C, F144A and Y158N displayed only 2%, 29% and 30% activity in a minigenome assay with respect to wt BUNV, and N-L co-precipitation of Y141C was only 36% with respect to wt BUNV (Eifan and Elliott, 2009). Further investigation of this region with regards to mapping N-L interactions would therefore be warranted. Additionally, L protein alignment residues 2241-2253 which contain a 23 aa insertion by Tete serogroup viruses, may also be of interest. Tete serogroup viruses encode larger N proteins and the L protein alignment 2241-2253 is flanked by fully conserved residues (Figure 5-3A). High levels of conservation were also present in L protein alignment residues 1441-1463 (88.8%) and 1493-1546 (83.2%), compared with 60.6% conservation across the complete L protein. This region is not yet associated with any known motif or function.

In this study only 5 viruses, GAMV, WELV, MPOV, NDV and EREV were found to encode ORFs containing NSs pfam domain 01104. Excluding Brazoran virus, which encodes an NSs protein of 173 aa in an overlapping reading frame that precedes that of the N protein, 'traditional' orthobunyavirus NSs proteins previously ranged from 83 – 109 aa (Mohamed *et al.,* 2009; Lanciotti *et al.*, 2013). Sequencing of the 'neglected' serogroups reveals greater diversity in NSs protein size as Gamboa serogroup viruses are predicted to encode NSs proteins at 130 - 137 aa, whilst the shortest at only 79 aa is encoded by MPOV a member of the Turlock serogroup (Nunes *et al.*, 2014). Despite this large variation in size small conserved sequence motifs are still present, with additional conserved aa within serogroups. The conserved motifs map to NSs aa alignment positions 95-114, a region with 57.3% conservation, in comparison to only 28.9% when the entire NSs protein is considered. Conservation within NSs proteins may arise as a consequence of conservation within the overlapping N protein ORF. However, the conserved NSs sequence motifs map to regions of the genome not associated with high N protein conservation. For example, NSs alignment residues 95-114 share coding sequence with aa 75-93 in BUNV N protein, a region with only

57.7% conservation compared to the 81.0% conservation for BUNV N residues 68 - 87, which are associated with RNA binding.

Studying the conserved motifs within NSs proteins may help elucidate the mechanisms by which NSs proteins act. The interaction of BUNV NSs with Med8 has been mapped to alignment residues 110-118, which contains the Bunyamwera serogroup conserved sequence VLPSTxxVD. A truncated version of this motif (xLPSTxxxD) is also present in Bwamba and California serogroups, whilst MPOV NSs truncates prior to this region and Gamboa serogroup NSs proteins contain a divergent sequence (xxPxTxxxD). Moreover, BUNV NSs is subject to proteosomal degradation yet Gamboa viruses and some Simbu serogroup NSs proteins lack a single lysine residue.

Truncated NSs ORFs were first identified in Bunyamwera (Wyeomyia) group viruses in 2009 and have subsequently been detected in the Anopheles A, Anopheles B, Mapputta and Tete serogroups (Mohamed *et al.,* 2009; Mores *et al.*, 2009). Mohamed *et al*. (2009) also demonstrated that the severely truncated NSs ORFs of Anopheles A, Anopheles B and Tete serogroup viruses were non-functional when S segment clones were expressed in cell free expression systems. This study now reports that viruses belonging to the Capim, Guama and Minatitlan serogroups also contain severely truncated and presumably non-functional NSs ORFs (Table 5-2). We also report that the presence/absence of the NSs ORF is not conserved within current serogroups, as a full length NSs ORF (114 aa) was detected in WELV, a member of the Tete serogroup that was previously thought to contain only non-NSs viruses. It has been hypothesised that non-NSs viruses may exhibit greater diversity in N protein sequences as the S segment ORF is no longer constrained by the need to encode both N and NSs proteins (Mohamed *et al.,* 2009). There is, as yet, no evidence of this as there is greater sequence diversity amongst the N proteins of Simbu serogroup viruses (e.g. 49.8% aa identity between Leanyer virus and Facey's Paddock virus) than non-NSs viruses of the same serogroup. However, it should be noted that the largest non-NSs serogroup, Wyeomyia, contains only 7 named viruses with sequence data and low levels of sequencing may mask true levels of diversity (Chowdhary *et al.*, 2012).

Prior to the work of Mohamed *et al,* (2009), NSs, the primary virulence factor of orthobunyaviruses, was thought to be essential in overcoming vertebrate innate

immune defence (Hart *et al.,* 2009). However, 6 non-NSs viruses (TCMV, BORV, GMAV, Tataguine virus, BUCV and Tucunduba virus) belonging to the Anopheles A, Anopheles B, Guama, Mapputta, and Wyeomyia serogroups have been linked with human infection (de Souza Lopes and de Abreu Sacchetta, 1974; Moore *et al.*, 1975; Calisher *et al.*, 1980; Chowdhary *et al.*, 2012; Gauci *et al.*, 2015). The mechanism by which these viruses are able to overcome the human innate immune response is yet to be identified. It was initially postulated that the N or M protein of non-NSs orthobunyaviruses may play a role in IFN antagonism as the N proteins of hantaviruses have been shown to antagonise the type I IFN response through the inhibition of PKR dimerisation (Wang and Mir, 2015). Additionally, the cytoplasmic tails of Gn proteins in virulent hantaviruses suppress ISRE and IFN-β promoter activation in a mechanism thought to involve regulation of the TBK-1 complex (Alff *et al.*, 2008). Viruses in the Anopheles A, Anopheles B and Tete serogroups encode larger N proteins (243-258 aa) than NSs encoding orthobunyaviruses; however, this characteristic is not conserved among the other non-NSs viruses. Interestingly, viruses within the Capim, Guama, Minatitlan and Wyeomyia viruses have incredibly long A-rich antigenomic 3'UTRs and it is unclear what role these play in the virus replication (Table 5-2). Although more prevalent in non-NSs viruses, they are not exclusive to this subset of orthobunyaviruses with GAMV, a member of the Gamboa serogroup, encoding an S segment UTR of 438 nt (Table 5-2). Therefore, there is no clear conserved genome trait in the newly sequenced non-NSs viruses that may account for pathogenicity in man.

The M polyprotein of orthobunyaviruses is post-translationally cleaved to form the 2 virus glycoproteins, Gn and Gc, and the non-structural protein NSm. There is striking conservation of cysteine residues throughout the polyprotein and Gn zinc finger motifs and the Gc fusion peptide remain fully conserved (Figure 5-3). It is also of note that of the 5 conserved histidine residues present in the ectodomain of orthobunyavirus Gc proteins studied by Shi *et al.* (2005) only alignment position 1267 (BUNV 1029), the histidine residue closest by sequencing to the Gc fusion peptide, remains conserved in all orthobunyaviruses with the exception of Khurdun virus.

Bunyavirus glycoproteins form Gn Gc heterodimers and both Golgi-targeting and Golgi-retention signals map to the Gn protein. Further to this, N-glycosylation of Gn has been demonstrated to have an essential role in virus replication with BUNV N60Q

mutants misfolding and being retained in the ER (Shi *et al.,* 2005). However, this study has found that viruses in the Anopheles A serogroup are predicted to lack a single glycosylation site in the Gn protein, a feature that has only previously been reported for Maprik virus (MPKV), a member of the Mapputta serogroup. Anopheles A serogroup M proteins, excluding TCMV, are also smaller than those of other orthobunyaviruses at 1405 - 1415 aa, a feature they also share with Mapputta serogroup viruses (1370-1379 aa). However, unlike the severely truncated M proteins of Khurdun virus and the insect only bunyaviruses Herbert, Tai and Kibale, Anopheles A and Mapputta serogroup viruses are still predicted to encode full length NSm proteins (Figure 5-4) (Marklewitz *et al.*, 2013).

The role of NSm proteins in virus replication has yet to be fully elucidated. NSm localises to the Golgi complex and is thought to act as a scaffold protein as the N terminal region of BUNV NSm has been shown to be important in glycoprotein folding and virus assembly (Shi *et al.*, 2006). Yet recombinant viruses with large NSm deletions are able to replicate in cell culture and remain virulent in animals, with rSBVΔNSm causing viraemia and seroconversion comparable to wt SBV in cattle (Kraatz *et al.*, 2015). In this study all viruses were found to encode NSm proteins but of note is GAMV, which encodes an M polyprotein of 1592 aa. This finding is in agreement with Nunes *et al.* (2014) who published complete coding sequences for 5 members of the Gamboa serogroup. Analysis now shows the additional aa map to the N terminal region of NSm with a 149 aa insertion at alignment positions 350 - 498 (Figure 5-3A). Moreover, Gamboa serogroup viruses are predicted to have altered transmembrane topology, with 2 additional transmembrane domains located in the predicted NSm protein (Appendix 3). Gamboa serogroup viruses have been isolated from Aedeomyia sp. of mosquitoes in South America, although little is known about their host range and it remains unclear what role the additional aa in NSm may play. Further studies of the non-NSm viruses, such as Khurdun virus and Gamboa serogroup viruses, would therefore be of interest, especially in studying mechanisms of glycoprotein folding and trafficking.

5.4.2 Impact on Orthobunyavirus Classification

Orthobunyavirus species are primarily defined by cross neutralisation and complement fixation relationships, with viruses considered distinct when Ht/Ho titres differ by ≥ 4-fold. Further to this N protein aa sequences, if available, should differ by > 10% and current data implies that 1 species should not be able to reassort with any other (Elliott and Blakqori, 2011; Plyusnin *et al.*, 2012). Applying this to viruses within the Capim serogroup highlights problems with current classification methods. JDV, CAPV and MORV are currently classified as members of distinct Capim serogroup species: JDV (Bushbush sp.), CAPV (Capim sp.), MORV (Acara sp.). There is no cross-reaction between the viruses in neutralisation (NT) assays, supporting their distinct classification. However, JDV and MORV are indistinguishable in CFTs and their N proteins show only 6.8% aa difference (Table 5-4C). Phylogeny supports the serological data, with JDV and MORV N and L proteins branching together, whilst their M proteins are separated by CAPV (Figure 5-6, Figure 5-7 and Figure 5-8). Moreover, although CAPV does not cross-react in either NT or CFT assays with JDV and MORV, it does cross-react with GMAV (CFT Ht/Ho 1/64) and PLSV (CFT Ht/Ho 1/16), members of the Guama and Minatitlan serogroups (Berge, 1975; Calisher *et al.*, 1983). Cross-reaction between serogroups has long been known, complicating classification by serological methods and highlighting the complicated interrelatedness of orthobunyaviruses (Srihongse *et al.,* 1966; Zarate *et al.*, 1968; Zeller *et al.*, 1989). As more sequencing data for closely related and serologically characterised viruses become available it may be possible to map antigenic sites, shedding light on antigenic cross-reactions and crucially facilitating the development of a robust method of orthobunyavirus classification.

As the classification of orthobunyaviruses is reviewed phylogenetics will play a key role in unravelling the genetic relationships within the orthobunyavirus genus. Orthobunyaviruses have long been known to reassort, with classical reassortment studies utilising viruses in the Bunyamwera and California serogroups yeilding novel viruses from mixed infections (Gentsch *et al.,* 1979). More recently, sequencing and phylogenetic analysis has provided evidence of reassortants in the Bunyamwera, Wyeomyia, Simbu, Group C and California groups (Yanase *et al.*, 2006; Yanase *et al.,* 2010; Aguilar *et al.*, 2011; Blitvich *et al.*, 2012; Chowdhary *et al.*, 2012; Yanase *et al.*,

2012; Briese *et al.,* 2013; Hontz *et al.*, 2015; Tilston-Lunel *et al.*, 2015). This study continues to detect reassortant viruses, with evidence of reassortment in the Anopheles A, Tete and, as described above, Capim serogroups. In agreement with previous studies S and L segments share a common ancestor with divergence occurring at the M segment. However, classification by phylogenetics alone is not without its constraints as due to the prevalence of reassortant viruses, the provision of complete sequences for all 3 virus segments is essential in elucidating true genetic relatedness.

There have been well documented but conflicting findings regarding the phylogenetic placement of Group C viruses. The resolution of these discrepancies is hindered by the viruses innate capability to reassort, the lack of complete genome sequences for all isolates concerned, different sources of virus isolates, and the ever changing name/classification of these viruses (Nunes *et al.*, 2005; Forshey *et al.,* 2014; Hang *et al.*, 2014; Hontz *et al.*, 2015). This study also reports divergent findings with previously published sequences. MPOV strain ArB365 and NDV strain ArB16055 N protein sequences, accession numbers AM711133 and AM70978, fell within the Bunyamwera serogroup on phylogenetic analysis, sharing 81.9% and 86.4% identity with BUNV N protein (NC001927), respectively (Yandoko *et al.*, 2007) (Appendix 4). However, in this study MPOV branched separately, sharing a common ancestor with Gamboa serogroup viruses, and Nyando serogroup viruses NDV and EREV branched with the NDV sequences published by Groseth *et al*., (2014). MPOV strain ArB365 (AM711133) and NDV strain ArB16055 (AM70978) were sourced from the Institut Pasteur de Bangui, Central African Republic, whereas all viruses sequenced in this study were sourced from the Emerging Viruses and Arboviruses Reference Collection, University of Texas Medical Branch (Yandoko *et al.*, 2007).

Furthermore, it has been proposed that I612045, Oyo and Sedlec viruses form a new serogroup termed the Sedlec serogroup (Bakonyi *et al.*, 2013). However, at the time of the proposed classification only S segment sequences were available for Tete serogroup viruses and, although I612045 virus grouped with these on phylogenetic analysis, the classification was taken forward on the phylogenetic relationship of partial L segment sequences for I612045, Sedlec and Oyo only. It is now clear that I6120245 not only shares genetic and biological features comparable to viruses within the Tete serogroup (N protein of 258 aa, truncated NSs ORF, isolated from birds), it also has high

sequence identity across all 3 genome segments and clusters on phylogenetic analysis within the Tete serogroup (Shchetinin *et al.*, 2015). Unfortunately, there is no serological data relating to I612045 but genomic data strongly support the classification of I612045 as a member of the Tete serogroup. Complete sequencing of WELV M and L segments, along with complete sequencing of Sedlec virus, will further clarify the relationships within this group of viruses and emphasises the need to determine genome sequences for recognised orthobunyavirus species across all serogroups prior to the creation of new groups.

In addition to phylogenetic analysis, the presence of conserved invertedly complimentary terminal sequences has provided a quick and simple method of identifying bunyavirus genera. However, as predicted by Elliott and Blakqori (2011), as more viruses are sequenced diversity in previously conserved sites is uncovered with changes at the 8th nt position in S and M segment reported for AKAV, SBV, Brazoran and MPKV (Table 5-3). The viruses sequenced in this study further contribute to UTR sequence divergence and support the reduction of conserved terminal sequences from 9 to 7 nucleotides in the S and M segments of orthobunyaviruses, and the removal of segment specific sequences (Table 5-3). Crucially, the presence of terminal sequences can no longer be relied upon as a quick method of classification. This can be shown by the out grouping of Khurdun virus on phylogenetic analysis. Khurdun virus isolated from a Eurasian coot, *Fulica atra*, in Russia in 2001 contains complete orthobunyavirus terminal sequences in the S, M and L segments, with only a single change at the 8th nt position in the S segment (Galkina *et al.*, 2005; Al'kovskhovskiĭ *et al.*, 2013). Khurdun is phylogenetically distant from other orthobunyaviruses, branching as an outlier in N, M and L phylogenetic trees. Moreover, it encodes a severely truncated M polyprotein that lacks the NSm domain, a feature shared with the, as yet, unclassified insect only bunyaviruses Kibale, Tai and Herbert. Kibale, Tai and Herbert viruses also contain terminal sequences comparable to those of orthobunyaviruses yet branch equidistant between tospoviruses and orthobunyaviruses on phylogenetic analysis (Marklewitz *et al.*, 2013).

5.4.3 Concluding Remarks

Complete genome sequences for viruses in the Anopheles A, Anopheles B, Capim, Gamboa, Guama, Gamboa, Minatitlan, Nyando, Tete and Turlock serogroups were determined prior to the publication of sequences by Groseth *et al*., (2014), Nunes *et al.,* (2014) or Al'kovskohovskii (2015). Sequencing viruses from the lesser studied serogroups challenges current classification methods and perceptions regarding NSs, as increasing numbers of orthobunyaviruses associated with human disease are found to lack a full length NSs ORF.

Chapter 6: Orthobunyaviruses and the Type I Interferon Response

6.1 Introduction

In this study, sequencing confirmed the presence of an NSs ORF in 5 of 20 sequenced orthobunyaviruses. The NSs positive viruses were Nyando serogroup viruses NDV and EREV, Gamboa serogroup virus GAMV, Turlock serogroup virus MPOV, and WELV, a Tete serogroup virus. In addition to these viruses, BWAV and SJV, which both belong to virus species with published sequences that contain full length NSs ORFs, were successfully propagated (Groseth *et al.*, 2014; Nunes *et al.*, 2014). The NSs protein is the primary virulence factor of orthobunyaviruses, antagonising the type I IFN system at the transcriptional level (Bridgen *et al.*, 2001; Weber *et al.,* 2002). The transcriptional blockade exerted by BUNV and LACV NSs proteins occurs, at least partly, at the mRNA elongation stage with inhibition of C-terminal domain serine 2 phosphorylation, blocking RNAPII activity and protein synthesis in mammalian cells (Thomas, 2004; Verbruggen *et al.*, 2011). The transcriptional blockade of the IFN-β promoter is profound and recombinant viruses lacking an NSs protein show increased sensitivity to type I IFNs (Blakqori *et al.*, 2007; Bridgen *et al*., 2001). Indeed it was thought that the NSs protein was a key factor in determining the zoonotic potential of orthobunyaviruses, enabling the virus to overcome the mammalian innate immune response (Hart *et al.,* 2009).

However, an increasing number of orthobunyaviruses that naturally lack an NSs ORF have been identified in sequencing studies. Through published reports and the work presented in this thesis non-NSs viruses have now been detected in the Anopheles A, Anopheles B, Bunyamwera (Wyeomyia), Capim, Guama, Minatitlan, Mapputta, Simbu and Tete serogroups (Chowdhary *et al.,* 2012; Gauci *et al.*, 2015; Ladner *et al.*, 2014; Mohamed *et al.,* 2009). Moreover, a small number of these non-NSs viruses have been linked with human infection, either through the presence of neutralising antibodies or, in the case of TCMV, GMAV and BUCV, virus isolation from febrile patients (Berge, 1975; Calisher *et al*., 1980; Wier, 2002). The mechanism by which these non-NSs viruses can overcome the host innate immune response has yet to be identified. It has been reported that TCMV antagonises the type I IFN system, whilst ANAV, another non-NSs orthobunyavirus in the Anopheles A serogroup that is not linked with human infection, induces high levels of IFN *in vitro* (Mohamed *et al.,* 2009). To date this

remains the sole publication investigating non-NSs orthobunyaviruses and the type I IFN system.

6.2 Aims

The aims of this part of my studies were to investigate the activation and antagonism of the type I interferon response by NSs encoding and naturally occurring non-NSs orthobunyaviruses. This would enable the correlation of the presence of an NSs ORF, the ability to antagonise the type I IFN system, and reported pathogenicity in man to be studied.

6.3 Results

To test for statistical significance, the results presented in this chapter were analysed by t-test or one-way ANOVA, followed by the Dunnett's multiple comparison test, with either BUNV or rBUNdelNSs2 as the control. Unless indicated, all p values in this chapter refer to these analyses and not any other form of statistical analysis.

6.3.1 Activation and Antagonism of the Type I IFN System by Viruses with an NSs ORF

Induction of biological IFN by viruses predicted to encode an NSs protein was tested *in vitro*. Briefly, serial dilutions of UV-inactivated supernatant from infected A549 cells were assayed for the presence of IFN by their ability to induce an anti-viral state in recipient cells, protecting them from subsequent infection by EMCV. The first dilution to show CPE, and therefore susceptibility to infection, was marked as the endpoint for each sample. BUNV, BWAV, NDV and EREV induced very low levels of biological IFN, ≤ 16 relative IFN U ml^{-1} (rIFN U ml^{-1}), during virus infection (Figure 6-1). Although > 3-fold higher levels were observed in MPOV infections, the level of IFN remained significantly lower ($p < 0.05$) than for rBUNdelNSs2, which induced approximately 256 rIFN U ml^{-1} (Figure 6-1).

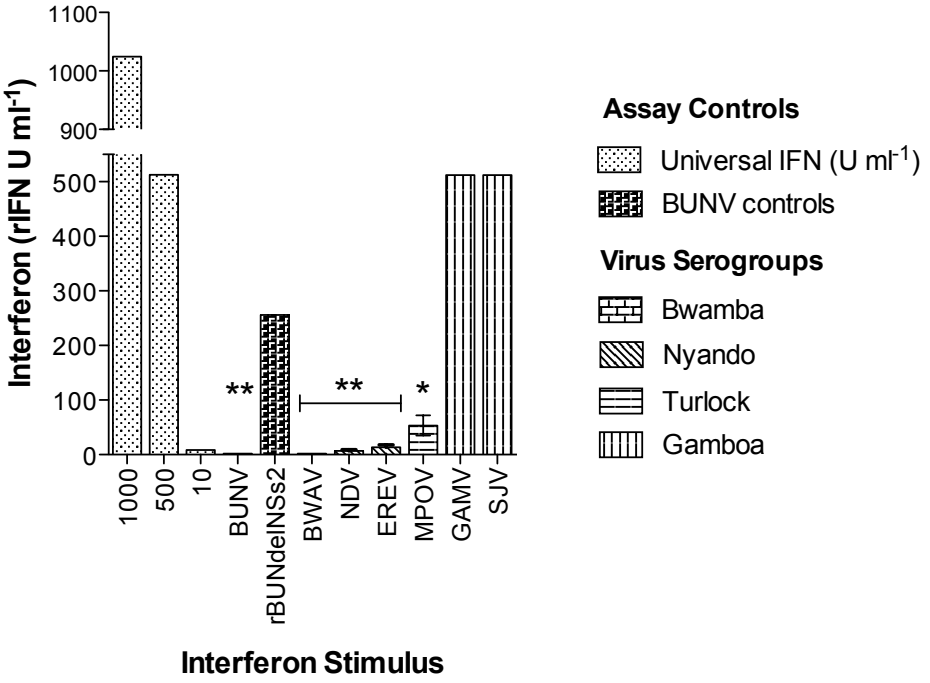


Figure 6-1: Biological Induction of Interferon by NSs Encoding Viruses
Relative levels of biological interferon (rIFN U ml^{-1}) produced by A549 cells 48 hours post induction with universal IFN (U ml^{-1}) or 48 hours post infection at multiplicity of infection 1. rIFN was measured by the level of protection given to A549-NPro cells from EMCV infection by 2-fold dilutions of UV-irradiated supernatant from IFN-induced and virus infected A549 cells. Mean ± standard deviation of triplicate experiments is shown. Samples with significantly lower levels of rIFN than rBUNdelNSs2 are marked with an asterisk, ** $p < 0.001$, * $p < 0.05$.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Bwamba virus (BWAV), Nyando virus (NDV), Eretmapodites virus (EREV), M'Poko virus (MPOV), Gamboa virus (GAMV), San Juan virus (SJV) and encephalomyocarditis virus (EMCV)

In GAMV and SJV infections approximately 512 rIFN U ml^{-1} were produced, significantly higher ($p < 0.0001$) than the other NSs encoding viruses and 2-fold higher than rBUNdelNSs2 (Figure 6-1).

Activation of the IFN-β promoter in a dual luciferase reporter assay mirrored the levels of biological IFN produced during virus infection (Figure 6-2A). Firefly activity, and thereby activation of the IFN-β promoter, was normalised to CMV-driven Renilla expression and relative light units (RLU) were adjusted so that mock equals 1. Using this formula activity of the IFN-β promoter in rBUNdelNSs2 infected cells (14.8 RLU) was significantly higher ($p < 0.01$) than in BUNV infections (0.7 RLU) (Figure 6-2A). This was despite similar levels of viral replication as mean virus titres were within 0.2 logs (Figure 6-2B). Indeed, IFN-β promoter activity in BUNV and BWAV, NDV and EREV infected cells was below background at only 0.45 – 0.85 RLU (Figure 6-2A). Although activity in MPOV infected cells was higher than in BUNV infected cells, values remained significantly lower ($p < 0.002$) than in rBUNdelNSs2 infected cells at only 4.29 ± 0.40 RLU (Figure 6-2A). GAMV and SJV infections induced significantly higher IFN-β promoter activity than rBUNdelNSs2 ($p < 0.002$) (Figure 6-2A).

To confirm that the low levels of IFN induction were due to antagonism of the type I IFN system, the reporter assay was repeated in the presence of exogenous IFN to prime the type I IFN response (Figure 6-3). In the presence of 10 U ml^{-1} IFN activation of the IFN-β promoter was significantly higher ($p < 0.005$) than in untreated cells at approximately 7.5 RLU (Figure 6-3A). BUNV and BWAV significantly antagonised activation of the IFN-β promoter in the presence exogenous IFN with RLU readings of only 2 ± 0.5 ($p < 0.005$). Antagonism by NDV and EREV was less pronounced with IFN-β promoter activity 4.5 ± 0.6 RLU higher than in infected, IFN untreated cells (Figure 6-3B). There was no evidence of antagonism by MPOV, with 10.5 ± 0.8 RLU in the presence of exogenous IFN, > 6.3 RLU higher than in MPOV infected IFN untreated cells (Figure 6-3). In GAMV and SJV infections IFN-β promoter activity was higher than in rBUNdelNSs2 infections, and > 7.3 RLU higher than in IFN untreated infected cells (Figure 6-3).

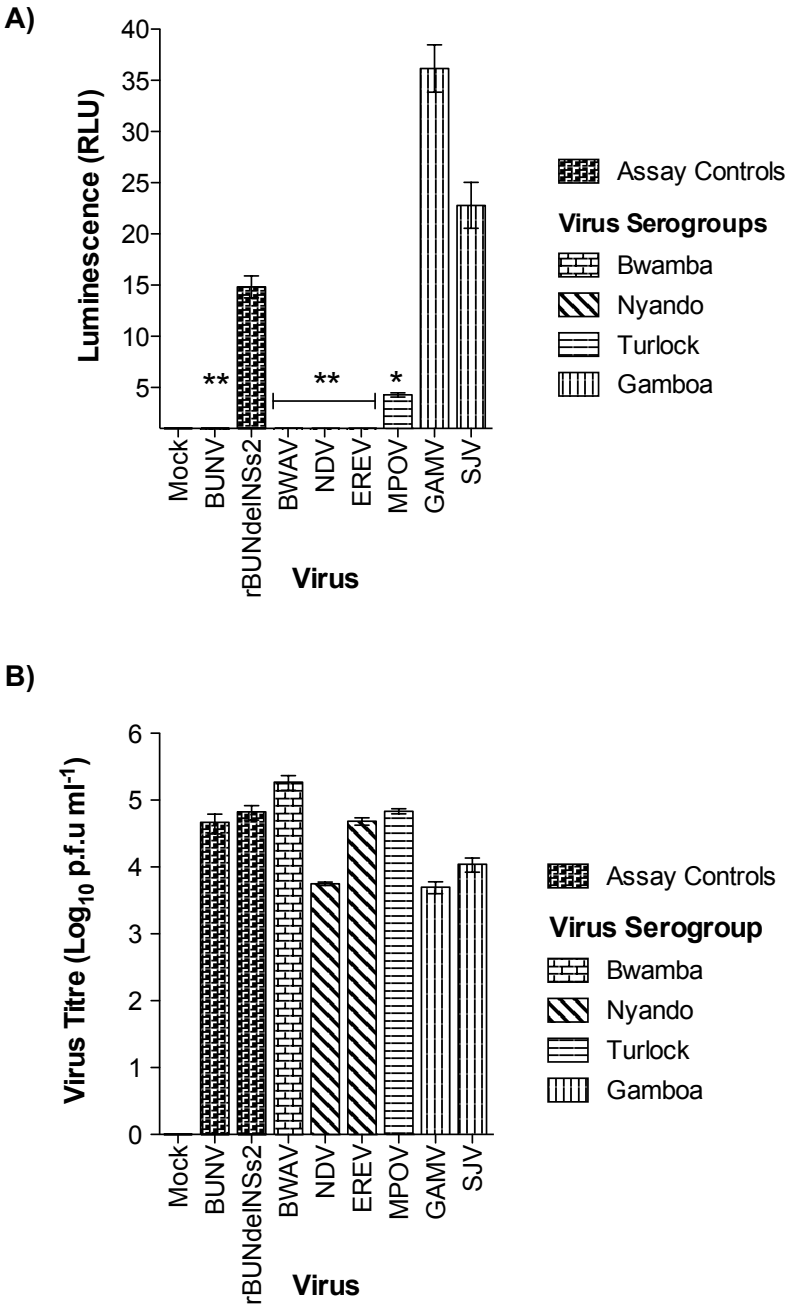


Figure 6-2: Activation of the Interferon-β Promoter in Virus Infection

A549 cells were co-transfected with IFN-β firefly and CMV Renilla reporter plasmids pIFΔ(-125)lucter and phRL-CMV. Cells were then infected at multiplicity of infection 1. **A)** At 18 hours post infection the cells were assayed for luciferase activity (relative light units, RLU). Mean ± standard deviation of triplicate experiments is shown. Samples with significantly lower levels of luminescence than rBUNdelNSs2 are marked with an asterisk, **$p < 0.001$, * $p < 0.002$. **B)** In addition to measuring luciferase activity virus titres of the corresponding cell supernatants were determined by plaque assay on Vero E6 cells. Mean ± standard deviation of triplicate experiments is shown.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Bwamba virus (BWAV), Nyando virus (NDV), Eretmapodites virus (EREV), M'Poko virus (MPOV), Gamboa virus (GAMV), and San Juan virus (SJV).

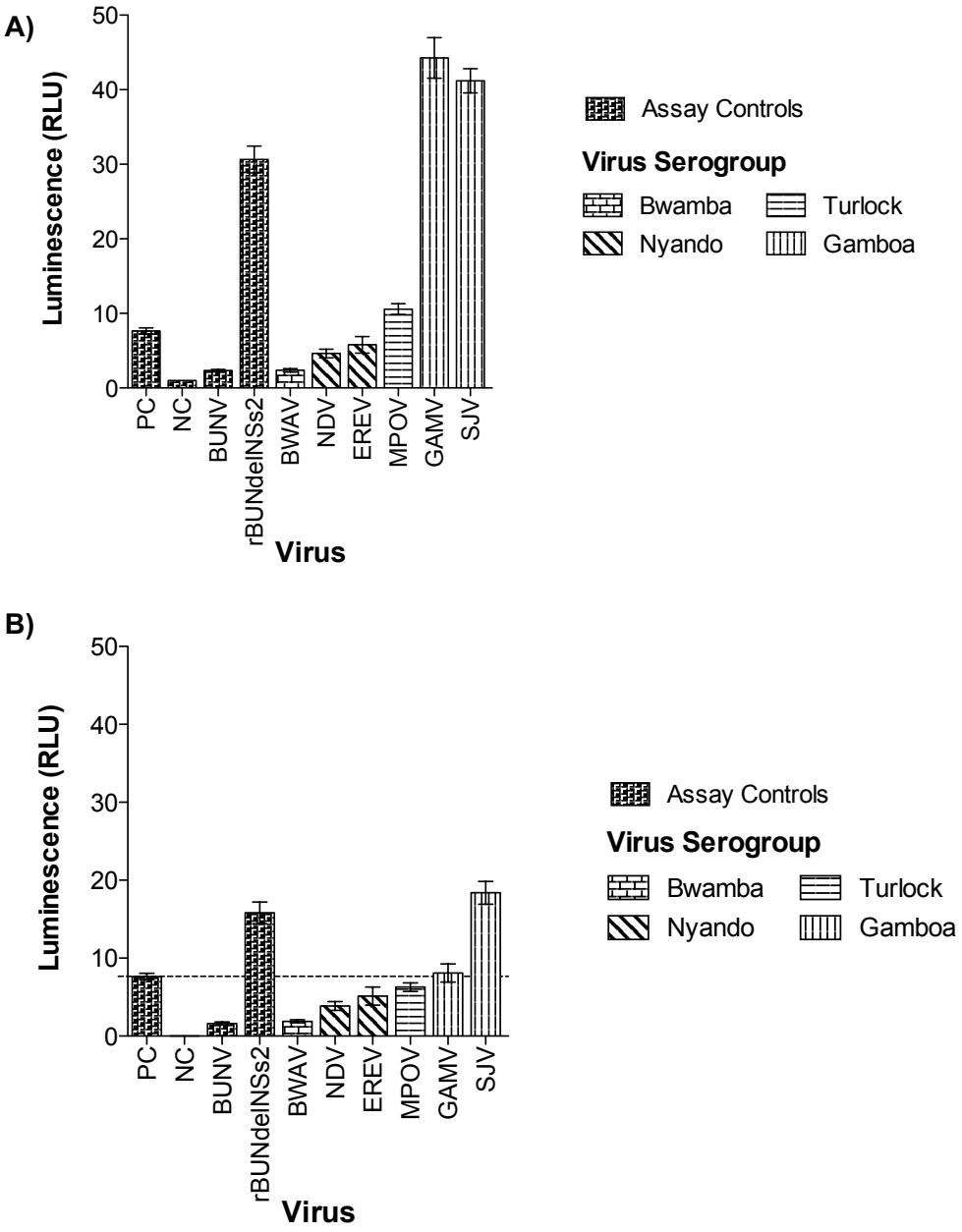


Figure 6-3: Antagonism of the Type I Interferon Response in Virus Infection

A549 cells were co-transfected with interferon (IFN)-β firefly and CMV Renilla reporter plasmids pIFΔ(-125)lucter and phRL-CMV. Cells were then infected at multiplicity of infection 1) or mock infected with an equal volume of PBS 2% (v/v) FCS. Two hours post infection (h p.i.) the mock infected positive control (PC) and virus infected cells were treated with 10 U ml^{-1} universal IFN. The mock infected negative control (NC) was treated with an equal volume of PBS in place of IFN. **A)** At 18 h p.i. cells were assayed for luciferase activity (relative light units, RLU). Mean ± standard deviation of triplicate infections is shown. **B)** To facilitate result interpretation RLU from infected cells not treated with IFN (Figure 6-2A) were subtracted from those presented in Figure 6-3A, thereby showing the difference in IFN-β promoter activity between infected and infected IFN treated cells. Mean ± standard deviation of triplicate experiments is shown.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Bwamba virus (BWAV), Nyando virus (NDV), Eretmapodites virus (EREV), M'Poko virus (MPOV), Gamboa virus (GAMV), and San Juan virus (SJV).

To assess whether the type I IFN antagonism observed during virus infection could be attributed to the NSs protein the NSs ORFs of BUNV, NDV, EREV, MPOV, GAMV, and WELV were cloned into CMV-driven expression plasmids. Due to time constraints, only viruses that were sequenced as part of my studies were included, therefore BWAV and SJV were omitted whilst WELV, a Tete serogroup virus that could not be titrated by plaque assay and was therefore not tested in previous assays, was included. The NSs expression plasmids were co-transfected with luciferase reporter plasmids and a plasmid encoding the 2CARD domain of RIG-I to activate the type I IFN system. Induction of the type I IFN response in this assay was confirmed by the positive control with ≥ 219-fold increase in IFN-β promoter activity compared with non-induced cells (Figure 6-4). In this assay BUNV, NDV and EREV NSs expression plasmids exerted strong antagonism, with significant ($p \leq 0.0002$) reductions in IFN-β promoter activity at only 10 ng (Figure 6-4).

Antagonism by the BUNV NSs expression plasmid significantly ($p < 0.01$) increased with transfection of 50 ng compared to 10 ng, and then levelled off with no significant differences in IFN-β promoter activity at higher concentrations (Figure 6-4). The antagonism exerted by the EREV NSs expression plasmid was dose-dependent with a steady increase in antagonism with increasing plasmid concentration (Figure 6-4). The NDV NSs expression plasmid showed strong antagonism at all concentrations (Figure 6-4). The putative NSs protein of WELV also had a strong antagonistic effect, in a dose-dependent manner, with a 2.5-fold reduction ($p < 0.004$) at 10 ng and a 16.8-fold reduction ($p \leq 0.0001$) at 250 ng (Figure 6-4). The MPOV NSs expression plasmid displayed weaker antagonistic activity than BUNV, NDV, and EREV, with only a 3.1-fold reduction ($p < 0.003$) in IFN-β promoter activity at 500 ng (Figure 6-4). Strikingly, the NSs ORF of GAMV showed no activity in this assay with no significant reductions in luciferase activity at any of the concentrations tested (Figure 6-4).

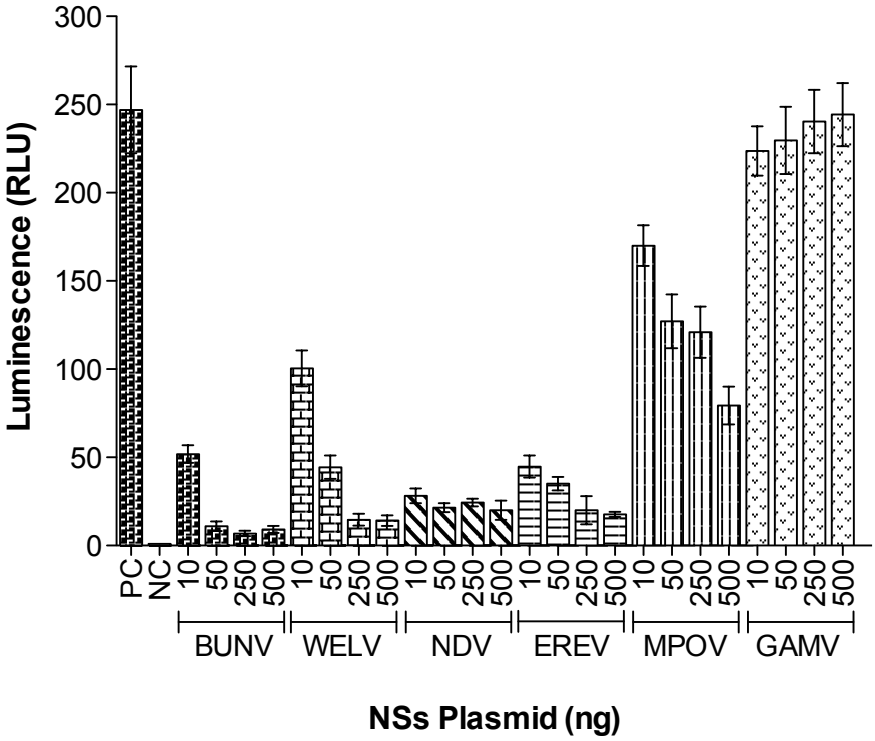


Figure 6-4: Antagonism of the Interferon-β Promoter by Virus NSs Proteins

293T cells were co-transfected with: interferon (IFN)-β firefly and CMV Renilla reporter plasmids, pIFΔ(-125)lucter and phRL-CMV; pCAGGs-2CARD to stimulate the type I IFN pathway; pCMV NSs encoding plasmids at specified concentrations; and pCMV-empty to normalise plasmid DNA concentrations across all reactions. At 18 hours post transfection cells were assayed for luciferase activity (relative light units, RLU). The NSs ORFs of BUNV, WELV, NDV, EREV, MPOV and GAMV were tested. The mean ± standard deviation of triplicate transfections is shown. The positive control (PC) contains IFN-β and CMV promoter reporter plasmids, pCAGGs-2CARD to stimulate type I IFN pathway and pCMV-empty in place of an NSs expression plasmid. The negative control (NC) contains IFN-β and CMV promoter reporter plasmids and pCMV-empty only, with no NSs expression plasmid or IFN induction plasmid, pCAGGs-2CARD.

Virus abbreviations: Bunyamwera virus (BUNV), Weldona virus (WELV), Nyando virus (NDV), Eretmapodites virus (EREV), M'Poko virus (MPOV) and Gamboa virus (GAMV).

6.3.2 Sensitivity of Viruses with an NSs ORF to Pre-treatment with IFN

Viruses predicted to encode an NSs protein were tested for sensitivity to exogenous IFN. Vero E6 cells were pre-treated with universal IFN for 24 hours prior to infection and virus replication measured by plaque assay on clarified culture supernatant 48 h p.i.. BUNV underwent a dose-dependent reduction in virus titre with ≥ 1.3 log reduction at 100 IFN U ml^{-1}, a 2.2 log reduction at 1 000 IFN U ml^{-1} and ≥ 2.4 log reduction at 10 000 IFN U ml^{-1} (Figure 6-5). The Nyando serogroup viruses, NDV and EREV, underwent similar reductions with ≥ 1.1 log drop at 100 IFN U ml^{-1}, ≥ 2.3 log drop at 1000 IFN U ml^{-1} and ≥ 2.6 log drop at 10 000 IFN U ml^{-1} (Figure 6-5). Gamboa serogroup viruses, GAMV and SJV, were also sensitive to IFN pre-treatment with > 2 log and > 3 log reductions in virus titres at 1 000 IFN U ml^{-1} and 10 000 IFN U ml^{-1}, respectively (Figure 6-5). BWAV, a member of the Bwamba serogroup, was slightly more resistant to IFN pre-treatment, with only a 0.8 log reduction in virus titre at 100 IFN U ml^{-1} and 1.7 log reduction at 1 000 IFN U ml^{-1} (Figure 6-5). However, of the NSs encoding viruses, MPOV showed the greatest resistance to IFN pre-treatment with < 1 log drop at 1 000 IFN U ml^{-1}, and < 2 log drop at 10 000 IFN U ml^{-1} (Figure 6-5).

To facilitate result interpretation and summarise the *in vitro* IFN phenotype of viruses predicted to encode an NSs protein the results presented thus far are collated in Table 6-1.

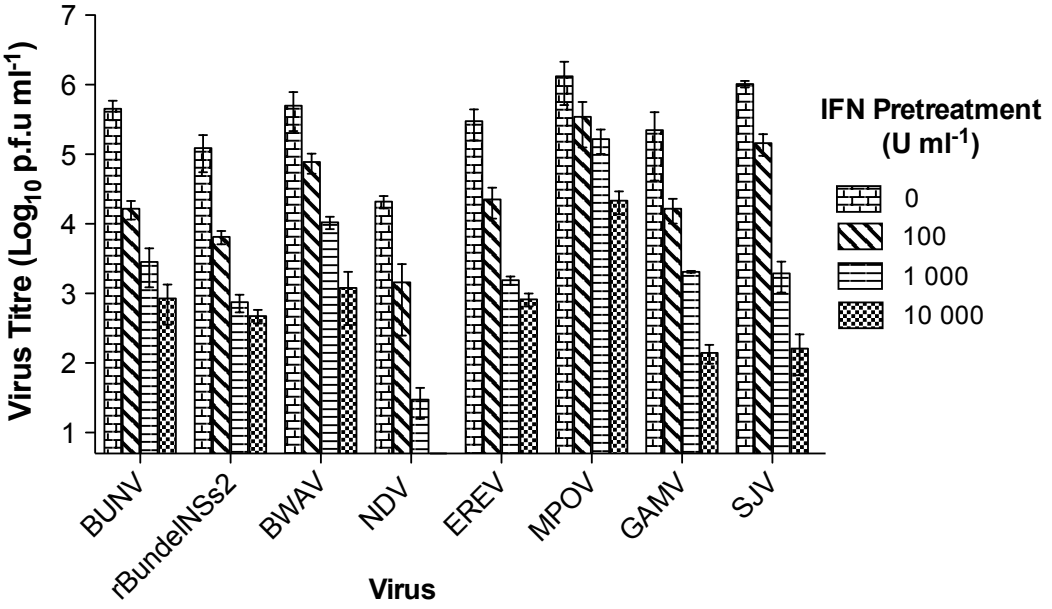


Figure 6-5: Virus Growth in Interferon Treated Cells

Vero E6 cells were pre-treated with universal interferon (IFN) (U ml^{-1}) 24 hours prior to infection at multiplicity of infection 1. Cell supernatants were collected 48 hours post infection and virus titre determined by plaque assay on Vero E6 cells. Mean ± range of duplicate experiments is shown.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Bwamba virus (BWAV), Nyando virus (NDV), Eretmapodites virus (EREV), M'Poko virus (MPOV), Gamboa virus (GAMV) or San Juan virus (SJV).

Table 6-1: *In vitro* Interferon Phenotype of Viruses with an NSs ORF

Virus	Serogroup	NSs ORF	Induction of Biological IFN	Activation of IFN-β Promoter	Sensitivity to IFN	Antagonism of IFN-β Promoter
BUNV	**Bunyamwera**	**+**	**-**	**-**	**+++**	**++++**
BWAV	Bwamba	+	-	-	++	N/A
NDV	Nyando	+	-	-	+++	+++
EREV	Nyando	+	+	-	+++	+++
MPOV	Turlock	+	++	+	+	+
GAMV	Gamboa	+	++++	++++	+++	-
SJV	Gamboa	+	++++	++++	++	N/A
WELV	Tete	+	N/A	N/A	N/A	+++

A summary of interferon (IFN) results for viruses predicted to encode an NSs protein. N/A; not available.

- Induction of biological IFN in virus infection: -, ≤ 10 rIFN U ml^{-1}; +, > 10 ≤ 50 rIFN U ml^{-1}; ++, > 50 ≤ 150 rIFN U ml^{-1}; +++, > 150 ≤ 300 rIFN U ml^{-1}; +++, > 300 rIFN U ml^{-1}.
- Activation of IFN-β promoter in virus infection: -, ≤ 1 RLU; +, 1 - 5 RLU; ++, 5 - 10 RLU; +++, 10 - 15 RLU; ++++, > 15 RLU.
- Sensitivity to IFN, lowest concentration IFN for ≥ 1 log reduction in virus titre: +++, 100 IFN U ml^{-1}; ++, 1 000 IFN U ml^{-1}; +, 10 000 IFN U ml^{-1}; -, <1 log reduction at 10 000 IFN U ml^{-1}.
- Antagonism of the IFN-β promoter by NSs expression plasmids at 250 ng: ++++, ≤ 10 RLU; +++, > 10 ≤ 25 RLU; ++, > 25 ≤ 100 RLU; +, > 100 ≤ 200 RLU; -, > 200 RLU.

6.3.3 Activation and Antagonism of the Type I IFN System by Viruses Lacking an NSs ORF

Activation of the type I IFN system was measured using the biological EMCV assay and IFN-β promoter reporter assays described in section 6.3.1. Anopheles A serogroup viruses, CPEV, LMV, TRMV and TUCV, induced approximately 128 rIFN U ml^{-1} to 256 rIFN U ml^{-1}, which was equal to, or within 1 dilution factor, of rBUNdelNSs2 (Figure 6-6). ANAV induced far higher levels of IFN at > 1 000 rIFN U ml^{-1}, whereas, TCMV, another Anopheles A serogroup virus, induced significantly lower ($p < 0.01$) levels of biological IFN than rBUNdelNSs2 at approximately 50 rIFN U ml^{-1} (Figure 6-6). Anopheles B serogroup viruses, ANBV and BORV, induced approximately 512 rIFN U ml^{-1}.

The Minatitlan serogroup virus, PLSV and Capim serogroup viruses, CAPV, JDV and MORV, induced a similar level of IFN as rBUNdelNSs2 at approximately 128 rIFN U ml^{-1} - 256 rIFN U ml^{-1} (Figure 6-6). The remaining Capim serogroup virus, GJAV, induced lower levels of biological IFN at approximately 64 rIFN U ml^{-1} (Figure 6-6). GMAV, a member of the Guama serogroup, induced approximately 27 rIFN U ml^{-1}, significantly less ($p < 0.01$) than rBUNdelNSs2. Tete serogroup viruses also induced low levels of IFN compared with rBUNdelNSs2, with significant reductions ($p < 0.01$) in BMAV, TETEV and TSUV infections (Figure 6-6).

Activation of the IFN-β promoter in a dual luciferase assay largely mirrored the results of the biological IFN assay. Anopheles A serogroup viruses CPEV, LMV, TRMV and TUCV induced similar levels of IFN-β promoter activity as rBUNdelNSs2, at 15.3 ± 3.0 RLU versus 14.9 ± 1.1 RLU (Figure 6-7A). ANAV induced significantly higher activity ($p < 0.02$) than rBUNdelNSs2, at approximately 33.5 RLU, and TCMV induced significantly lower levels ($p < 0.002$) at ≤ 4 RLU (Figure 6-7A). Differences in the level of induction by Anopheles A serogroup viruses could not be attributed to variation in virus replication as the mean virus titres of corresponding cell supernatants were within 0.2 logs (Figure 6-7B).

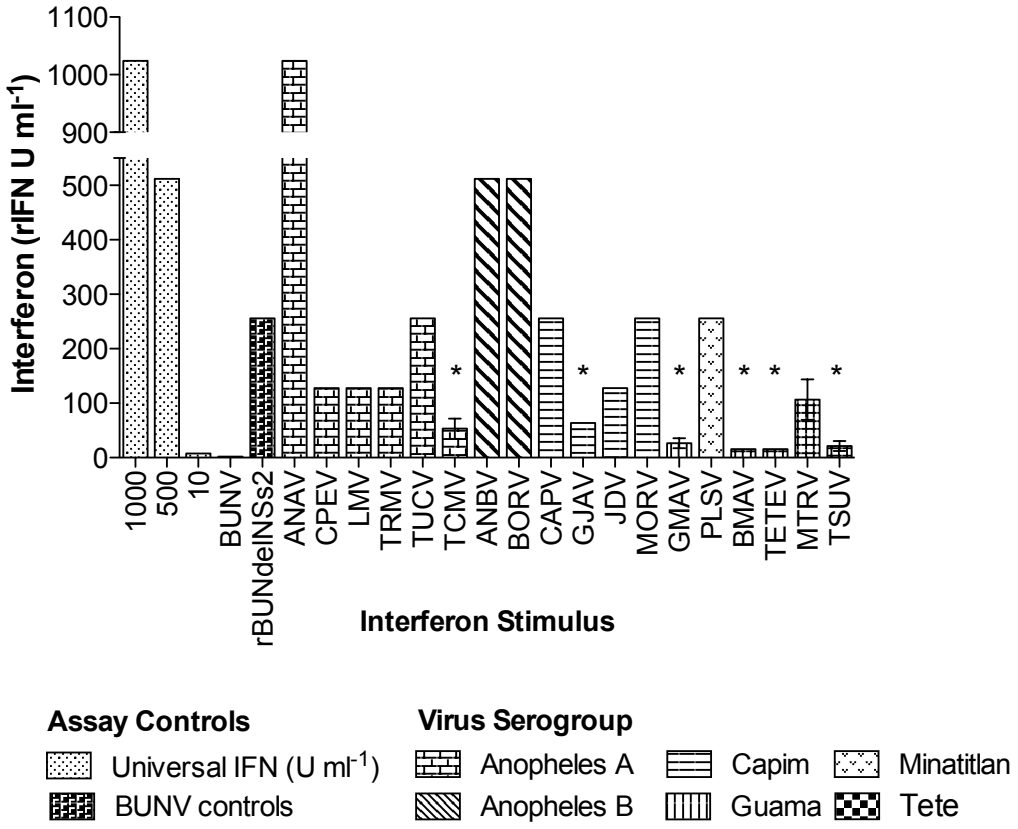


Figure 6-6: Biological Induction of Interferon by Non-NSs Viruses

Relative levels of biological interferon (IFN) (rIFN U ml^{-1}) produced by A549 cells 48 hours post induction with universal interferon (U ml^{-1}) or 24 hours post infection at multiplicity of infection 1. rIFN was measured by the level of protection given to A549-NPro cells from EMCV infection by 2-fold dilutions of UV-irradiated supernatant from induced and infected A549 cells. Mean ± standard deviation of triplicate infections is shown. Samples with significant ($p < 0.01$), ≥ 4-fold, lower induction of IFN are marked with a black asterisk (*).

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Anopheles A virus (ANAV), Caraipe virus (CPEV), Las Maloyas virus (LMV), Trombetas virus (TRMV), Tucurui virus (TUCV), Tacaiuma virus (TCMV), Anopheles B virus (ANBV), Boraceia virus (BORV), Capim virus (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV), Moriche virus (MORV), Guama virus (GMAV), Palestina virus (PLSV), Batama virus (BMAV), Tete virus (TETEV), Matruh virus (MTRV), Tsuruse-like virus (TSUV) and encephalomyocarditis virus (EMCV).

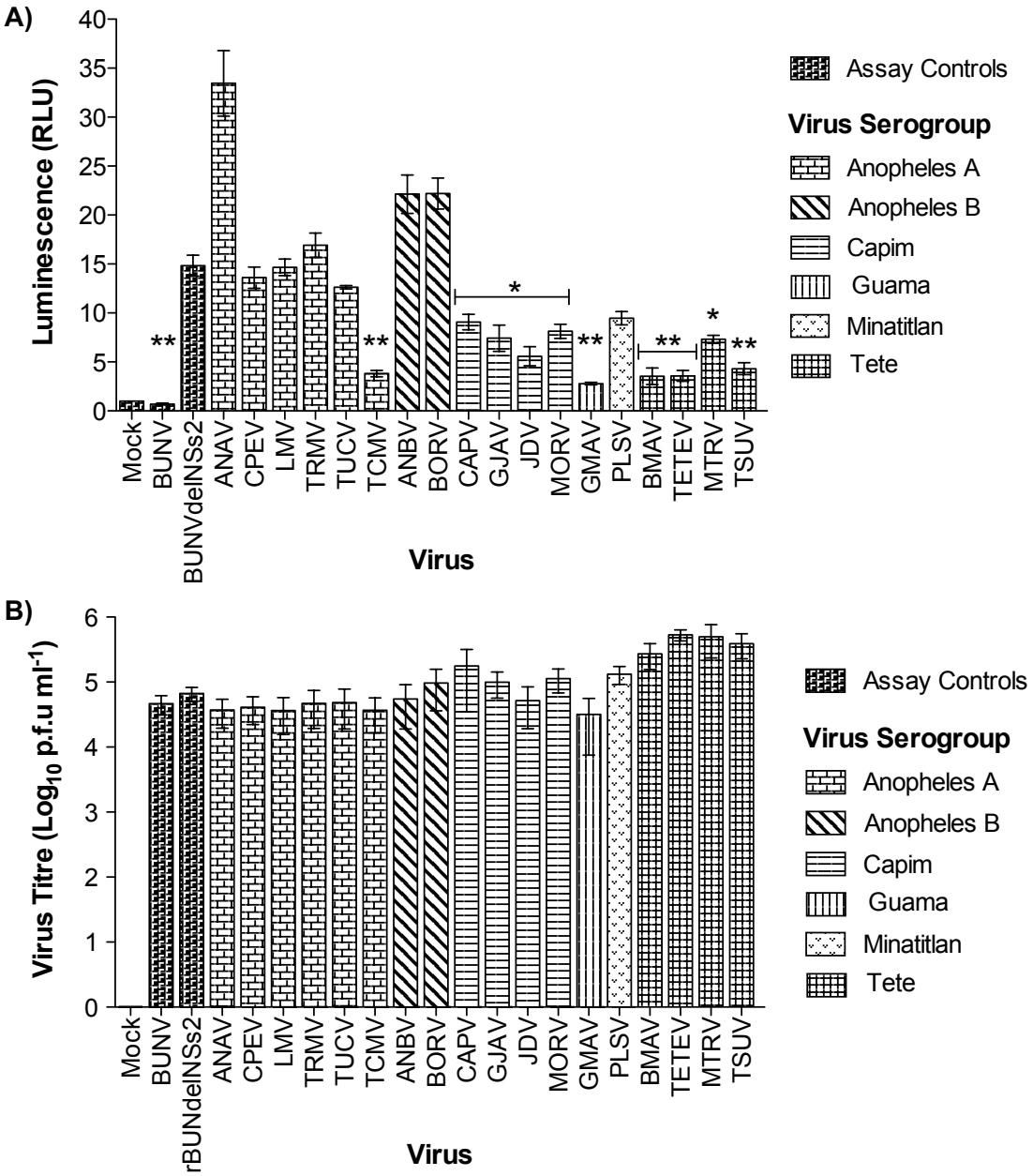


Figure 6-7: Activation of the Interferon-β Promoter in Non-NSs Virus Infection

A549 cells were transfected with interferon (IFN)-β and CMV promoter reporter plasmids pIFΔ(-125)lucter and phRL-CMV. Cells were then infected at multiplicity of infection 1. **A)** At 18 hours post infection cells were assayed for luciferase activity. Mean ± standard deviation of triplicate infections is shown, samples with significantly lower activity than rBUNdelNSs2 are marked with asterisks: **, $p \leq 0.002$; *, $p \leq 0.02$. **B)** In addition to measuring luciferase activity, virus titres of the corresponding cell supernatants were determined by plaque assay on Vero E6 cells, mean ± standard deviation are shown.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV engineered to lack an NSs protein (rBUNdelNSs2), Anopheles A virus (ANAV), Caraipe virus (CPEV), Las Maloyas virus (LMV), Trombetas virus (TRMV), Tucurui virus (TUCV), Tacaiuma virus (TCMV), Anopheles B virus (ANBV), Boraceia virus (BORV), Capim virus (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV), Moriche virus (MORV), Guama virus (GMAV), Palestina virus (PLSV), Batama virus (BMAV), Tete virus (TETEV), Matruh virus (MTRV), and Tsuruse-like virus (TSUV).

Anopheles B serogroup viruses, ANBV and BORV, induced significantly higher levels ($p < 0.005$) of IFN-β promoter activity than rBUNdelNSs2 at 22.1 ± 2.0 RLU. Capim and Minatitlan serogroup viruses induced lower levels of activity than rBUNdelNSs2 and, in contrast to the biological IFN assay, reductions were significant for CAPV, GJAV, JDV and MORV ($p<0.005$) (Figure 6-7A). Guama serogroup virus GMAV induced the lowest level of IFN-β promoter activity at only 2.8 ± 1.5 RLU (Figure 6-7A). Tete serogroup viruses also induced significantly lower ($p < 0.02$) levels of activity and, as with the biological assays, the biggest reductions were seen in BMAV, TETE and TSUV infected cells (Figure 6-7A).

Non-NSs viruses with lower activation of the type I IFN system in both the biological and IFN-β promoter reporter assay were tested for antagonism of the type I IFN response. The IFN-β promoter assay was repeated in the presence of 10 U ml^{-1} exogenous IFN, with BUNV and rBUNdelNSs2 as controls. In the presence of exogenous IFN BUNV antagonises the IFN response with IFN-β promoter activity of only 2.5 ± 0.2 RLU, notably lower than in the uninfected positive control (Figure 6-3A). In contrast, IFN treated cells infected with rBUNdelNSs2 virus had higher activity at 30.7 ± 1.5 RLU, approximately 15 RLU higher than infected cells that had not been treated with IFN (Figure 6-8). This also occurred in Tete serogroup infected cells, with no evidence of IFN antagonism by BMAV, MTRV, TETEV or TSUV as IFN-β promoter activity increased to > 15 RLU in the presence of exogenous IFN (Figure 6-8A). There was also no evidence of antagonism by Capim, Guama and Minatitlan serogroup viruses (Figure 6-8). However, in TCMV infected cells the difference in RLU between IFN treated and untreated cells was 5.8 ± 0.5, which was lower than that of the IFN PC (Figure 6-8B).

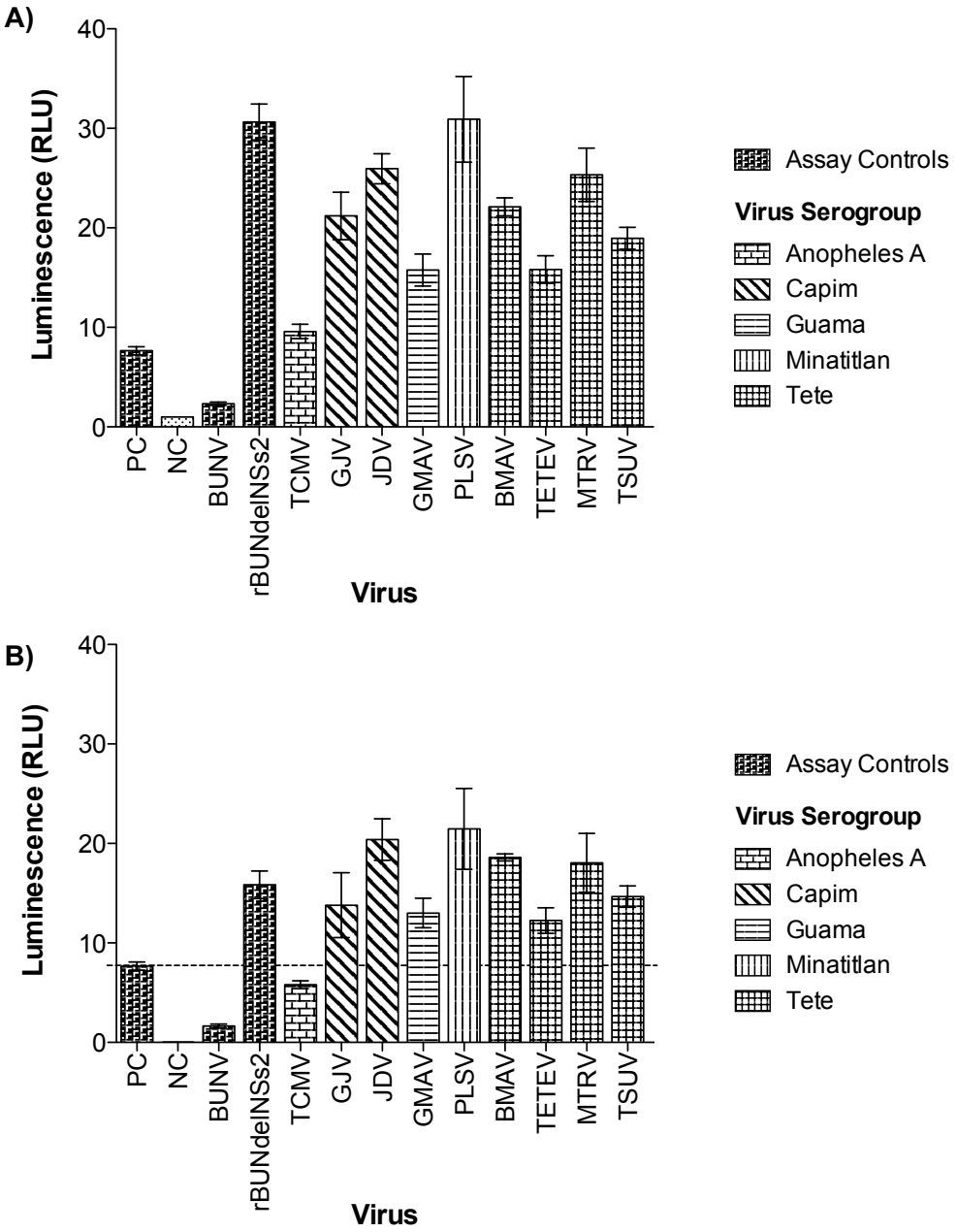


Figure 6-8: Antagonism of the Type I Interferon Response in Virus Infection

A549 cells were transfected with interferon (IFN)-β and CMV promoter reporter plasmids pIFΔ(-125)lucter and phRL-CMV. Cells were then infected at multiplicity of infection 1 and 2 hours post infection (h p.i.) 10 U ml^{-1} universal IFN, or an equal volume of PBS, were added to the cell supernatant. **A)** At 18 h p.i. cells were assayed for luciferase activity. Mean ± standard deviation of triplicate infections are shown, RLU (relative light units). **B)** To facilitate result interpretation normalised firefly luciferase values from infected cells not treated with IFN (Figure 6-6A) were subtracted from those presented in Figure 6-7A, mean ± standard deviation are shown.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV engineered to lack an NSs protein (rBUNdelNSs2), Tacaiuma virus (TCMV), Guajara virus (GJAV), Juan Diaz virus (JDV), Guama virus (GMAV), Palestina virus (PLSV), Batama virus (BMAV), Tete virus (TETEV), Matruh virus (MTRV) and Tsuruse-like virus (TSUV).

6.3.4 Sensitivity of non-NSs viruses to Pre-treatment with IFN

Non-NSs encoding viruses were tested for sensitivity to IFN pre-treatment as described in section 6.3.2. rBUNdelNSs2 had a similar sensitivity profile to BUNV with dose-dependent reduction in virus titre and 2.2 log reduction at 1 000 IFN U ml^{-1} (Figure 6-9A). Anopheles A serogroup viruses, excluding TCMV, were sensitive to IFN with titres > 1 log lower at 1 000 IFN U ml^{-1} (Figure 6-9A). TCMV however, was highly resistant, with virus titres at 1 000 IFN U ml^{-1} and 10 000 IFN U ml^{-1} within 1 log of untreated cells (Figure 6-9A). The Anopheles B serogroup viruses, ANBV and BORV, were also highly resistant to IFN with titres remaining within 1.5 logs of untreated cells, even at 10 000 IFN U ml^{-1} (Figure 6-9A).

Capim serogroup viruses CAPV, GJAV and JDV were sensitive to IFN with titres ≥ 1.5 log lower at 1 000 IFN U ml^{-1} (Figure 6-9B). MORV, also a member of the Capim serogroup, and PLSV, a Minatitlan serogroup virus, were more resistant with < 0.7 log reduction in virus titres at 1 000 IFN U ml^{-1} (Figure 6-9B). Guama serogroup virus GMAV and Tete serogroup viruses, excluding MTRV, were sensitive to IFN with a ≥ 2 log drop in virus titres at 1 000 IFN U ml^{-1}. MTRV was slightly more resistant with approximately a 1 log drop at 1 000 IFN U ml^{-1} (Figure 6-9B). To aid result interpretation IFN sensitivity and IFN activation data were collated and summarised in Table 6-2.

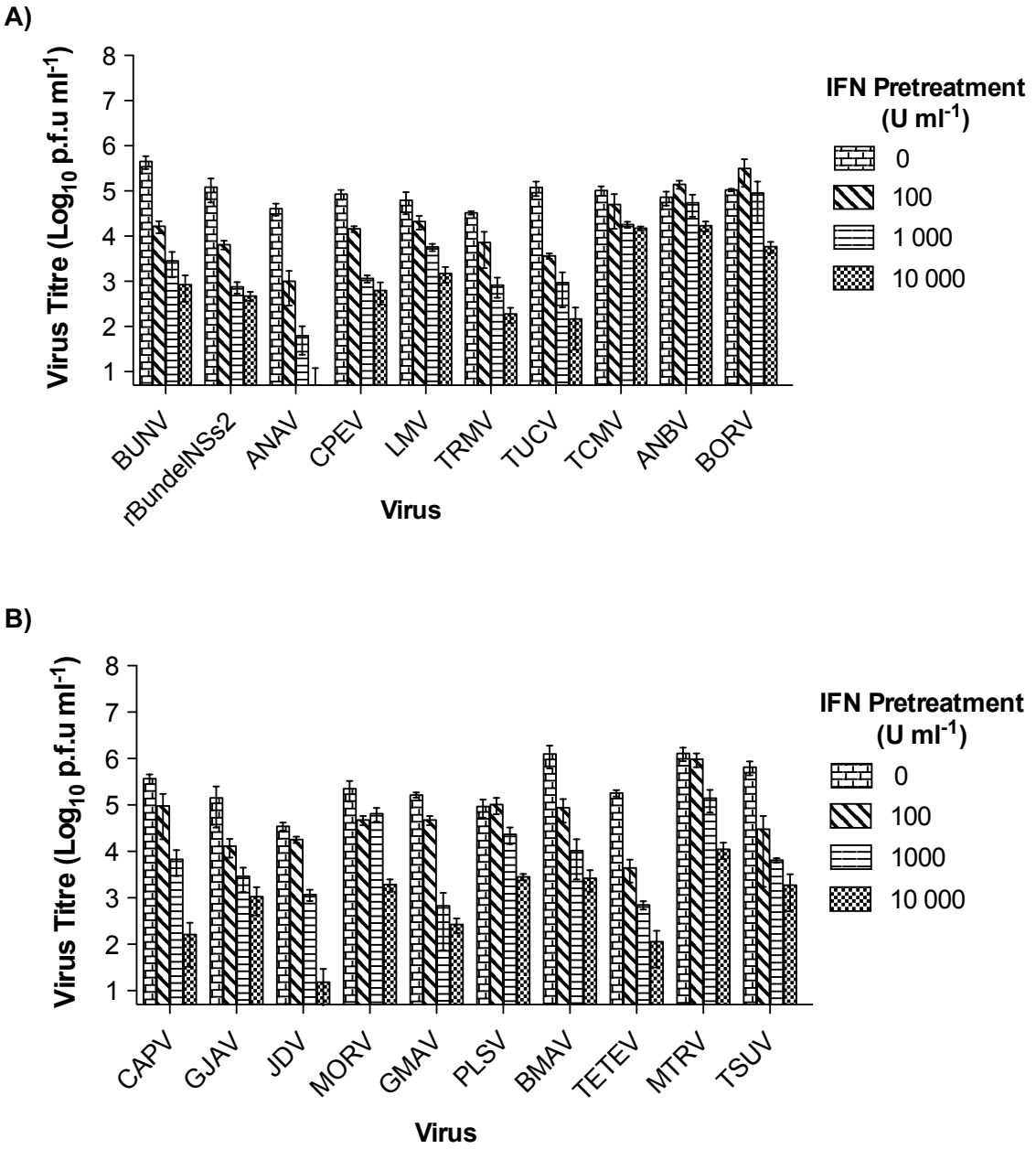


Figure 6-9: Virus Growth in Interferon Treated Cells

Vero E6 cells were pre-treated with universal interferon (IFN) (U ml^{-1}) 24 hours prior to infection at multiplicity of infection 1 with the specified viruses. Cell supernatants were collected 48 hours post infection and virus titre determined by plaque assay on Vero E6 cells. Mean ± range of duplicate experiments is shown. **A)** Viruses in the Anopheles A and Anopheles B serogroups were tested. **B)** Viruses in the Capim, Guama, Minatitlan and Tete serogroups were tested.

Virus abbreviations: Bunyamwera virus (BUNV), recombinant BUNV lacking an NSs protein (rBUNdelNSs2), Anopheles A virus (ANAV), Caraipe virus (CPEV), Las Maloyas virus (LMV), Trombetas virus (TRMV) and Tucurui virus (TUCV), Tacaiuma virus (TCMV), Anopheles B virus (ANBV) and Boraceia virus (BORV), Capim virus (CAPV), Guajara virus (GJAV), Juan Diaz virus (JDV), Moriche virus (MORV), Guama virus (GMAV), Palestina virus (PLSV), Batama virus (BMAV), Tete virus (TETEV), Matruh virus (MTRV), and Tsuruse-like virus (TSUV).

Table 6-2: *In vitro* Interferon Phenotype of Viruses lacking an NSs ORF

Virus	Serogroup	NSs ORF	Induction of Biological IFN	Activation of IFN-β Promoter	Sensitivity to IFN	Antagonism of IFN-β Promoter
BUNV	**Bunyamwera**	**+**	**-**	**-**	**+++**	**+++**
rBUNdelNSs2		**-**	**+++**	**+++**	**+++**	**-**
ANAV	Anopheles A	-	++++	++++	++++	N/A
CPEV	Anopheles A	-	++	+++	++	N/A
LMV	Anopheles A	-	++	+++	++	N/A
TRMV	Anopheles A	-	++	++++	++	N/A
TUCV	Anopheles A	-	+++	+++	+++	N/A
TCMV	Anopheles A	-	+	+	-	+
ANBV	Anopheles B	-	++++	++++	-	N/A
BORV	Anopheles B	-	++++	++++	+	N/A
CAPV	Capim	-	+++	++	++	N/A
GJAV	Capim	-	++	++	++	N/A
JDV	Capim	-	+++	++	++	-
MORV	Capim	-	+++	++	+	-
GMAV	Guama	-	+	+	++	-
PLSV	Minatitlan	-	+++	++	+	-
TETEV	Tete	-	+	+	+++	-
BMAV	Tete	-	+	+	+++	-
MTRV	Tete	-	++	++	++	-
TSUV	Tete	-	+	+	+++	-

A summary of interferon (IFN) results for viruses predicted to lack an NSs open reading frame (ORF). N/A; not available.

- Induction of biological interferon in virus infection: -, ≤ 10 rIFN U ml^{-1}; +, > 10 ≤ 50 rIFN U ml^{-1}; ++, > 50 ≤ 150 rIFN U ml^{-1}; +++, > 150 ≤ 300 rIFN U ml^{-1}; +++, > 300 rIFN U ml^{-1}.
- Activation of IFN-β promoter during virus infection: -, ≤ 1 RLU; +, 1 - 5 RLU; ++, 5 - 10 RLU; +++, 10 - 15 RLU; ++++, >15 RLU.
- Sensitivity to IFN, lowest concentration IFN for ≥ 1 log reduction in virus titre: +++, 100 IFN U ml^{-1}; ++, 1 000 IFN U ml^{-1}; +, 10 000 IFN U ml^{-1}; -, < 1 log reduction at 10 000 IFN U ml^{-1}.
- Antagonism of IFN-β promoter in the presence of exogenous IFN, Figure 6-8B: (-) ≥ 7 RLU; +, > 5 ≤ 7 RLU; ++, > 2 ≤ 5 RLU; +++, > 1 ≤ 2; ++++, ≤ 1 RLU.

6.4 Discussion

6.4.1 Viruses with an NSs ORF and Interferon

The NSs proteins of pathogenic orthobunyaviruses, including AKAV, BUNV, LACV, OROV (Personal communication, Natasha Tilston-Lunel, University of St Andrews) and SBV, have been shown to be potent antagonists of the type I IFN system and remain the primary virulence factor of orthobunyaviruses (Bridgen *et al.,* 2001; Weber *et al,.* 2002; Blakqori *et al.,* 2007; Ogawa *et al*., 2007; Barry *et al.,* 2014). In this study 7 viruses predicted to encode an NSs protein by genome sequencing were studied for activation, antagonism, and sensitivity to type I IFN. Bwamba serogroup virus, BWAV, mirrored the *in vitro* behaviour of BUNV, the type species of the genus, inducing undetectable levels of biological IFN and below background activation of the IFN-β promoter in a reporter assay (Figure 6-1, Figure 6-2). BWAV was also a strong antagonist of the IFN-β promoter in the presence of exogenous IFN (Figure 6-3). BWAV is widespread in Africa and causes a self-limiting febrile illness with exanthema and frequent meningeal involvement that is often misdiagnosed as malaria (Smithburn *et al.,* 1941; Smithburn, 1952; Lutwama *et al*., 2002). Expression of an NSs protein with potent anti-IFN activity would agree with our current understanding of orthobunyavirus pathogenicity.

Like BWAV, the Nyando serogroup viruses have been isolated from across Africa and are associated with a self-limiting febrile illness (Williams *et al,.* 1965; Digoutte *et al.,* 1972; Lutwama *et al.*, 1999; Groseth *et al,.* 2014). In this study Nyando serogroup viruses NDV and EREV, were found to induce very low levels of IFN during virus infection of A549 cells (Figure 6-1, Figure 6-2). Moreover, expression of the NSs ORF in a reporter assay significantly antagonised activation of the IFN-β promoter (Figure 6-4). Groseth *et al*. (2012) published the first complete sequences for Bwamba and Nyando serogroup viruses and studied the growth of these viruses in a range of mosquito (C6/36), mammalian (Vero E6) and bat (Tbu-L1 and RE05) cell lines. However, this is the first study confirming IFN antagonism by viruses in the Nyando

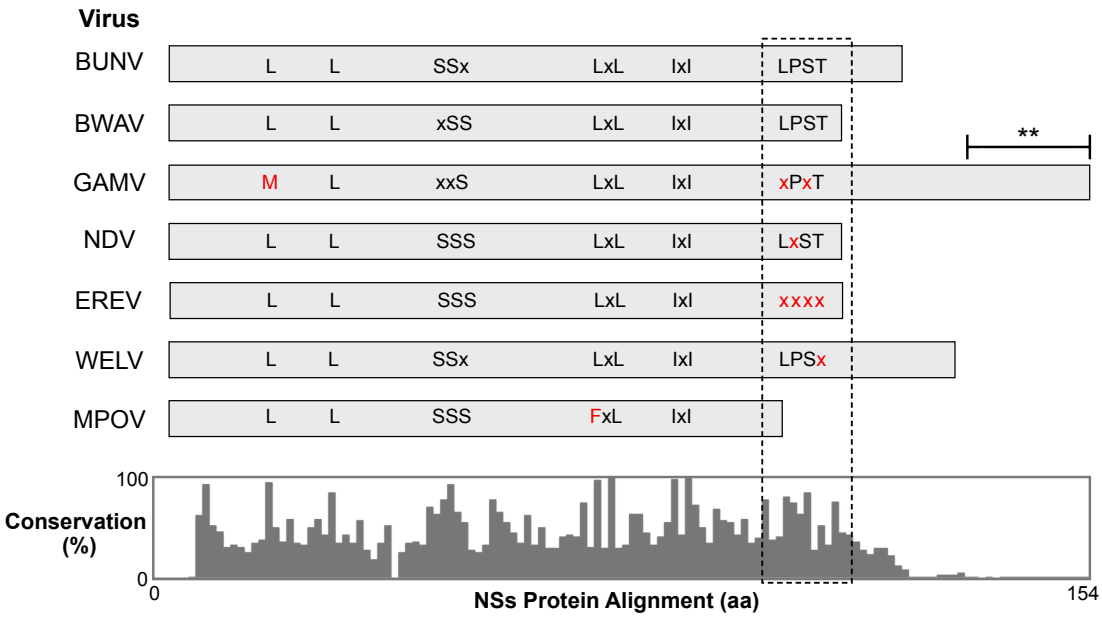


Figure 6-10: NSs Conservation

Schematic representation of NSs protein conservation highlighting Bunyamwera virus (BUNV) (NC001927), the type species of the genus and the viruses included in this study. Bwamba virus (BWAV) and Gamboa virus (GAMV) are representative of all sequenced Bwamba serogroup and Gamboa serogroup viruses (n=2, n=6). **, highlights the extended sequence of Gamboa serogroup viruses compared with other putative orthobunyavirus NSs proteins. Nyando serogroup viruses (n=6) are represented by Nyando virus (NDV) and Eretmapodites virus (EREV). Weldona virus (WELV) and M'Poko virus (MPOV) are the only sequenced viruses with NSs ORFs in the Tete and Turlock serogroups respectively. Published putative NSs protein sequences ≥ 50 amino acids (aa) for all named orthobunyaviruses isolates were combined with those obtained during this study. When more than 1 sequence for a named isolate was available 1 was selected. A complete list of virus isolates and accession numbers is detailed in Appendix 2. Protein sequences were aligned in CLC Genomics Workbench v7.5.1 (Qiagen) using the MUSCLE algorithm. A dashed box highlights the proposed Med8 interaction domain (BUNV NSs aa 81 - 93)(Léonard *et al.*, 2006). Non-conserved aa are highlighted in red.

and Bwamba serogroups. The NSs proteins of viruses in the Bwamba and Nyando serogroups are 92 aa in length, terminating just 1 aa short of the complete Med8 interaction domain, and all sequenced isolates contain the highly conserved LxL and IxI motifs (Figure 6-10).

The NSs protein of WELV, the only member of the Tete serogroup predicted to encode an NSs protein, also contains the highly conserved LxL and IxI motifs and the proposed Med8 interaction domain (Figure 6-10). Unfortunately, WELV could not be titrated by plaque assay and was therefore omitted from all experiments requiring virus titration. However, expression of the WELV NSs ORF in a reporter assay did provide evidence that, if expressed, the NSs protein would antagonise the IFN-β promoter (Figure 6-4). WELV has not yet been associated with human infection but limited prevalence studies in Colorado detected neutralising antibodies in birds and live virus in unidentified ceratopogonid midge species (Calisher *et al.,* 1990). It would be of interest to determine expression of the predicted NSs ORF during virus replication and to contrast the biological characteristics of WELV with those of the non-NSs Tete serogroup viruses.

The NSs protein of MPOV, a member of the Turlock serogroup, terminates prior to the Med8 interaction domain and contains FxL in place of the conserved LxL motif (Figure 6-10). MPOV induced higher levels of biological IFN and IFN-β promoter activity than BUNV, BWAV, NDV and EREV (Figure 6-1, Figure 6-2). Moreover, the putative MPOV NSs protein appeared less active than the other NSs proteins when expressed in an IFN-β reporter assay (Figure 6-4). Expression of the NSs ORF in this assay was not confirmed as no antibodies were available, and expression levels would need to be assessed prior to drawing conclusions about actual NSs activity. To circumvent the lack of NSs antibodies, tagged proteins could be made but this was not done due to time constraints. Interestingly, of the NSs encoding viruses MPOV showed the greatest resistance to pre-treatment with IFN *in vitro* (Figure 6-8, Table 6-1). This may play a compensatory role in virus infection if the NSs protein is unable to completely shut down the cellular type I IFN response.

Gamboa serogroup viruses GAMV and SJV induced high levels of biological IFN and IFN-β promoter activity during virus infection *in vitro* (Figure 6-1, Figure 6-2).

Moreover, the GAMV NSs ORF expressed under a CMV promoter failed to show any activity in a IFN antagonism assay (Figure 6-4). Although expression of NSs in this assay was not confirmed as no antibodies targeting GAMV NSs were available, the results correspond with experiments utilising infectious virus, as GAMV induced high levels of IFN with no antagonism of IFN-β promoter activity (Figure 6-2). It is also of note that GAMV and SJV struggled to replicate at low MOI in the IFN-competent A549 cell line (Figure 4-7). Interestingly, all sequenced Gamboa viruses contain the highly conserved LxL and IxI motifs and at 130-137 aa extend past the Med8 interaction domain, although there is sequence variation in this region (Figure 6-10). There is a high level of conservation in the extended C-terminus, with 6 out of 6 sequenced isolates encoding a serine-rich sequence that terminates in STGSMEQ (Figure 5-2B) (Nunes *et al.,* 2014). To further investigate the NSs ORF of Gamboa serogroup viruses, priority should be given to establishing NSs expression during virus infection. If expression is confirmed, development of a reverse genetics system would greatly facilitate protein function studies, allowing recombinant viruses with NSs deletions and substitutions to be made. There are no reported cases of human infections caused by Gamboa serogroup viruses. Neutralising antibodies to Calchaqui virus have been found in horses although it is unclear if these relate to the Gamboa serogroup Calchaqui virus sequenced by Nunes *et al.* (2014) or the Calchaqui virus reported to belong to the Vesicular stomatitis antigen group (Berge, 1975).

6.4.2 Viruses without an NSs ORF and Interferon

Naturally occurring non-NSs viruses were first discovered in 2009 (Mohamed *et al.,* 2009; Mores *et al.,* 2009). These viruses would be predicted to induce high levels of IFN, but one, TCMV, induced significantly lower levels of IFN than rBUNdelNSs (Mohamed *et al.,* 2009; Mores *et al.,* 2009). Moreover, co-infection assays with TCMV and rBUNdelNSs implied an as yet unidentified NSs-independent mechanism of IFN antagonism by TCMV (Mohamed *et al.,* 2009). To date, Mohamed *et al.* (2009) remains the only published study investigating activation of the type I IFN response by naturally occurring non-NSs viruses. To expand our understanding of non-NSs viruses, viruses predicted to lack an NSs ORF from the Anopheles A (n=6), Anopheles B (n=2), Capim (n=4), Guama (n=1), Minatitlan (n=1) and Tete (n=4) serogroups were

investigated for type I IFN induction and antagonism. Strikingly, 8 viruses were found to induce significantly lower levels of biological IFN and IFN-β promoter activity than rBUNdelNSs2 during virus infection. These were the Anopheles A serogroup virus TCMV, Capim serogroup viruses GJAV and JDV, Guama serogroup virus GMAV, and the Tete serogroup viruses BMAV, TETEV, MTRV, and TSUV (Figure 6-6, Figure 6-7, Table 6-1).

TCMV was the only virus in the Anopheles A and Anopheles B serogroups to induce significantly lower levels of biological IFN and IFN-β promoter activity than rBUNdelNSs2 (Figure 6-6, Figure 6-7). Moreover, in support of the antagonism reported by Mohamed *et al.* (2009), TCMV antagonised IFN-β promoter activation in the presence of exogenous IFN (Figure 6-8). As discussed in Chapter 5, sequence analysis revealed TCMV lacks homology to other Anopheles A serogroup viruses, and appears to bridge the Anopheles A and Anopheles B serogroups on phylogenetic analysis. However, there are no clear genome characteristics that could account for the IFN phenotype observed *in vitro*, as sequence variation among Anopheles A and Anopheles B serogroup viruses occurs throughout the genome with no clear region of divergence. TCMV circulates in South America and remains the only Anopheles A serogroup virus associated with human disease (Calisher *et al.,* 1980). In addition to antagonism of the type I IFN system, TCMV was highly resistant *in vitro* to pre-treatment with universal IFN (Figure 6-9). Previous studies have also shown that i.p. administration of ≤ 100 000 IU ml^{-1} IFN-α 24 hours before infection failed to protect new born mice from fatal infection with TCMV, despite protecting 16/16 mice infected with OROV, a pathogenic NSs encoding orthobunyavirus (Livonesi *et al.*, 2007).

In this study resistance to pre-treatment with IFN was also observed for viruses in the Anopheles B serogroup (Figure 6-9). Anopheles B serogroup viruses have been isolated in South America and BORV has been linked with human infection (Lopes *et al.*, 1966; de Souza Lopes and de Abreu Sacchetta, 1974). BORV induced high levels of IFN and IFN-β promoter activity during virus infection and resistance to IFN may therefore play a key role in virus pathogenicity (Figure 6-6, Figure 6-7, Table 6-2).

Capim and Guama serogroup viruses GJAV, JDV and GMAV induced lower levels of biological IFN and IFN-β promoter activity during virus infection than rBUNdelNSs2.

However, there was no clear evidence of IFN antagonism in the presence of exogenous IFN, suggesting lower induction or evasion of the IFN pathway rather than active antagonism (Figure 6-7). Complete genome sequencing revealed viruses in the Capim and Guama serogroups contain exceptionally long M and/or S segment UTR sequences leading to the hypothesis that these may contribute to the observed IFN phenotype. However, other Capim serogroup viruses, CAPV and MORV, induced the same level of IFN as rBUNdelNSs2 during virus infection, which suggests that the presence of long UTRs in itself does not account for the observed *in vitro* IFN phenotype of GJAV, JDV and GMAV. It may be that RNA sequence and structure in the extended UTRs play a role (Davis *et al.*, 2012). Interestingly, of the Capim and Guama serogroup viruses only those belonging to the Guama group have been associated with human infection (Berge, 1975). GMAV and Catu virus (CATUV) have been isolated from sentinel primates and human sera in Brazil and are associated with fever and myalgia in man. Antibodies to a third Guama serogroup virus, Bimiti virus, have also been detected in human sera in Trinidad (Berge, 1975).

Viruses in the Tete serogroup were also found to induce lower levels of IFN and IFN-β promoter activity than rBUNdelNSs2 (Figure 6-6, Figure 6-7, Table 6-2). BMAV, TETEV, MTRV and TSUV encode larger N proteins than NSs encoding orthobunyaviruses. As discussed in Chapter 5, the additional amino acids align to the amino terminal, a region shown through x-ray crystallography of BUNV, SBV, LACV and LEAV N proteins to bind RNA (Ariza *et al.,* 2013; Dong *et al.,* 2013a; Dong *et al.,* 2013b; Reguera *et al.,* 2013). It is hypothesised that the larger N proteins of Tete serogroup viruses may bind RNA with differing topology, altering RNP formation which may in turn decrease activation of type I IFN by shielding viral RNA from host PRRs. This hypothesis is further investigated and discussed in Chapter 7 "Batama Virus Reverse Genetics and Interferon Induction".

6.4.4 Concluding Remarks

The NSs proteins from orthobunyavirus, phlebovirus and hantavirus share a common function, antagonising the type I IFN response at the transcriptional level (Weber *et al.,* 2002; Billecocq *et al.,* 2004; Jääskeläinen *et al.,* 2007). This is despite differing coding

strategies and large divergence in sequence, with no pan-bunyavirus conserved domains. However, the results presented in this chapter may challenge this dogma as there was no evidence of IFN antagonism by MPOV or Gamboa serogroup viruses. Lack of antagonism by MPOV NSs may be attributed to truncation prior to the proposed Med8 interaction. However, the Gamboa serogroup viruses are predicted to encode full length NSs proteins with a conserved serine-rich C terminus. To fully challenge our current understanding expression of the Gamboa serogroup NSs proteins must first be confirmed in virus replication, work could then begin to elucidate their role in virus replication.

Characterising activation and antagonism of the type I IFN system by divergent orthobunyaviruses also revealed that TCMV is not the only non-NSs orthobunyavirus to induce low levels of IFN during virus infection *in vitro*. Viruses in the Capim, Guama and Tete serogroup also showed significantly lower activation of the IFN-β promoter. Interestingly, these viruses did not appear to antagonise the type I IFN system in the presence of exogenous IFN suggesting a strategy of evasion rather than antagonism. TCMV on the other hand did show evidence of antagonism, supporting the findings of Mohammed *et al.* (2009), and highlighting the need for further research into NSs-independent mechanisms of IFN antagonism.

Chapter 7: Batama Virus Reverse Genetics and Interferon Induction

7.1: Introduction

The Tete serogroup currently contains 2 virus species, Tete and Batama. The Batama virus species contains only 1 named isolate, BMAV, whilst the Tete virus species contains BAHV, MTRV, TETEV, TSUV and WELV (Plyusnin *et al.*, 2012). Tete serogroup viruses have been isolated from wild and domestic birds although very little is known of their ecology (Berge, 1975). WELV, the only Nearctic Tete serogroup virus, has been isolated from unidentified pools of biting midges. BAHV and MTRV have been isolated from hard bodied ticks in Egypt and Italy (Converse *et al.*, 1974; Moussa *et al.*, 1974; Calisher *et al.*, 1990).

Genome sequencing of BMAV, MTRV TETEV, TSUV, and WELV revealed that all, except WELV, lacked a full length NSs ORF (Table 5-2). The orthobunyavirus NSs protein is a strong antagonist of the type I IFN system and it was therefore surprising that BMAV, and other non-NSs Tete serogroup viruses, induced only low levels of IFN and IFN-β promoter activity during virus infection *in vitro* (Figure 6-6, Figure 6-7). Moreover, there was no evidence of IFN-β promoter antagonism, leading to the hypothesis that Tete serogroup viruses may be low inducers rather than antagonists of the type I IFN system.

In addition to the unexpected IFN phenotype a notable feature of non-NSs Tete serogroup viruses is the size of their N protein, which at 258 aa is larger than that of most other orthobunyaviruses (Table 5-2). Moreover, WELV, the only sequenced Tete serogroup virus with a full length NSs ORF, encodes a smaller N protein (248 aa) and branches ancestrally to the group on N protein phylogeny (Figure 5-6). This suggests that the larger N proteins of non-NSs Tete serogroup viruses, such as BMAV, may have evolved over time as loss of the NSs ORF occurred. Investigating the role of the larger N protein in virus replication and IFN induction would therefore be of interest.

7.2 Aims

The aims of this part of my studies were to further investigate activation of type I IFN by Tete serogroup viruses and to determine the role of the virus N protein in this

context. This would be facilitated by the development of a reverse genetics system and the creation of recombinant viruses containing N protein mutations. These investigations were carried out using BMAV, the first Tete serogroup virus with a complete genome sequence.

7.3 Results

To test for statistical significance the results in this chapter were analysed by one-way ANOVA and the Dunnett's multiple comparison test with either BUNV or rBUNdelNSs2 as the control, or by t-test. Unless indicated all p values in this chapter refer to this analysis only and not any other form of statistical analysis.

7.3.1 Interferon Antagonism by Batama Virus Proteins

In Chapter 6 it was shown that BMAV induced significantly lower levels of IFN than other non-NSs viruses yet there was no evidence of IFN antagonism in the presence of exogenous IFN (Figure 6-6, Figure 6-7, Figure 6-8). To follow up on this observation, the ORFs encoding BMAV N, M and L proteins were cloned into CMV-driven expression plasmids and tested using a reporter assay for antagonism of the IFN-β promoter (Figure 7-1). Expression plasmids encoding the N and NSs proteins of WELV, a Tete serogroup virus with a full length NSs ORF, were included as controls. The WELV NSs expression plasmid significantly ($p < 0.01$) inhibited IFN-β promoter activity in a dose-dependent manner, with approximately a 2.3-fold reduction at 10 ng, 5.2-fold reduction at 50 ng, and 15.4-fold reduction at 250 ng (Figure 7-1). However, there was no clear evidence of antagonism by the expression plasmids encoding WELV N protein or BMAV N, M or L proteins (Figure 7-1). Although there was a small drop in IFN-β promoter activity at high concentrations (≥ 250 ng), values remained within 2-folds of the positive control at ≥ 130 RLU (Figure 7-1).

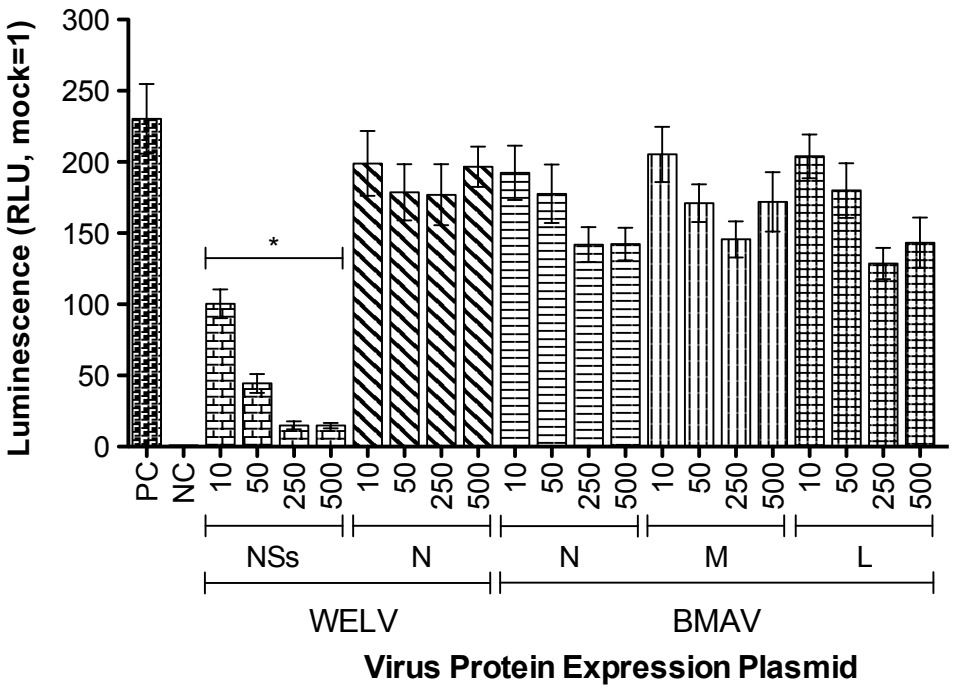


Figure 7-1: Interferon-β Promoter Antagonism by Batama Virus Proteins

293T cells were co-transfected with: Interferon (IFN)-β firefly and CMV Renilla reporter plasmids, pIFΔ(-125)lucter and phRL-CMV; pCAGGs-2CARD to stimulate the type I IFN pathway; pCMV plasmids encoding the virus proteins; and pCMV-empty to normalise plasmid DNA concentrations across all reactions. Eighteen hours post transfection cells were assayed for luciferase activity. The NSs and N ORFs of Weldona virus (WELV), and the N, M and L ORFs of Batama virus (BMAV) were tested. The positive control (PC) contains IFN-β and CMV promoter reporter plasmids, pCAGGs-2CARD to stimulate type I IFN pathway and pCMV-empty in place of an NSs expression plasmid. The negative control (NC) contains IFN-β and CMV promoter reporter plasmids and pCMV-empty only, with no NSs expression plasmid or IFN induction plasmid, pCAGGs-2CARD. The mean ± standard deviation of triplicate transfections is shown. Significance ($p < 0.01$) is indicated by a black asterisk (*).

7.3.2 Batama Virus N Protein Structural Model

BMAV and the other non-NSs Tete serogroup viruses, TETEV, MTRV, and TSUV, encode N proteins of 258 aa. As discussed in Chapter 5.3.1, N protein alignments show the additional aa form a 22 aa - 25 aa extension at the N terminus and a short, 3 aa, extension at the C terminus relative to other orthobunyaviruses (Figure 7-2A). A structural model of BMAV N protein with high coverage (88%) and confidence (100%) was generated by Phyre2 using the published crystal structure of Bunyamwera N protein (Figure 7-2B) (Kelley and Sternberg, 2009; Li *et al.*, 2013a). Overlaying the BMAV N protein structural model on the crystal structure of SBV N protein bound to RNA (PDB 4JNG) emphasises the high level of homology that BMAV N protein is predicted to share with other orthobunyavirus N proteins (Figure 7-2B) (Dong, *et al.* 2013a). However, the model omits the first 25 aa of the BMAV N protein, beginning only at I26 (Figure 7-2B). The first 25 aa of BMAV N protein are also omitted in structural models based on the solved crystal structures of BUNV, LACV and LEAV N proteins (data not shown). Interestingly, the N and C terminal aa of BMAV that are excluded from structural models are predicted to be highly disordered, whereas the rest of the protein displays a similar disorder prediction to BUNV N protein (Figure 7-2C).

To fully elucidate the role of the larger N protein in virus replication and to direct future mutagenesis studies structural determination of the full length protein, including the highly disordered termini, is required. To that end the BMAV N protein ORF was cloned into the expression vector pEHISTEV and sent to Professor Changjiang Dong, University of East Anglia for structural studies. Unfortunately, due to changes in research staff, commencement of structural studies was delayed and results are not yet available.

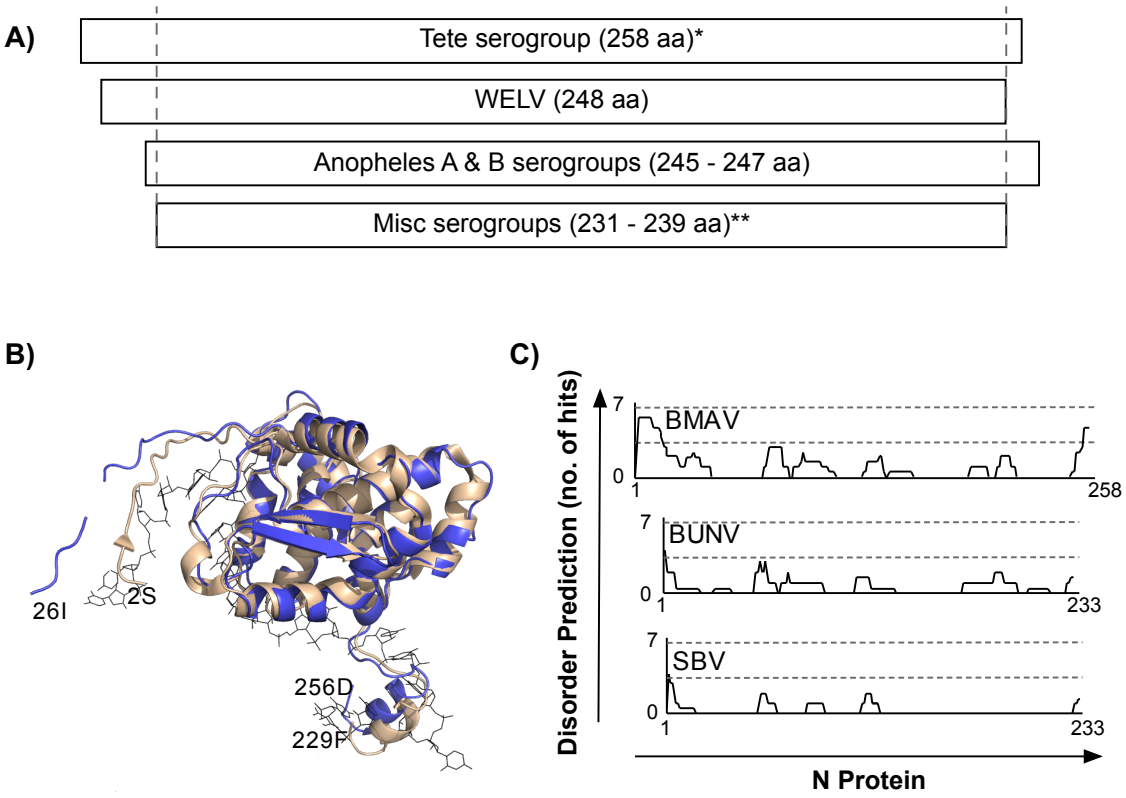


Figure 7-2: Structural Modelling of Batama Virus N Protein

A) Scaled schematic of N protein amino acid (aa) alignment showing N terminal and C terminal extensions of viruses in the Tete, Anopheles A and Anopheles B serogroups. (*) Tete serogroup viruses, except Weldona virus (WELV), which is shown separately as it encodes a smaller protein at 248 aa, are represented by a single bar, as are viruses from the Anopheles A and Anopheles B serogroups. (**) Viruses from the Bunyamwera, Bwamba, California, Capim, Gamboa Group C, Guama, Minatitlan, Nyando, Simbu and Turlock serogroups with 231 – 239 aa N proteins align together and are shown as single bar labelled Misc serogroups. **B)** Phyre2 model of Batama virus (BMAV) N protein based on Bunyamwera virus (BUNV) N protein (PDB 4IJS, chain B) aligned to the RNA-bound Schmallenberg virus (SBV) N protein (PDB 4JNG) in PyMOL, Molecular Graphics System, Version 1.7.4 Schrödinger, LLC (Kelley and Sternberg, 2009). **C)** Disorder predictions of BMAV, BUNV (NC001927) and SBV (JX853181) by Dismeta (Huang *et al.*, 2014).

7.3.3 Batama Virus Reverse Genetics

Viral genome segments were amplified by RT-PCR on virion RNA and cloned, in the antigenomic sense, into the pTVT7 backbone (Figure 7-3A). The N and L protein ORFs were also cloned into pTM-1 expression plasmids to allow both the 3 and 5 plasmid rescue systems to be tested. Plasmids were co-transfected into subconfluent BSRT-7 cells and clarified culture supernatant collected at > 80% CPE or 7 days post transfection (Figure 7-3B). Rescue supernatant was then screened for infectious particles by plaque assay on Vero E6 cells (Figure 7-3C). The 3 plasmid rescue system was robust and recombinant wild type BMAV (rBMAV) was rescued from 8 out of 8 independent experiments, with variations in plasmid concentration (500 ng - 1000 ng), cell density (1 x 10^5 cells ml^{-1} - 5 x 10^5 cells ml^{-1}) and transfection reagent (LT1, Lipofectamine 2000). Virus titres in rBMAV p1 stocks were comparable to wild type BMAV, referred to simply as BMAV from this point forward, at > 1 x 10^7 p.f.u ml^{-1}. Plaque morphology of rBMAV and BMAV on Vero E6 cells was also comparable, at 2 mm - 4 mm in diameter, with complete clearing of the cell monolayer and blurred edges (Figure 7-3C). However, it should be noted that the 5 plasmid rescue consistently failed, with no evidence of CPE and no plaques on virus titration. Moreover, both the 3 and 5 plasmid rescue systems failed when Huh7-Lunet T7 cells were used in place of BSRT-7 cells (Figure 7-3B).

Once the reverse genetics system had been established a panel of recombinant viruses was made by QuickChange® PCR and excision PCR (Figure 7-4, Table 7-1). The recombinant viruses contained mutations in the N protein N and C termini to allow the role of the larger N protein in virus replication to be studied. As the N protein termini of orthobunyavirus N proteins are known to be involved in RNA binding and N-N protein interactions a number of approaches were tested. These included the introduction of point mutations, deletions at conserved sites, and bulk aa deletions coupled with aa substitution to mirror the terminal aa sequence of BUNV and WELV N proteins (Figure 7-4).

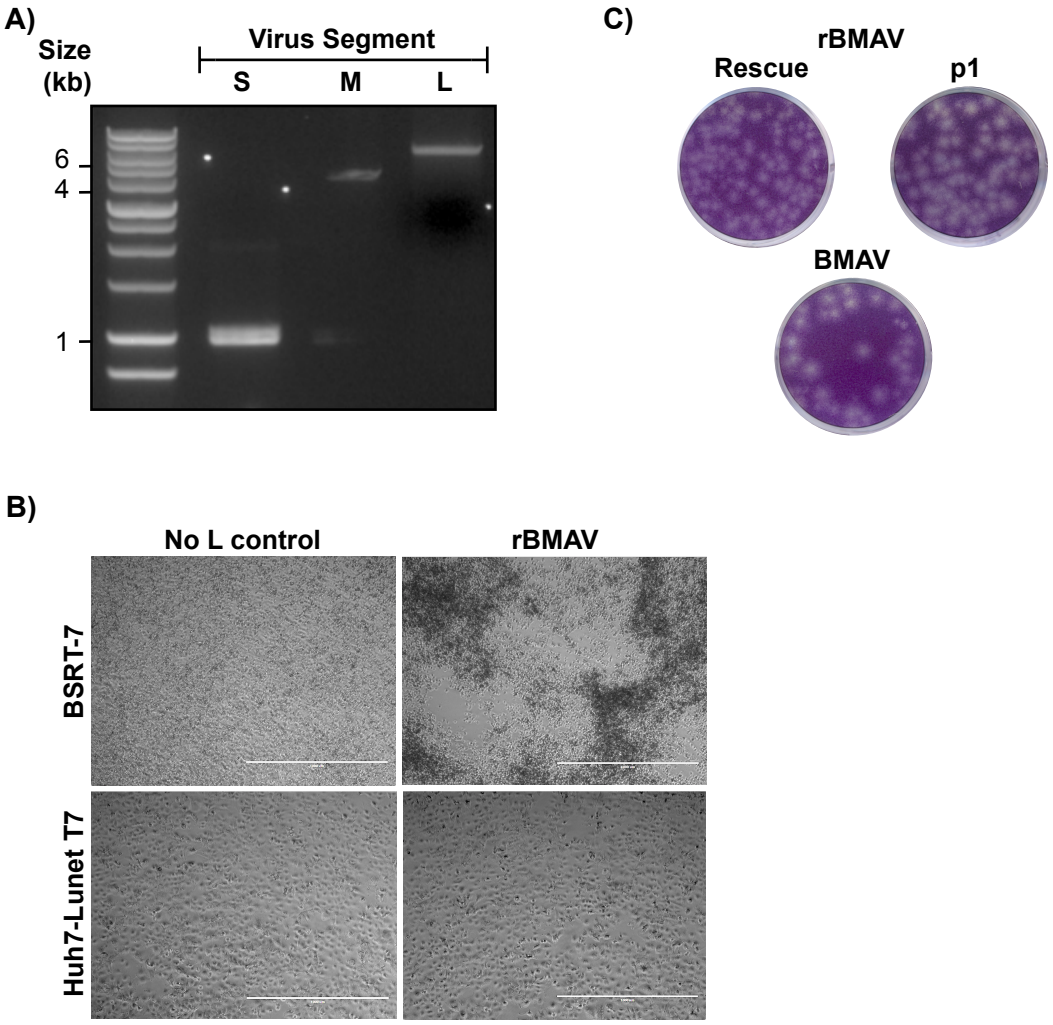


Figure 7-3: Batama Virus Rescue System

A) Batama virus (BMAV) genome segments were amplified by RT-PCR on virion RNA. PCR products were visualised by gel electrophoresis and gel-purified for restriction free cloning into the pTVT7 plasmid. **B)** pTVT7 plasmids encoding BMAV S, M and L cRNA were co-transfected into BSRT-7 and Huh7-Lunet T7 cells. Cytopathic effect (CPE) 7 days post transfection is shown, scale bars represent 1 000 μm. No L control, pTVT7-BMAVS and pTVT7-BMAVM only; recombinant BMAV (rBMAV), pTVT7-BMAVS, pTVT7-BMAVM and pTVT7-BMAVL. **C)** Plaque morphology of rescue supernatant (Rescue), Vero E6 passage 1 (p1) and wild type BMAV (BMAV) on Vero E6 cells 7 days post infection with a 0.6% (w/v) Avicel overlay.

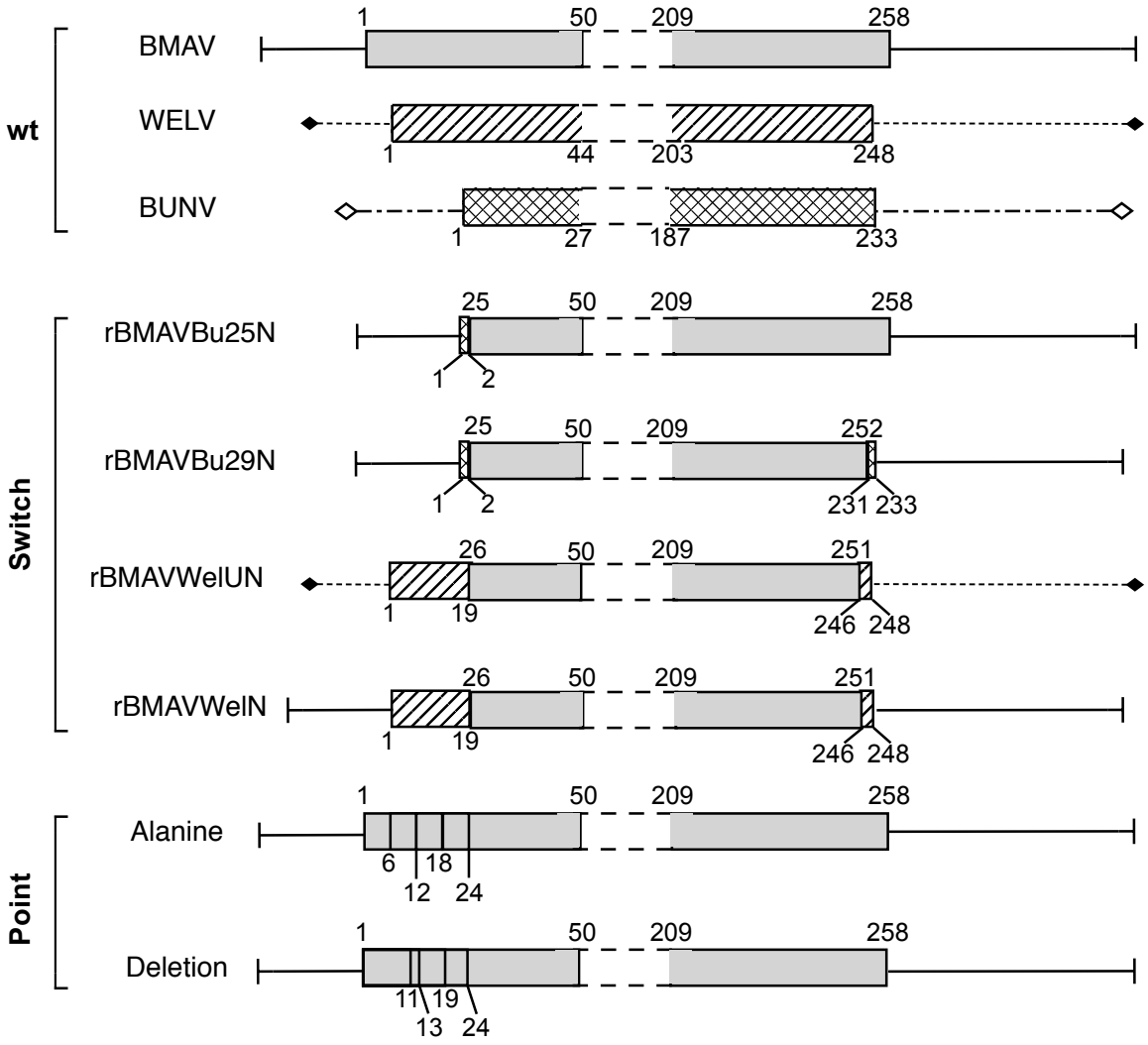


Figure 7-4: Batama Virus N Protein Constructs for Virus Rescue

Scaled schematic of cRNA S segment constructs for virus rescue. Wild type (wt) Batama virus (BMAV), Weldona virus (WELV) and Bunyamwera virus (BUNV, NC001927) S segments are shown for comparison. Recombinant viruses are grouped into those with BUNV or WELV N protein termini (Switch), and those with point mutations (Point). A complete list of recombinant viruses is detailed in Table 7-1 and S segment cRNA nucleotide sequences are in Appendix 5.

Table 7-1: Recombinant Batama Virus Titres

rBMAV	N protein Mutation	Rescue Titre	Working Stock Titre
rBMAV	-	2.75×10^6	1.70×10^7
Point Mutations			
rBMAVn6A	R6A	1.10×10^6	8.50×10^7
rBMAVn12A	S12A	6.00×10^4	3.50×10^7
rBMAVn18A	S18A	5.00×10^4	8.50×10^7
rBMAVn24A	D24A	5.00×10^5	5.50×10^7
rBMAVn2aA	R6A.S12A	3.85×10^6	9.50×10^6
rBMAVn2bA	R6A.S18A	2.73×10^6	7.00×10^7
rBMAVn2cA	R6A.D24A	1.10×10^7	5.95×10^7
rBMAVn2dA	S12A.S18A	5.00×10^6	1.05×10^8
rBMAVn2eA	S12A.D24A	6.50×10^6	3.85×10^7
rBMAVn2fA	S18A.D24A	9.00×10^5	6.00×10^7
rBMAVn3aA	R6A.S12A.S18A	8.00×10^6	3.73×10^7
rBMAVn3bA	R6A.S12A.D24A	7.50×10^6	1.60×10^7
rBMAVn3cA	R6A.S18A.D24A	2.30×10^7	3.50×10^7
rBMAVn3dA	S12A.S18A.D24A	2.80×10^6	9.50×10^7
rBMAVn4xA	R6A.S12A.S18A.D24A	8.00×10^6	6.50×10^7
rBMAVn11Δ	P11Δ	5.50×10^4	5.50×10^7
rBMAVn13Δ	I13Δ	6.00×10^2	-
rBMAVn19Δ	A19Δ	2.10×10^5	9.50×10^7
rBMAVn24Δ	D24Δ	9.75×10^3	3.50×10^7
rBMAVn2aΔ	P11Δ.I13Δ	-	-
rBMAVn2bΔ	P11Δ.A19Δ	1.80×10^6	5.30×10^7
rBMAVn2cΔ	A19Δ.D24Δ	2.75×10^6	6.20×10^7
rBMAVn2dΔ	I13Δ.A19Δ	-	-
rBMAVn2eΔ	I13Δ.D24Δ	-	-
rBMAVn2fΔ	P11Δ.D24Δ	2.15×10^6	5.45×10^7
rBMAVn3aΔ	P11Δ.I13Δ.A19Δ	-	-
rBMAVn3bΔ	P11Δ.I13Δ.D24Δ	-	-
rBMAVn3cΔ	P11Δ.A19Δ.D24Δ	1.55×10^6	3.00×10^7
rBMAVn3dΔ	I13Δ.A19Δ.D24Δ	-	-
rBMAVn4xΔ	P11Δ.I13Δ.A19Δ.D24Δ	-	-
Termini Switched			
rBMAVBu25N	BUNV N terminal	-	-
rBMAVBu29N	BUNV N and C termini	-	-
rBMAVWelN	WELV N and C termini	-	-
rBMAVWelUN	WELV N and C termini and UTRs	-	-

The virus titres (p.f.u ml^{-1}) of recombinant Batama viruses (rBMAV) are listed. N protein mutations include: point mutations, amino acid substitutions and deletions (Δ), which are numbered by the amino acid position in the wild type N protein; termini switched, recombinant viruses with N protein termini modified to match those of Bunyamwera virus (BUNV) or Weldona virus (WELV). Virus titre was determined by plaque assay on Vero E6 cells, (-) no plaques at 6 or 14 days post infection.

Firstly, recombinant 'switch' viruses with large mutations in the N protein termini, and UTRs for rBMAVWelUN, were tested (Figure 7-4, Table 7-1). All 'switch' recombinant virus rescues failed, with no CPE at 7 days post transfection and no plaques at 6 or 14 d p.i. (data not shown).

Virus rescues with point mutants were then tested (Figure 7-4, Table 7-1). The rescue supernatant from rBMAVn13Δ, containing a virus in which N protein aa I13 had been deleted, produced small pinprick plaques that diluted out on titration (Figure 7-5A). However, p1 and p2 stocks were negative by plaque assay (Figure 7-5B). This happened repeatedly, with separate plasmid stocks, and increasing the length of incubation to 14 days did not notably increase plaque size or improve visualisation. Passaging rescue supernatant on BSRT-7 cells, instead of Vero E6 cells, also failed to propagate rBMAVn13Δ further (data not shown).

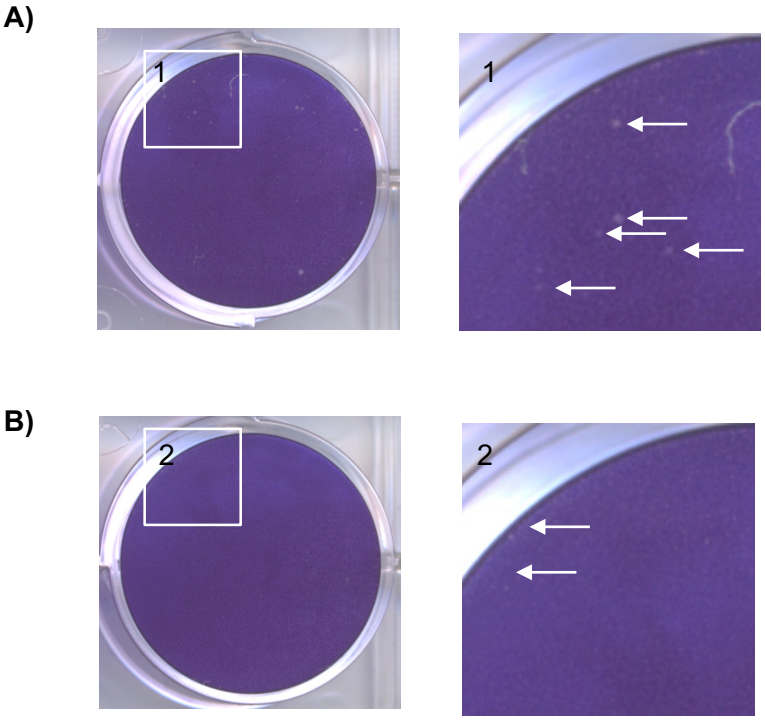


Figure 7-5: rBMAVn13Δ Plaque Morphology

A) rBMAVn13Δ rescue supernatant titrated on Vero E6 cells with a 0.6% (w/v) Avicel overlay, 7 days post infection. **B)** rBMAVn13Δ passage 1 supernatant titrated on Vero E6 cells with a 0.6% (w/v) Avicel overlay, 10 days post infection. Box 1 and 2 show an enlarged images of selected regions, white arrows highlight pin-prick clearings in the cell monolayer.

All other recombinant viruses with ≤ 4 point mutations showed strong CPE 6 – 7 days post transfection, formed clear plaques on virus titration and p1 stocks had titres ≥ 9.5 x 10^6 p.f.u ml^{-1} (Table 7-1, Figure 7-6, Figure 7-7).

Plaque morphology of recombinant viruses with alanine substitutions varied. rBMAVn2fA and rBMAVn3dA formed plaques comparable to rBMAV, whilst rBMAVn24A, rBMAVn3bA, rBMAVn3cA and rBMAVn4xA formed clear plaques that were slightly reduced in size at 1.5 mm - 3 mm (Figure 7-6). rBMAVn6A, rBMAVn12A, rBMAVn18A, rBMAVn2bA, rBMAVn2dA, rBMAVn2eA and rBMAVn3aA formed plaques 1 mm - 2 mm, with indistinct serrated edges. rBMAVn2aA formed the smallest plaques at ≤ 1 mm with incomplete clearing of the cell monolayer.

Recombinant viruses with point deletions also showed variations in plaque morphology. rBMAVn3cΔ formed large clear diffuse plaques whilst rVMAVn2bΔ formed small sharp plaques approximately 1.5 mm in size. (Figure 7-7). The remaining recombinant viruses with point deletions, rBMAVn11Δ, rBMAVn19Δ, rBMAVn24Δ, rBMAVn2cΔ, and rBMAVn2fΔ, formed plaques 1.5 mm - 3 mm in diameter (Figure 7-7).

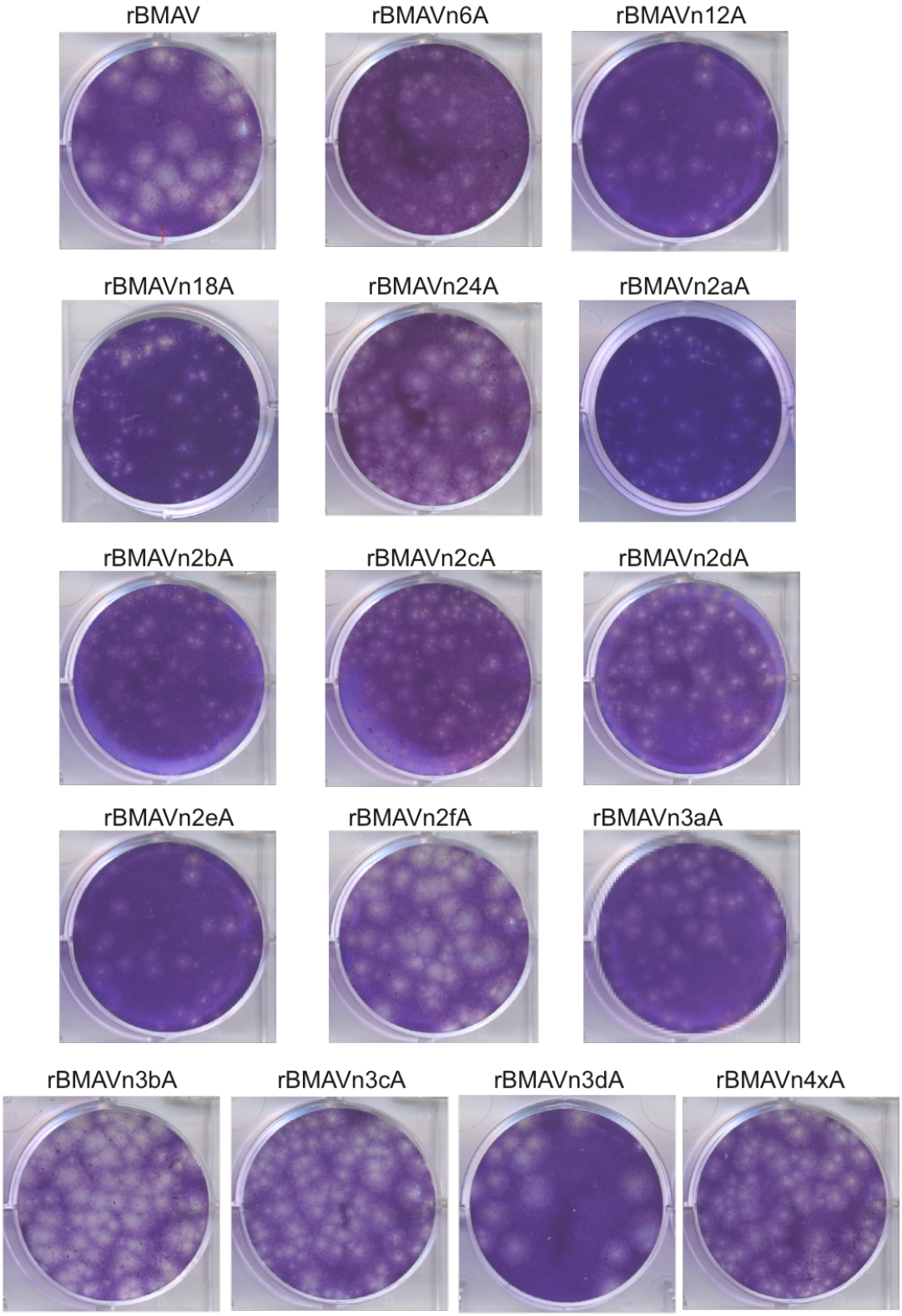


Figure 7-6: Plaque Morphology of Recombinant Viruses with Alanine Substitutions

Plaque morphology of passage 1 recombinant Batama viruses (BMAV) with alanine substitutions are shown. Viruses were titrated on Vero E6 cells with a 0.6% (w/v) Avicel overlay and fixed and stained 7 days post infection. Recombinant virus names are given, descriptions of mutations are listed in Table 7-1, and S segment sequences are included in Appendix 5.

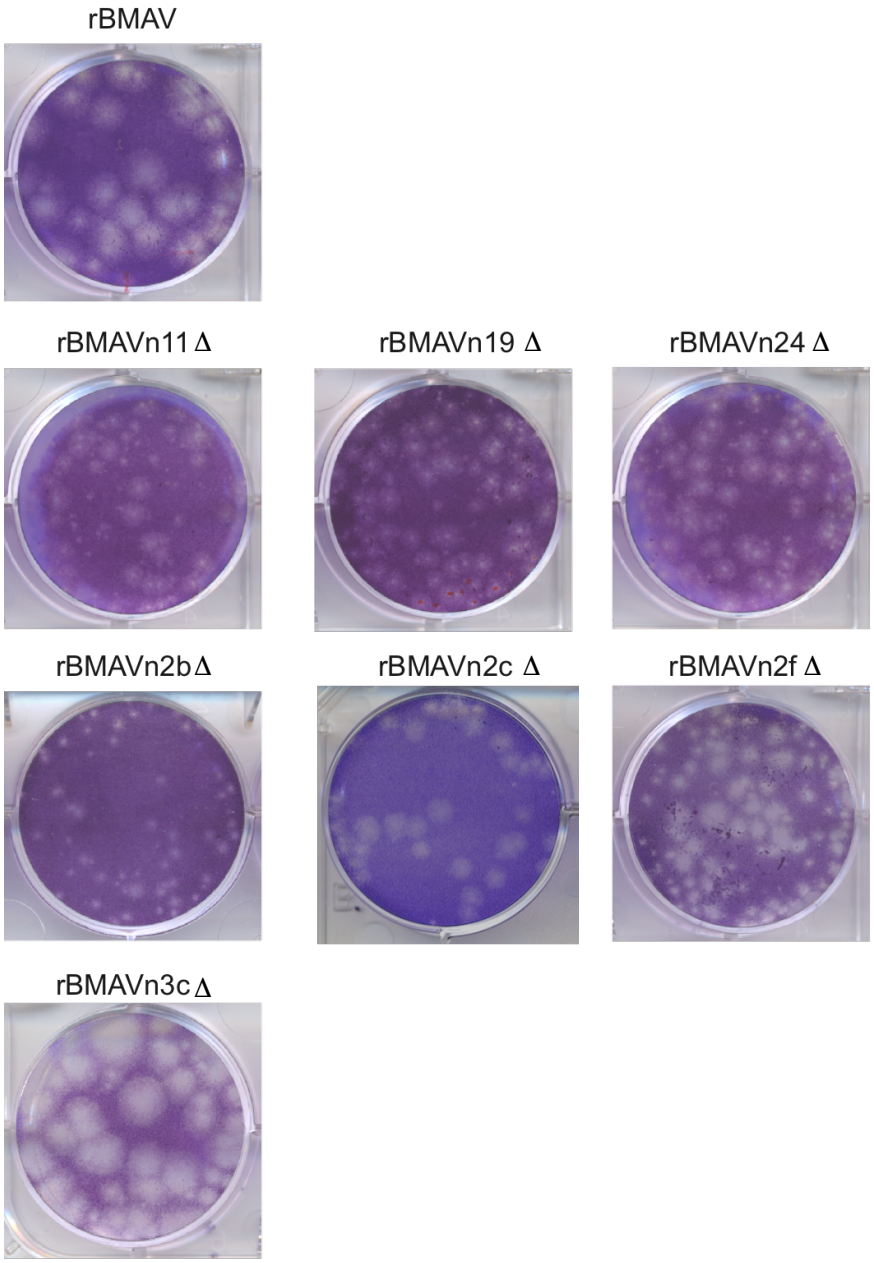


Figure 7-7: Plaque Morphology of Recombinant Viruses with Point Deletions
Plaque morphology of passage 1 recombinant Batama viruses (BMAV) with point deletions are shown. Viruses were titrated on Vero E6 cells with a 0.6% (w/v) Avicel overlay and fixed and stained 7 days post infection. Recombinant virus names are given, descriptions of mutations are listed in Table 7-1, and complete S segment sequences are included in Appendix 5.

7.3.5 Growth Kinetics of Recombinant Batama Viruses

To further characterise the recombinant viruses, growth kinetics in IFN-competent A549 and IFN incompetent Vero E6 cell lines were determined. rBMAV displayed similar growth kinetics as BMAV in both A549 and Vero E6 cells, growing to 6.5 x 10^4 p.f.u ml^{-1} and 8.5 x 10^6 p.f.u ml^{-1} at 48 h p.i. (Figure 7-8).

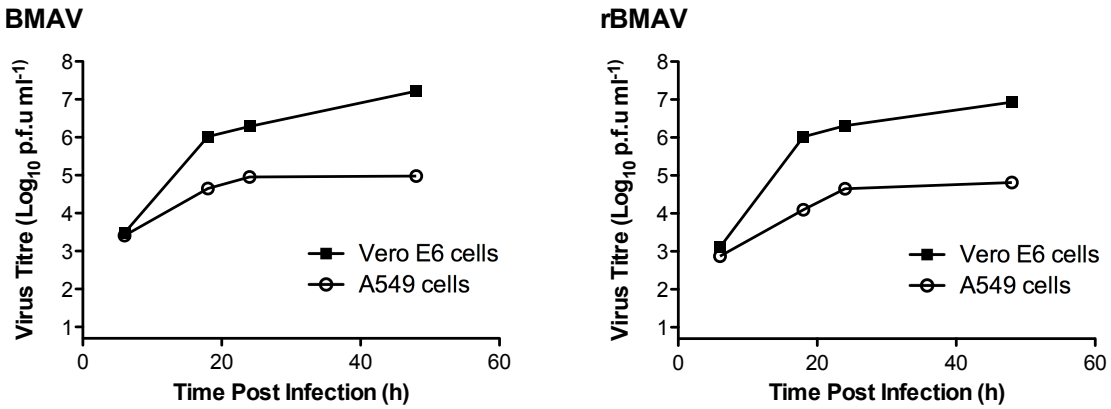


Figure 7-8: Growth Kinetics of Wild Type Batama Viruses
A549 and Vero E6 cell lines were infected at multiplicity of infection 0.01 with wild type Batama virus (BMAV) and recombinant wild type BMAV (rBMAV). Clarified culture supernatants were harvested at specified hours (h) post infection and virus titre determined by plaque assay on Vero E6 cells.

At early time points recombinant viruses with single point mutations demonstrated delayed growth, with lower titres than rBMAV in both A549 and Vero E6 cells. However, at 48 h p.i. titres for all viruses, except rBMAVn6A and rBMAVn11Δ, were within 0.5 log of rBMAV in both Vero E6 and A549 cells (Figure 7-9). At 48 h p.i rBMAVn6A and rBMAVn11Δ virus titres were > 1 log lower than rBMAV in Vero E6 cells, at 5.0 x 10^5 p.f.u ml^{-1} and 2.1 x 10^5 p.f.u ml^{-1}, respectively. At 48 h p.i. in A549 cells rBMAVn6A and rBMAVn11Δ virus titres were 0.5 log lower than rBMAV titres (Figure 7-9).

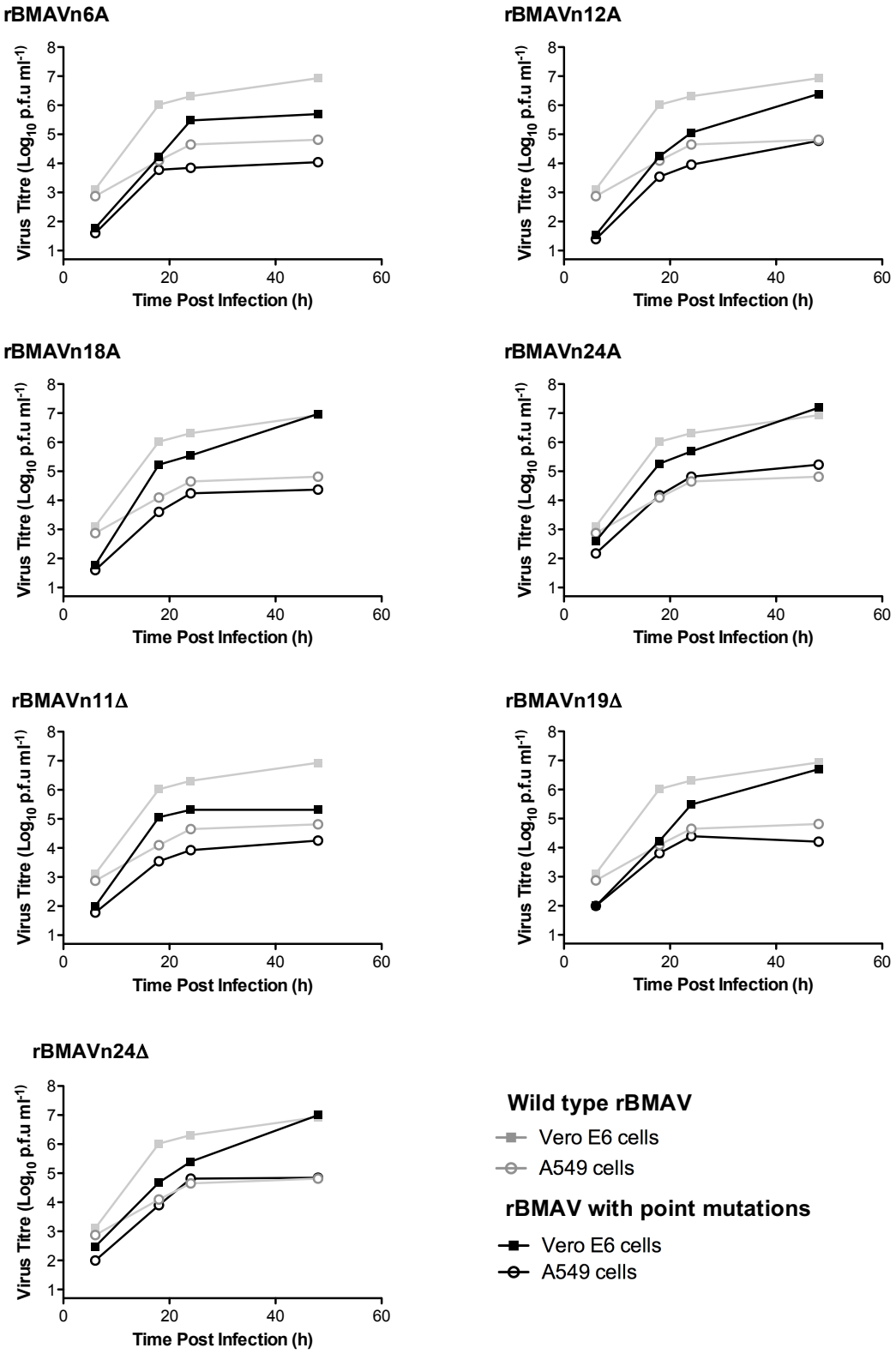


Figure 7-9: Growth Kinetics of Recombinant Viruses with Single Point Mutations
A549 and Vero E6 cell lines were infected at multiplicity of infection 0.01. Clarified culture supernatants were harvested at specified hours (h) post infection and virus titre determined by plaque assay on Vero E6 cells. Descriptions of recombinant viruses are listed in Table 7-1.

Recombinant viruses with 2 alanine substitutions generally exhibited greater attenuation in virus growth than single point mutants (Figure 7-10, Figure 7-11). rBMAVn2aA and rBMAVn2fA showed the largest reductions, with a > 1 log decrease in both Vero E6 and A549 cells at 48 h p.i. compared with rBMAV (Figure 7-10). Whereas rBMAVn2cA and rBMAVn2dA had lower titres in both Vero E6 and A549 cells at early time points, at 48 h p.i. only titres in A549 cells remained ≥ 0.5 log lower than rBMAV (Figure 7-10). rBMAVn2bA and rBMAVn2eA virus titres were only slightly suppressed at early time points and equalled or exceeded titres of rBMAV in Vero E6 cells at 48 h p.i., although titres remained approximately 0.4 log lower in A549 cells at 48 h p.i. (Figure 7-10).

Recombinant viruses with 3 or 4 alanine substitutions, excluding rBMAVn3cA, showed evidence of growth attenuation with lower titres in Vero E6 and A549 cells < 48 h p.i. (Figure 7-11). rBMAVn3aA had the greatest attenuation with a 1.4 log reduction in virus titre in A549 cells at 48 h p.i. At early time points (< 48 h p.i.) in Vero E6 cells rBMAVn3aA virus titre was also > 1 log lower than rBMAV (Figure 7-11). rBMAVn3bA also showed evidence of attenuation with a ≥ 1 log drop in virus titre at early time points in both Vero E6 and A549 cells and a 0.7 log reduction in A549 cells at 48 h p.i. (Figure 7-11). rBMAVn3dA and rBMAVn4xA had evidence of attenuation early in infection, although at 48 h p.i virus titres were within 0.5 log of rBMAV in both Vero E6 and A549 cells (Figure 7-11).

Interestingly, recombinant viruses with 2 and 3 point deletions had comparable titres to rBMAV in Vero E6 cells at 48 h p.i. At early time points (< 48 h p.i.) rBMAVn2bΔ, rBMAVn2fΔ and rBMAVn3cΔ showed attenuation with ≥ 1 log reduction in virus titre (Figure 7-12). In A549 cells only rBMAVn2fΔ, which contains deletions at P11 and D24, showed reduced growth at 48 h p.i. with a 0.9 log reduction in virus titre compared with rBMAV, although other deletant viruses showed attenuation at earlier time points (Figure 7-12).

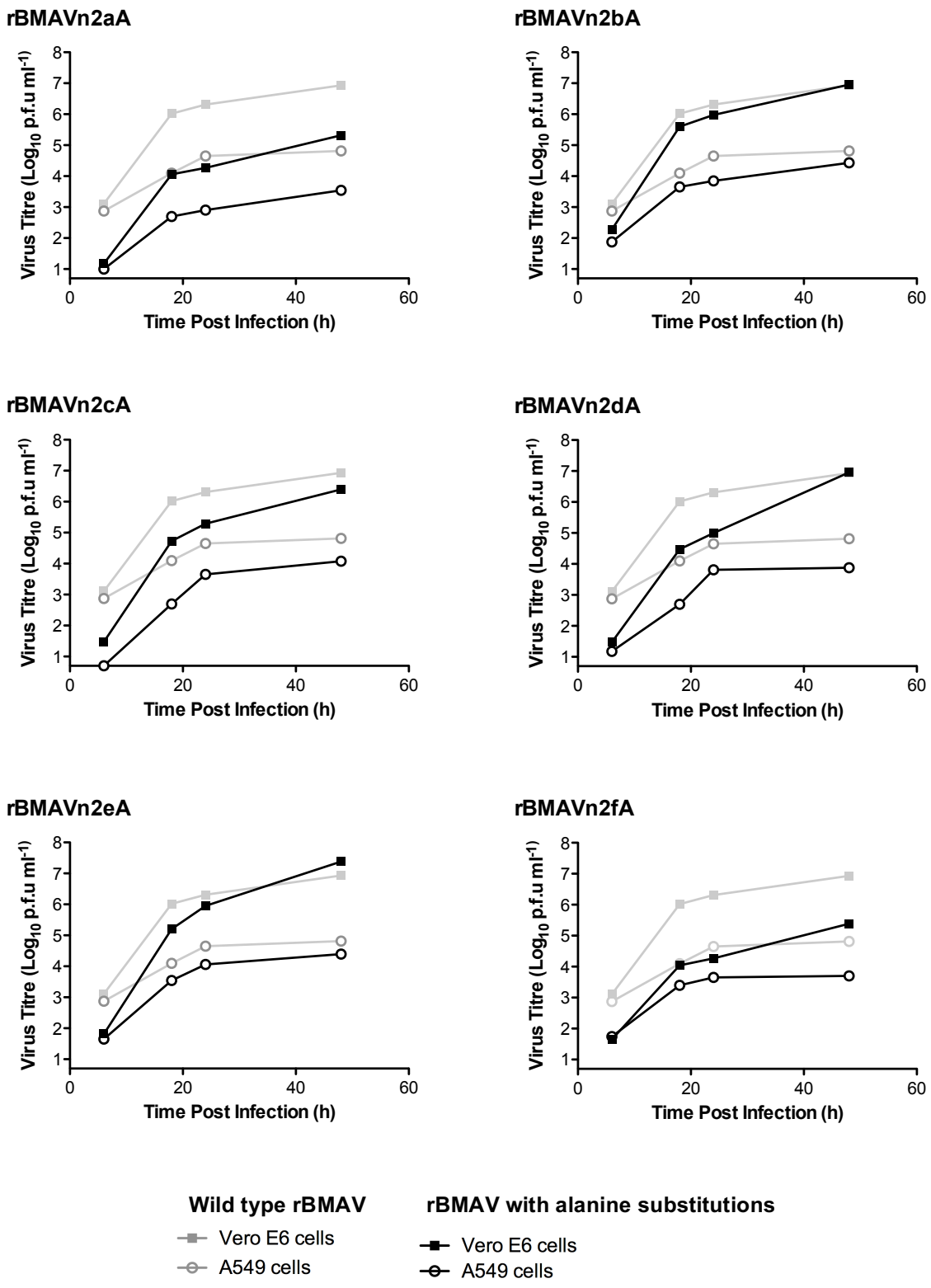


Figure 7-10: Growth Kinetics of Recombinant Viruses with Double Alanine Substitutions

A549 and Vero E6 cell lines were infected at multiplicity of infection 0.01. Clarified culture supernatants were harvested at specified hours (h) post infection and virus titre determined by plaque assay on Vero E6 cells. Descriptions of recombinant viruses are listed in Table 7-1.

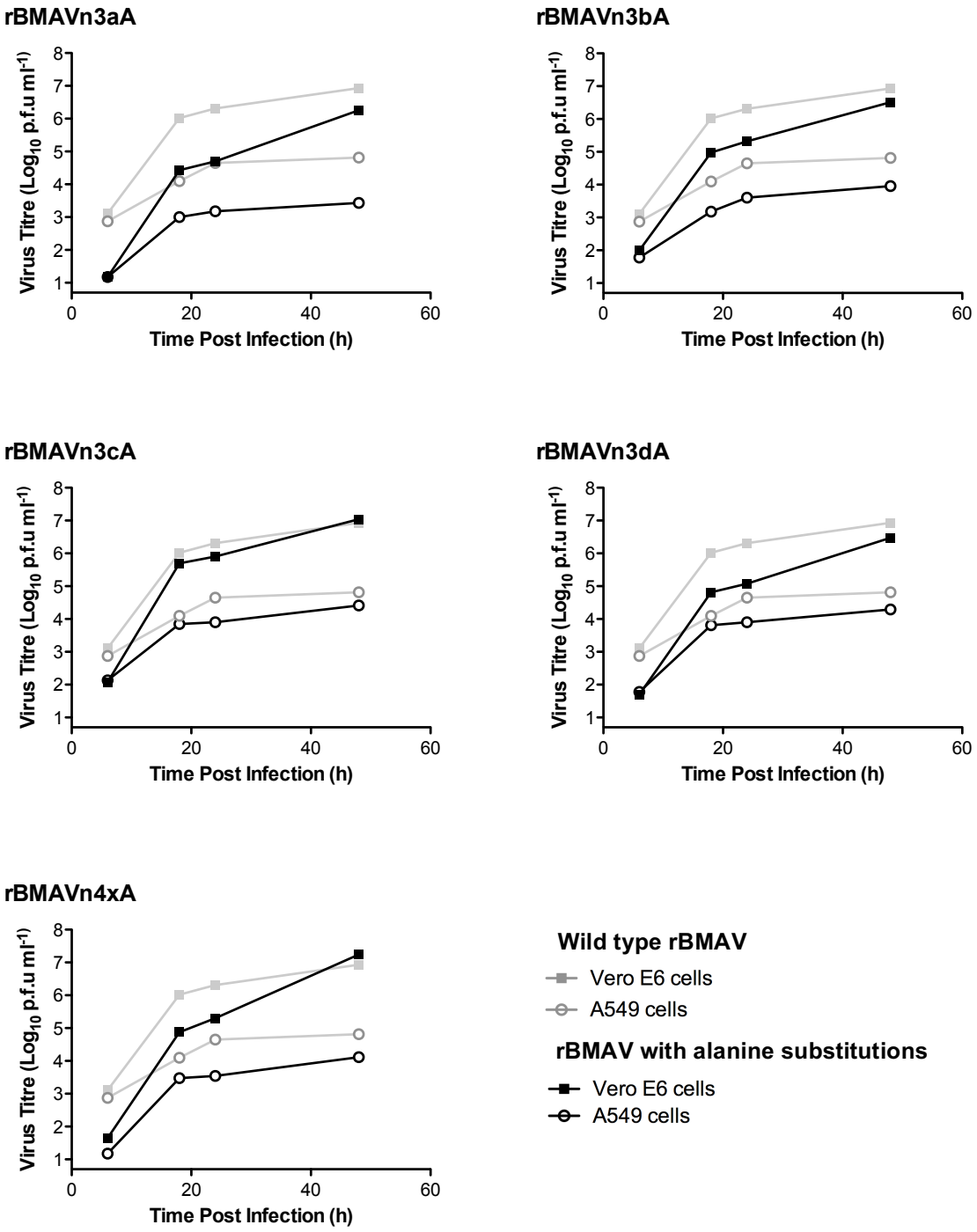


Figure 7-11: Growth Kinetics of Recombinant Viruses with Triple and Quadruple Alanine Substitutions

A549 and Vero E6 cell lines were infected at multiplicity of infection 0.01. Clarified culture supernatants were harvested at specified hours post infection and virus titre determined by plaque assay on Vero E6 cells. Descriptions of recombinant viruses are listed in Table 7-1.

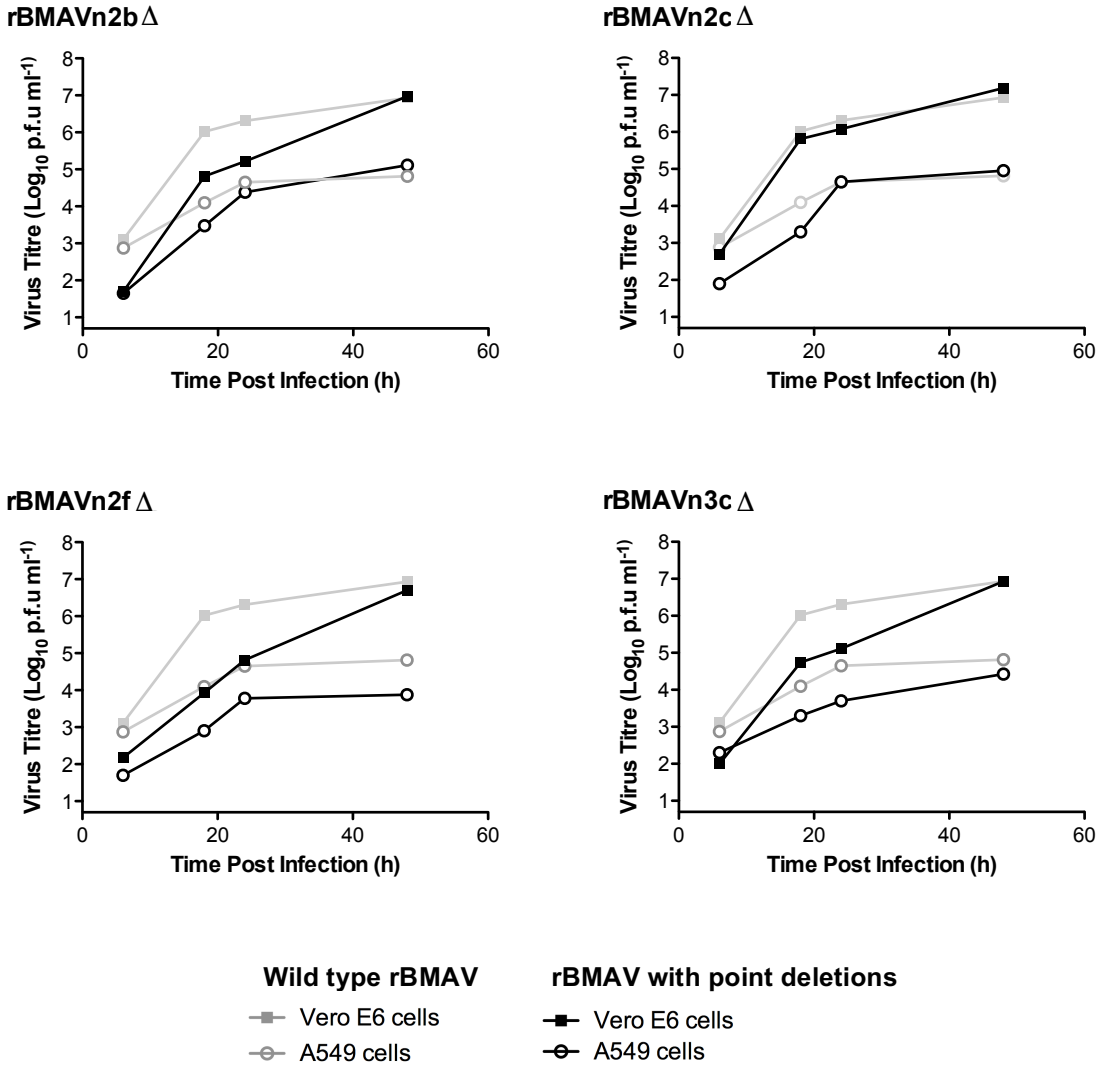


Figure 7-12: Growth Kinetics of Recombinant Viruses with Multiple Point Deletions

A549 and Vero E6 cell lines were infected at multiplicity of infection 0.01. Clarified culture supernatants were harvested at specified hours (h) post infection and virus titre determined by plaque assay on Vero E6 cells. Descriptions of recombinant viruses are listed in Table 7-1.

7.3.6 Interferon Induction by Recombinant Batama Viruses

Recombinant viruses were tested for the induction of biological IFN and the IFN-β promoter during virus infection of A549 cells. The level of biological IFN and IFN-β promoter activity induced by rBMAV infection was comparable to BMAV, at approximately 16 rIFN U ml^{-1} and 5.4 RLU respectively (Figure 7-13).

Recombinant viruses with N protein mutations induced higher levels of biological IFN than rBMAV and BMAV *in vitro* (Figure 7-13A). The largest rise in IFN production (> 4-fold) occurred in rBMAVn2aA, rBMAVn3aA and all recombinants with ≥ 2 point deletions (Figure 7-13A). In agreement with the biological IFN assay rBMAVn2aA and rBMAVn3aA induced high levels of IFN-β promoter activity, ≥ 13.5 RLU (Figure 7-13B). The only other recombinant viruses with higher activity were rBMAVn3cA, rBMAVn4xA, and 2 deletant viruses rBMAVn24Δ and rBMAVn2fΔ. Interestingly rBMAVn2fΔ induced approximately 18.7 RLU, which was higher than rBUNdelNSs2 which induced only 17.2 RLU (Figure 7-13B).

To aid interpretation of results plaque size, growth kinetics in A549 cells and induction of biological IFN and IFN-β promoter activity have been scored relative to rBMAV and summarised in Table 7-2.

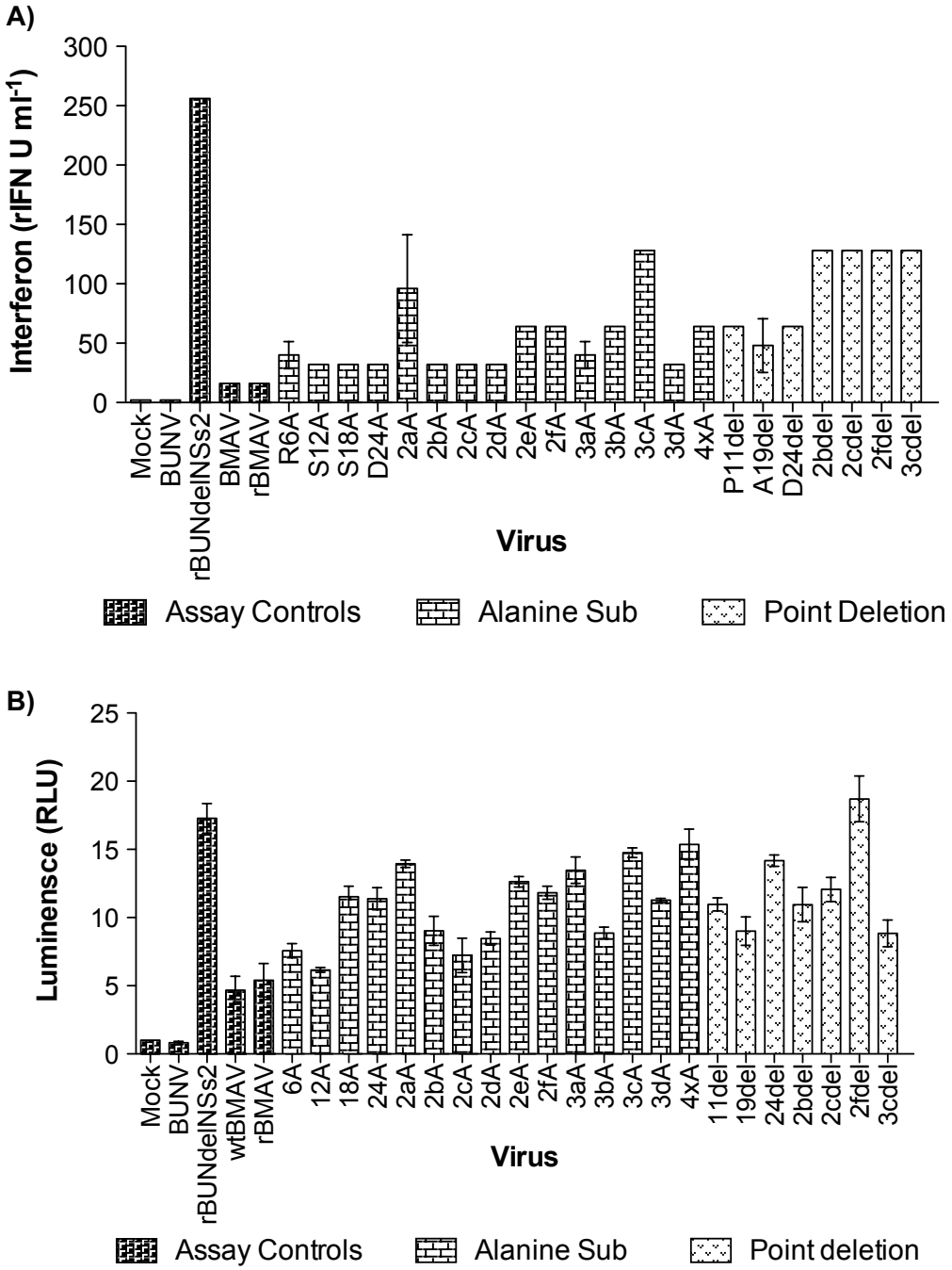


Figure 7-13: Interferon Induction by Recombinant Batama Viruses

Descriptions of recombinant viruses are listed in Table 7-1. **A)** Relative levels of biological interferon (rIFN U ml^{-1}) produced by A549 cells 48 hours post induction with universal IFN (U ml^{-1}) or 48 hours post infection (h p.i.) at multiplicity of infection (MOI) 1. Mean ± standard deviation of triplicate experiments is shown. **B)** A549 cells were co-transfected with IFN-β firefly and CMV Renilla reporter plasmids pIFΔ(-125)lucter and phRL-CMV, respectively, and infected at MOI 1. At 18 h p.i. the cells were assayed for luciferase activity (relative light units, RLU). Mean ± standard deviation of triplicate experiments is shown.

Table 7-2: Biological Characteristics of Recombinant Batama Viruses

rBMAV	Plaque Size in Vero E6 cells	Reduced Titres in A549 cells	Biological IFN Production	IFN-β Promoter Activity
rBMAV	2 – 4 mm	1.25 x 10^4 p.f.u ml^{-1}	16 rIFN U ml^{-1}	5.4 RLU
rBMAVn6A	++	+	+	-
rBMAVn12A	++	+	+	-
rBMAVn18A	++	+	+	++
rBMAVn24A	+	-	+	++
rBMAVn2aA	+++	+++	++	++
rBMAVn2bA	+++	+	+	+
rBMAVn2cA	++	+++	+	-
rBMAVn2dA	++	+++	+	+
rBMAVn2eA	+/++	+	++	++
rBMAVn2fA	-	++	++	++
rBMAVn3aA	+	+++	+	++
rBMAVn3bA	+	++	++	+
rBMAVn3cA	+	-	+++	++
rBMAVn3dA	-	-	+	++
rBMAVn4xA	+	++	++	+++
rBMAVn11Δ	+	++	++	++
rBMAVn19Δ	+	-	+	+
rBMAVn24Δ	+	-	++	++
rBMAVn2bΔ	++	++	+++	++
rBMAVn2cΔ	-	++	+++	++
rBMAVn2fΔ	+	+++	+++	+++
rBMAVn3cΔ	-	++	+++	+

- Plaque morphology of passage 1 isolates on Vero E6 cells: -, comparable to rBMAV; 2 mm – 4 mm; +, 2 mm – 3 mm; ++, 1 mm – 2 mm; +++, ≤ 1 mm.
- Virus titres in A549 cells 18 hours post infection, multiplicity of infection 0.01, compared with rBMAV: -, ± 0.5 log; +, 0.5 – 0.75 log lower; ++, 0.75 -1 log lower; +++, > 1 log lower.
- Induction of biological IFN during virus infection compared with rBMAV: -, < 2-fold difference; +, 2-fold higher; ++, 4-fold higher; +++, 8-fold higher.
- IFN-β promoter activity, relative light units (RLU): -, ≤ 8 RLU; +, > 8 RLU; ++, > 10 RLU; +++, > 15 RLU.

7.4 Discussion

Bunyavirus N proteins play a critical role in virus replication encapsidating nascent cRNA and vRNA to form the RNP, which is the template for genome transcription and replication, and interacting with virus glycoproteins and the viral polymerase (Elliott, 2014). The crystal structure of orthobunyavirus N proteins from viruses in the Bunyamwera, Simbu and California serogroups have been determined both in the presence and absence of RNA (Ariza *et al.*, 2013; Dong *et al.*, 2013a; Dong *et al.*, 2013b; Niu *et al.*, 2013; Reguera *et al.*, 2013). Strikingly, despite low sequence identity (39.7% - 43.2% aa identity) structural confirmation is highly conserved with the presence of a unique fold that has not been seen in the N proteins of other negative sense viruses (Li *et al.*, 2013a). In the presence of RNA the N protein assembles into tetramers with RNA binding occurring within the positively charged inner ring (Ariza *et al.*, 2013; Dong *et al.*, 2013a; Dong, *et al.*, 2013b; Niu *et al.*, 2013; Reguera *et al.*, 2013). Oligomerisation of the tetramer occurs via protruding N and C terminal arms; the C terminal arm binds the hydrophobic cavity in the neighbouring N molecule, whilst the N terminal arm extends away from the inner ring to interact with the adjacent N monomer, stabilising the tetramer and shielding viral RNA. Access to viral RNA for virus replication is then thought to occur via a hinge like mechanism, with assembly of the initiation complex triggering further release of viral RNA (Ariza *et al.*, 2013; Dong *et al.*, 2013a; Dong *et al.*, 2013b; Niu *et al.*, 2013; Reguera *et al.*, 2013). Given the critical role the N terminal arm plays in N-N oligomerisation and RNA binding it was hypothesised that the larger N proteins found in Tete serogroup viruses may possess a novel mechanism of RNA binding, and possibly N-N oligomerisation, as the additional amino acids align to the N-terminal arm of the protein (Figure 7-2A, Figure 7-2B).

7.4.1 Structural Modelling of Batama Virus N Protein

BMAV N protein shares ≤ 35% aa sequence identity with the N proteins of non-Tete serogroup orthobunyaviruses. Despite low sequence identity a structural model with high confidence and high homology to other orthobunyavirus N proteins was generated (Figure 7-2B). However, the model does not cover the first 25 aa of BMAV N protein. The N terminal arm of orthobunyavirus N proteins has been shown to be disordered in

RNA-free solutions; however, upon the addition of RNA N proteins oligomerise and the previously disordered N terminal arm interacts with RNA and the neighbouring N protomer to form an ordered structure that stabilises the oligomer and shields RNA from the external environment. The N terminal arm of Tete serogroup N proteins are predicted to be highly disordered and it would therefore be of great interest to determine the role of these aa in RNA binding and RNP formation (Figure 7-2C). To this end the BMAV N protein ORF was cloned into the pEHISTEV expression vector for structural studies by Professor Changjiang Dong at The University of East Anglia, UK, whilst a reverse genetics system was developed to allow functional studies of recombinant viruses with mutations targeting the N terminal arm of the N protein.

7.4.2 Batama virus Reverse Genetics and Recombinant Virus Rescue

Utilising the complete genome sequence of BMAV, obtained in the course of my studies, a 3 plasmid reverse genetics system was successfully established. However, recombinant viruses with large deletions in the N and C terminal arms could not be rescued. This is perhaps not surprising given the crucial role these domains play in RNP formation, although rescue of a recombinant BUNV encoding an additional 10 aa at the C terminal arm (BUNV ts19) has been reported (Ariza *et al*., 2013).

The only other recombinant virus in this study that could not be propagated was rBMAVn13Δ, which contained a single point deletion at I13. I13 is conserved within sequenced Tete serogroup viruses, namely BMAV, TETEV, MTRV, TSUV and WELV, although it is not present in I612045 virus. As discussed in Chapter 5, the unclassified, I612045 virus shares high sequence identity to Tete serogroup viruses and falls within the Tete serogroup clade on phylogenetic analysis (Figure 5-6). Determining the structure of BMAV N protein may shed light on the critical role I13 has in virus replication. Eifan *et al*., (2009) carried out targeted mutagenesis of conserved aa in BUNV N protein and were unable to rescue a virus containing a F17A point mutation. Structural studies subsequently mapped F17 to the RNA binding groove and it is hypothesised that mutating this residue would decrease RNA binding affinity (Ariza *et al.* 2013). Interestingly recombinant viruses which contained single point deletions A19 and D24, which are conserved in all Tete serogroup viruses and

I612045, showed little attenuation in either plaque morphology or growth kinetics. Thus, the conservation of single amino acids does not always correlate with a critical role in virus replication *in vitro*.

7.4.3 Recombinant Viruses Growth Kinetics and Plaque Morphology

All rescued recombinant viruses, at p1, had comparable titres to BMAV and rBMAV at $\geq$ 9.5 x 10^6 p.f.u ml^{-1} (Table 7-1). However, the titres of rescue supernatant for rBMAVn12A, rBMAVn18A, rBMAVn24A, rBMAVn11Δ, rBMAVn19Δ and rBMAVn24Δ were $\leq$ 5.0 x 10^5 p.f.u ml^{-1}, with p1 titres increasing by > 2 logs. In comparison, the virus titre of all other recombinant viruses increased by only 1 log between rescue supernatants and p1 (Table 7-1). In this study only the N protein ORF of p1 recombinant viruses was confirmed by sequencing and mutations in the UTRs, M or L segments will not have been detected. It would therefore be prudent to carry out complete genome sequencing of the recombinant viruses to ensure that observed phenotypes are due to the intended mutations only and not mutations acquired during virus replication, especially in viruses that underwent a large rise in virus titre in 1 passage.

Recombinant viruses generally showed attenuation in plaque morphology with respect to BMAV and rBMAV, with smaller or more opaque plaques than wild type virus (Figure 7-6, Figure 7-7). The exception, however, was rBMAVn3cΔ which formed large clear plaques that were bigger than those of BMAV or rBMAV (Figure 7-7). rBMAVn3cΔ contains 3 point deletions, P11, A19 and D24, whilst recombinant viruses with only 1 or 2 of these mutations formed smaller slightly opaque plaques. Plaque morphology of recombinant viruses with multiple point mutations can therefore not be predicted by the behaviour of recombinant viruses containing the corresponding single point mutations. Plaque size also failed to correlate with virus titre as although rBMAVn2aA formed the smallest plaques at $\leq$ 1 mm in size and had a virus titre of 9.5 x 10^6 p.f.u ml^{-1}, and rBMAVn3cΔ, which formed the largest plaques had a titre of 3.0 x 10^7 p.f.u ml^{-1}, rBMAVn2dA, which formed plaques < 2 mm in size had a titre of 1.05 x 10^8 p.f.u ml^{-1}.

Additionally, some recombinant viruses gave mixed plaque morphology (e.g. rBMAVn2fΔ), a phenomenon that has been previously reported in the literature (Figure 7-7). Eifan *et al* (2009) rescued a panel of recombinant Bunyamwera viruses with N protein point mutations, and plaques of mixed size were repeatedly found on virus titration despite plaque purification. It was postulated that mutations in BUNV N protein may influence polymerase fidelity. This warrants further investigation, and complete genome sequencing of virus inoculum and isolated plaques over virus passage would help address this hypothesis.

Looking in more detail at the growth kinetics of recombinant viruses in Vero E6 cells it is notable that although titres of p1 isolates were comparable to those of BMAV and rBMAV titres of all except rBMAVn2bA and rBMAVn2cdel were reduced early in infection. The greatest reductions occurred in rBMAVn11Δ, rBMAVn2aA and rBMAVn2fA infections with ≥ 1.4 log reduction in virus titre at 48 h p.i. compared with rBMAV (Figure 7-9, Figure 7-10). In IFN-competent A549 cells BMAV and rBMAV grew to lower titres than in IFN-deficient Vero E6 cells (Figure 7-9). In this study the growth kinetics of recombinant viruses were also attenuated in A549 cells when compared to growth in Vero E6 cells. Furthermore, all except rBMAVn24A and rBMAVn2cΔ had lower titres than rBMAV (Figure 7-10, Figure 7-11, Figure 7-12, Figure 7-13). The largest decrease (> 0.9 log at 48 h p.i.) was observed in recombinant viruses with ≥ 2 point mutations, namely rBMAVn2aA, rBMAVn2dA, rBMAVn2fA, rBMAVn3aA, rBMAVn3bA and rBMAVn2fΔ (Figure 7-11, Figure 7-12).

The N protein and RNP plays a crucial role in virus infection, and subtle modifications in N protein structure may lead to sub-optimal primary transcription by the virion associated RdRp. Structural information would greatly aid the interpretation of biological phenotypes, although in the absence of this replicon assays with agarose-urea gel electrophoresis could be used to investigate and differentiate between disruption to transcription or genome replication (Barr and Wertz, 2005; Ariza *et al.*, 2013). Moreover, protein-protein interactions, including N-N oligomerisation and N-L interactions could be investigated using chemical cross-linking and co-immunoprecipitation assays (Eifan and Elliott, 2009). These experiments should be conducted only on single point mutants as the combined effect of multiple mutations cannot be predicted without structural information. To fully map the role of each amino

acid additional mutants with single point mutations could easily be constructed through QuickChange® PCR.

7.4.4 Recombinant Viruses and the Type I IFN System

BMAV and other non-NSs Tete serogroup viruses induce very low levels of IFN during virus infection *in vitro*, significantly lower than rBUNdelNSs2 and other non-NSs viruses (Table 6-2). However, there was no evidence of active antagonism of the type I IFN system, leading to the hypothesis that Tete serogroup viruses may be low inducers rather than active antagonisers of the IFN response (Figure 6-8, Figure 7-1). Recombinant viruses were therefore investigated for their induction of biological IFN and activation of the IFN-β promoter during virus infection of A549 cells. There was a general trend with the level of IFN induction increasing as the number of N protein point mutations increased, with the highest induction by rBMAVn4xA and rBMAVn3cΔ (Table 7-2, Figure 7-13). This would agree with the hypothesis that disrupting RNA binding and or RNP formation by the N terminal arm of the N protein increases the exposure of viral RNA to the host cell, leading to increased induction of the type I IFN system. Transfecting purified RNPs and measuring the induction of type I IFN would help address this theory, whilst sensitivity of RNA-bound in RNPs to RNase treatment may also help assess exposure of RNA to the external environment (Ariza *et al*., 2013; Li *et al*., 2013a; Reguera *et al*., 2013).

7.4.5 Concluding Remarks

Altering BMAV N protein sequence and presumably structure through the introduction of point mutations in the N terminal extension led to attenuation in growth kinetics and increased induction of type I IFN. In lieu of structural data a number of experimental methods, as discussed above, could be employed to further investigate the role of this protein in virus replication and the function of the extended N terminal domain. However, it is hoped that the crystal structure of BMAV N protein will be available soon, allowing a more targeted approach to future experiments.

Chapter 8: Antagonism by Interferon Stimulated Genes

8.1 Introduction

The type I IFN response is a key anti-viral component of the innate immune response, stimulating the expression of hundreds of ISGs. ISG products known to have anti-bunyavirus activity include MxA, the 2'-5' OAS system and viperin (Frese *et al.*, 1996; Kanerva *et al.,* 1996; Hefti *et al.*, 1999; Andersson *et al.*, 2004; Bridgen *et al.*, 2004; Carlton-Smith and Elliott, 2012). The activity of these proteins has been widely studied and varies across the *Bunyaviridae* family. For example, MxA binds LACV N protein forming copolymers that localise to the perinuclear compartment, reducing the availability of N and inhibiting virus replication (Haller and Kochs, 2002; Kochs, 2002; Reichelt *et al.,* 2004). Yet MxA has little activity against BUNV, the type species of the *Orthobunyavirus* genus and *Bunyaviridae* family (Bridgen *et al.,* 2004). An ISG recently identified as possibly having anti-BUNV activity is IFN-induced protein 44 (IFI44) (Carlton-Smith and Elliott, 2012).

IFI44, also known as microtubule aggregate protein 44 (MTAP44), is highly conserved amongst mammals (Hallen *et al.,* 2007). A previous study utilising Flp-In^{TM} T-REx^{TM} 293 cells with tetracycline inducible expression of individual ISGs identified IFI44 as inhibiting BUNV replication in a virus release assay (Carlton-Smith and Elliott, 2012). However, very little is known about the structure or function of IFI44. First identified in 1990 as a protein physically associated with microtubule aggregates in the liver of chimpanzees infected with hepatitis C virus (HCV) it was not until 1994 that IFI44 was characterised as an ISG (Honda *et al.,* 1990; Kitamura *et al.,* 1994). Kitamura *et al.,* (1994) identified 2 ISREs in the 5' flanking region and demonstrated that expression was induced by IFNα but not IFNγ in a PLC/PRF/5 human hepatoma-derived cell line. IFI44 has since been shown to be significantly upregulated in a number of clinical conditions including infections and inflammatory autoimmune conditions, such as systemic lupus erythematosus (SLE) and Sjogrens syndrome (Nzeusseu Toukap *et al.*, 2007; Wildenberg *et al.,* 2008; Bochkov *et al.*, 2010; Farina *et al.*, 2010). In 2007 IFI44 was identified as a cytoplasmic protein with a GTP binding site, leading to the hypothesis that IFI44 may sequester intracellular GTP, inhibiting extracellular signal-regulated kinases (ERK) and leading to cell cycle arrest and establishment of an antiproliferative state (Hallen *et al.*, 2007).

In addition to IFI44, 2 groups of membrane bound ISGs, bone marrow stromal antigen 2 (BST2) and the IFITM family (IFN-induced transmembrane protein), have been shown to have inhibitory roles in the replication of enveloped RNA viruses. IFITM1, IFITM2 and IFITM3 are transmembrane proteins that are upregulated by type I and type II IFN (Friedman *et al.*, 1984; Jaffe *et al.*, 1989; Lewin *et al.*, 1991). The proteins have been shown to have wide ranging anti-viral activities, restricting the replication of influenza A virus (IAV), SARS coronavirus (CoV), Ebola, Dengue, HIV-1 and the bunyaviruses ANDV, HTNV, LACV and RVFV (Brass *et al.*, 2009; Huang *et al.,* 2011; Lu *et al.,* 2011; Mudhasani *et al.*, 2013). IFITM proteins also interact with VAPA (vesicle-membrane-protein-associated protein A), antagonising the VAPA-OSBP (VAPA-oxysterol-binding protein) interaction, which leads to the accumulation of cholesterol in late endosomal compartments (Amini-Bavil-Olyaee *et al.,* 2013). Disrupted cholesterol homeostasis and increased membrane rigidity inhibits membrane fusion, leading to the retention of virus particles in endosomal compartments, thus inhibiting virus entry (Feeley *et al.,* 2011; Amini-Bavil-Olyaee *et al.,* 2013; Li *et al.*, 2013b).

In contrast to the IFITM proteins which act early in the virus life cycle BST2, also known as tetherin or CD317, antagonises a later stage, inhibiting the release of virus particles from infected cells. BST2 is a type I IFN-induced type II transmembrane protein that localises to lipid rafts in the plasma membrane, endocytic compartments and trans-Golgi network (Kupzig *et al.,* 2003; Blasius *et al.*, 2006). It has a broad activity against enveloped viruses and expression of BST2 leads to the accumulation of virions at the cell surface (Neil et al., 2008; Jouvenet *et al.,* 2009; Sakuma *et al.*, 2009; Radoshitzky *et al.*, 2010; Weidner *et al.,* 2010; Mangeat *et al.,* 2012). It is hypothesised that BST2 simultaneously spans both viral and cellular membranes 'tethering' viruses to the host cell, and thereby inhibiting virus spread and release. In addition, it has been proposed that BST2 may play a role in host restriction as over-expression of human BST2, but not ovine BST2, leads to a reduction in titres of SBV, an orthobunyavirus that infects ruminants but has no association with human infection (Personal communication, Dr Mariana Varela, University of Glasgow).

8.2 Aims

The aims of the research presented in this chapter were first to confirm the anti-bunyavirus activity of IFI44 by studying the effects of IFI44 over-expression on the replication of BUNV and rBUNdelNSs2, a recombinant BUNV that lacks a functional NSs protein (Bridgen *et al.,* 2001). My second aim was to investigate possible antiviral mechanisms of IFI44 and finally I aimed to investigate the anti-orthobunyavirus activity of the membrane bound IFITM and BST2 proteins.

8.3 Results

To test for statistical significance the results in this chapter were analysed by either an individual t-test or one-way ANOVA followed by the Dunnett's multiple comparison test. The t-test was used to determine statistical significance of differences when 2 conditions at 1 time point were being compared. One-way ANOVA and the Dunnett's multiple comparison test were used when there were > 2 data sets, for example when virus titres from 4 cell lines were being compared. The cut-off for significance was set at $p < 0.05$. Unless indicated all p values in this chapter refer to these analyses only and not any other form of statistical analysis.

8.3.1 Inducible Expression of IFI44

Following on from the work presented by Carlton-Smith and Elliott (2012) a Flp-In™ T-REx™ 293 cell line with tetracycline inducible expression of FLAG-tagged IFI44 (T-REx IFI44) was used to investigate IFI44 orthobunyavirus antagonism. Tetracycline induced expression of FLAG-tagged IFI44 was confirmed by western blot anaylsis with anti-FLAG and anti-IFI44 antibodies, with a clear band at approximately 50 kDa (Figure 8-1A). Expression of IFI44 was detected with the anti-FLAG antibody as early as 8 h after the addition of tetracycline, with band strength increasing over the 48 h induction period. An additional band at approximately 115 kDa was also visible and present in both induced and non-induced cells (Figure 8-1A). This protein was also intermittently detected by the anti-IFI44 antibody, which detected IFI44 48 h after the

addition of tetracycline to the cell culture medium (Figure 8-1A). Detection by anti-IFI44 required the primary antibody to be incubated for 2 to 4 h at room temperature. Incubation for less than 2 h at room temperature, or over night incubation at 4°C, failed to result in detection (data not shown). Altering the blocking buffer to BSA or PBS with 2% (w/v) skimmed milk and 0.01% (v/v) Tween-20 also failed to improve detection (data not shown). Following induction, FLAG-tagged IFI44 remained detectable by western blot analysis with anti-FLAG antibody for at least 72 h with a clear band visible at approximately 50 kDa (Figure 8-1B). No proteins were detected by either anti-FLAG or anti-IFI44 antibodies in T-REx CAT cells, the control Flp-In™ T-REx™ 293 cell line which contains chloramphenicol acetyl transferase (CAT) in place of a FLAG-tagged ISG (Figure 8-1C).

To investigate the cellular localisation of FLAG-tagged IFI44 immunofluorescence (IF) and cellular fractionation, followed by western blot analysis, were carried out. In tetracycline induced cells IF with the anti-FLAG antibody showed predominantly cytoplasmic staining (Figure 8-2A). However, cross-reaction with the anti-FLAG antibody can occur and cellular fractionation was therefore carried out to confirm this observation. Cellular fractionation on induced and non-induced cells again indicated that FLAG-tagged IFI44 localises to the cytoplasm in uninfected cells, with a clear band at approximately 50 kDa (Figure 8-2B). However, IFI44 was not detected in either the cytoplasmic or nuclear fraction of cells infected at MOI 3 with BUNV (Figure 8-2A). Failure to detect FLAG-tagged IFI44 is unlikely to be due to experimental error as BUNV N protein and tubulin were detected in the cytoplasmic fraction, and uninfected and infected cell lysates were probed for FLAG-tagged IFI44 on the same membrane during western blotting.

In addition to the tetracycline inducible system human cell lines were screened for IFN inducible expression of IFI44 by western blot analysis. Cells were treated with 1 000 U ml^{-1} IFN-2α and harvested at 4, 8, 12 and 24 h post IFN treatment. Endogenous IFI44 expression following IFN treatment was undetectable in Huh7, HeLa, A549 or HEK-293 cells (Figure 8-3). This was despite confirmation of an active IFN response by detection of MxA in A549 and Huh7 cells (Figure 8-3). As no cell line with detectable expression of IFI44 following IFN treatment was available the T-REx IFI44 cell line with tetracycline inducible IFI44 expression was used for all future work.

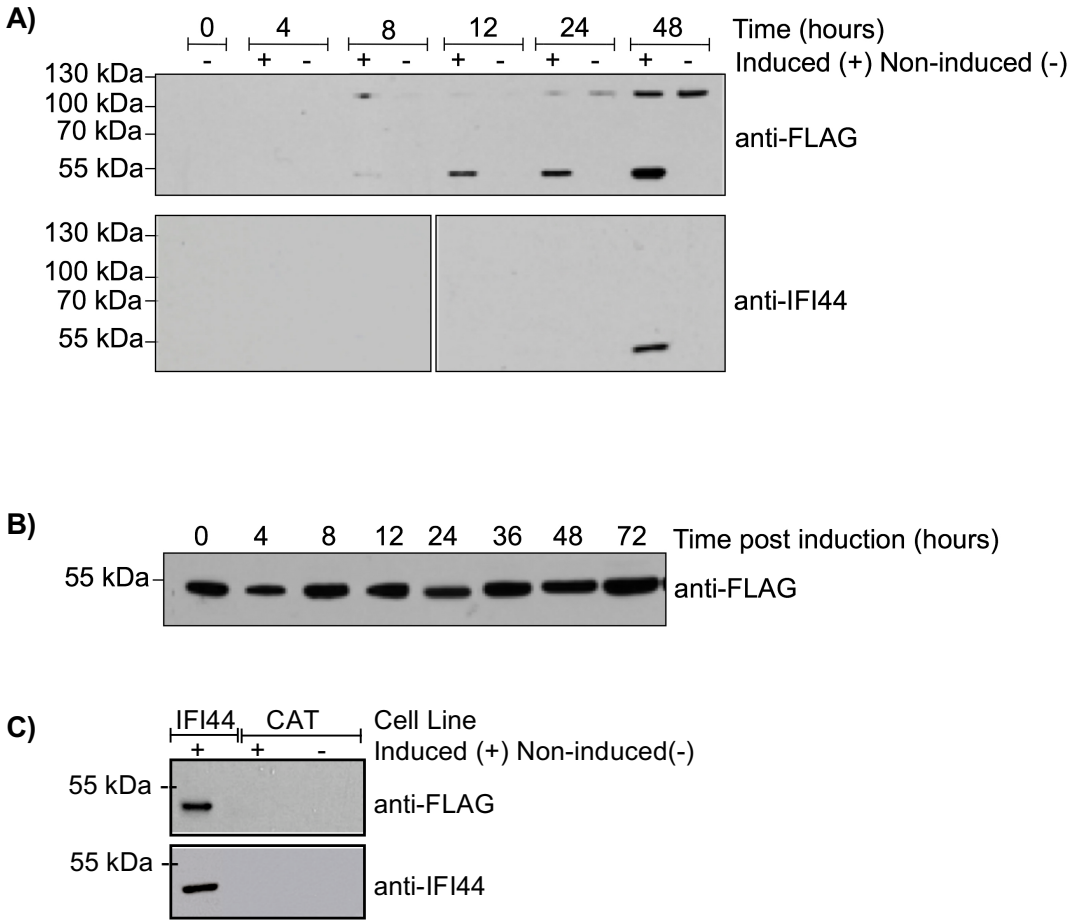
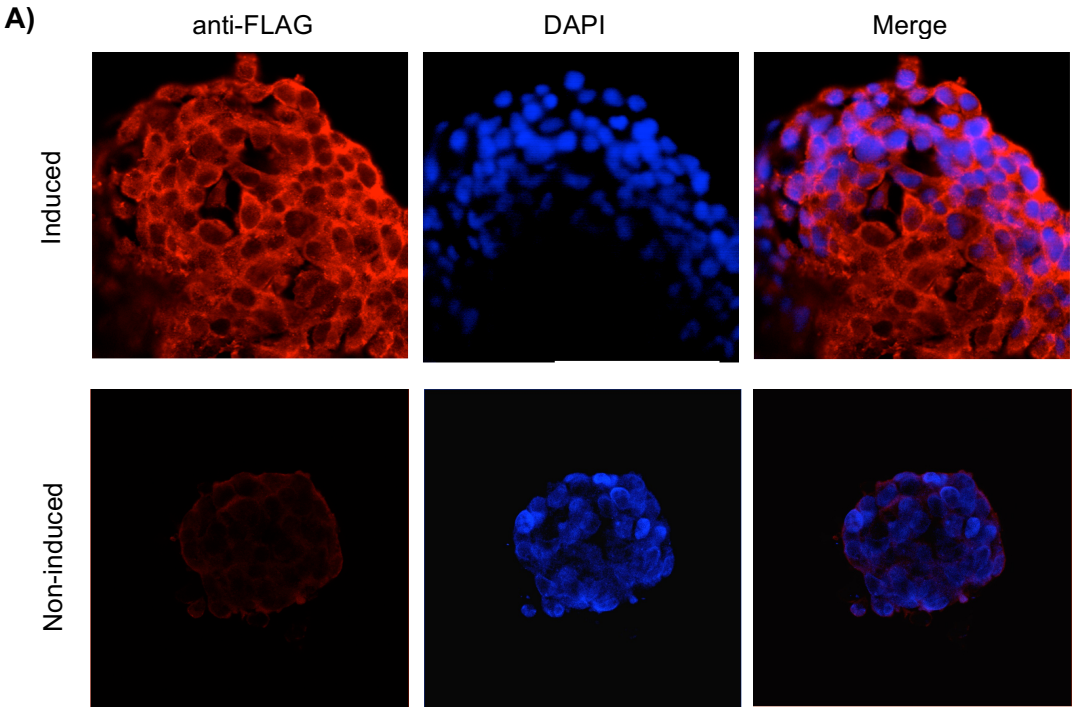


Figure 8-1: Tetracycline Induction of IFI44

A) Expression of IFI44 was induced by the addition of tetracycline at a final concentration of 1 μg ml^{-1} to cell media, or an equal volume of PBS in non-induced cells, for 48 h. Expression of FLAG-tagged IFI44 during the 48 h induction period was monitored by harvesting cell lysates at the specified time points. Cell lysates were then probed with anti-FLAG and anti-IFI44 antibodies. **B)** Following 48 h induction with 1 μg ml^{-1} tetracycline T-REx IFI44 cells were harvested at specified time points post induction and FLAG-tagged IFI44 expression detected with anti-FLAG antibody. **C)** T-REx CAT cell lysates were collected 48 h after the addition of 1 μg ml^{-1} tetracycline, or an equal volume of PBS, to the cell medium. Cell lysates were tested for FLAG-tagged gene expression using anti-FLAG antibody, with tetracycline induced T-REx IFI44 cell lysate included as a positive control.



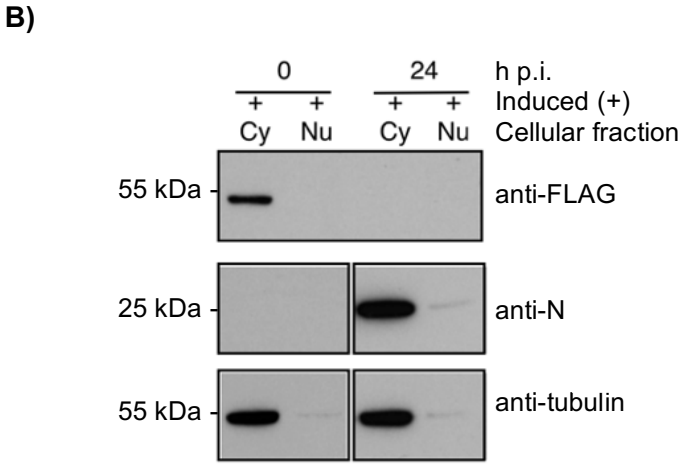


Figure 8-2: Cellular Localisation of FLAG-tagged IFI44

A) T-REx IFI44 cells were induced with 1µg ml^{-1} tetracycline or PBS for 48 h. Cells were then fixed and stained with anti-FLAG antibody to detect IFI44 expression. Coverslips were mounted in Mowiol, supplemented with DAPI, for nuclear staining. Scale bars represent 100 µm. **B)** T-REx IFI44 cells were induced with 1µg ml^{-1} tetracycline for 48 h. Cells were then infected with Bunyamwera virus (BUNV) at multiplicity of infection 3 and monolayers harvested at 0 and 24 h post infection (h p.i.). Cell lysates were then fractionated and tested by western blot analysis the presence of FLAG-tagged IFI44 using anti-FLAG antibody. Samples were also probed with anti-N and anti-tubulin antibodies to detect BUNV N protein and tubulin, respectively.

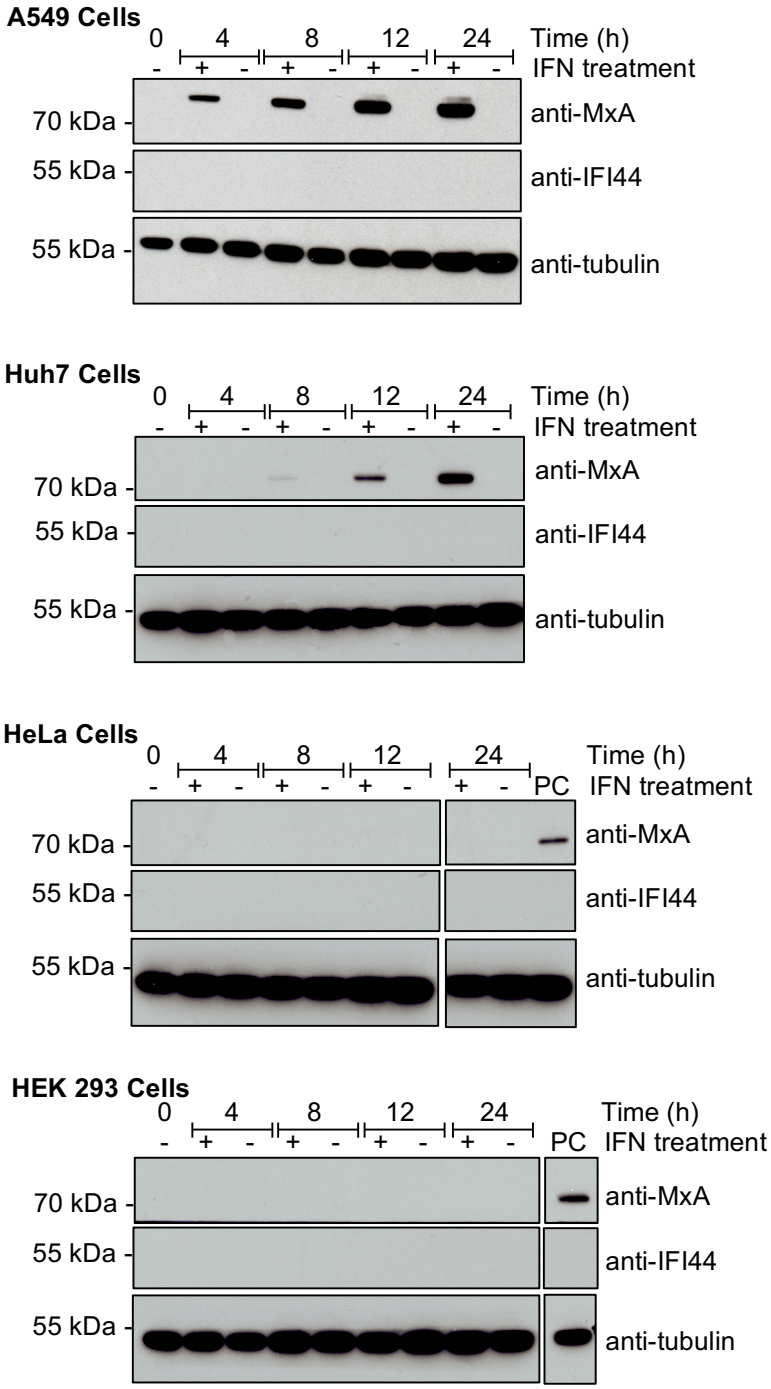


Figure 8-3: Investigation of IFN Inducible Expression of IFI44

A549, Huh7, HeLa and HEK 293 cells were treated with 1 000 U ml^{-1} IFN2α for 4, 8, 12, and 24 h. Cells were then harvested and analysed for expression of IFI44 and MxA by western blot analysis using anti-IFI44 and anti-MxA antibodies. Samples were also probed with anti-tubulin antibody to detect tubulin as a loading control. A Positive control (PC) of IFN-stimulated A549 cell lysate was included in HeLa and HEK 293 blots.

8.3.2 IFI44 Expression and Virus Replication

Following induction by tetracycline FLAG-tagged IFI44 was detected during low MOI with BUNV and rBUNdelNSs2 in T-REx IFI44 cells (Figure 8-4A, Figure 8-5A). During virus infection a reduction in the detection of viral N protein occurred, with fainter bands in both BUNV and rBUNdelNSs2 infections at 24 h p.i. and 36 h p.i. in induced cells compared to non-induced cells (Figure 8-4B, Figure 8-5B). A reduction in viral N protein was not detected in the T-REx CAT control cells (Figure 8-6). Induced T-REx IFI44 cells over-expressing FLAG-tagged IFI44 also displayed a reduction in virus release, quantified by plaque assay of clarified culture supernatants, when compared to non-induced cells (Figure 8-4C, Figure 8-5C). The reduction in virus titre was most evident and statistically significant ($p<0.05$) early in infection, with no virus detected at 8 h p.i. in induced cells infected with BUNV (Figure 8-4C). This was followed by a significant 9-fold reduction in virus titre at 12 h p.i. ($p < 0.05$). This reduction was not maintained at later time points, with titres equalling or exceeding that of non-induced cells (Figure 8-4C). The reduction in virus yield during rBUNdelNSs2 infection was also most notable early in infection with a 5-fold reduction at 12 h p.i. (Figure 8-5C). rBUNdelNSS2 virus titres remained suppressed in induced cells until 48 h p.i, when titres then equalled those of non-induced cells (Figure 8-5C).

To further investigate the effect of IFI44 over-expression during BUNV infection the virus release assay was carried out at a range of MOIs (0.001, 0.01, 0.1, 1 and 10). Once again at MOI 0.01 there was a reduction in virus titre at early time points with a 16-fold lower virus titre in induced cells at 8 h p.i. (Figure 8-7). The only other notable reduction in virus release occurred at the lower MOI of 0.001 with no virus detected at 8 h p.i. in T-REx IFI44 induced cells. This was followed by a 6-fold reduction in virus titre at 16 h p.i., however, virus titres equalled those of non-induced cells at 24 h p.i (Figure 8-7). In agreement with these virus titre results a reduction in the level of virus N protein detected by western blotting was visible only at low MOI (Figure 8-8). At MOI 0.1, 1 and 10 a reduction in the amount of IFI44 detected occurred. The level of FLAG-tagged IFI44 detected decreased with increasing MOI, with no visible bands at 24 h p.i. or 48 h p.i. at MOI 10, suggesting degradation of IFI44 upon virus infection (Figure 8-8).

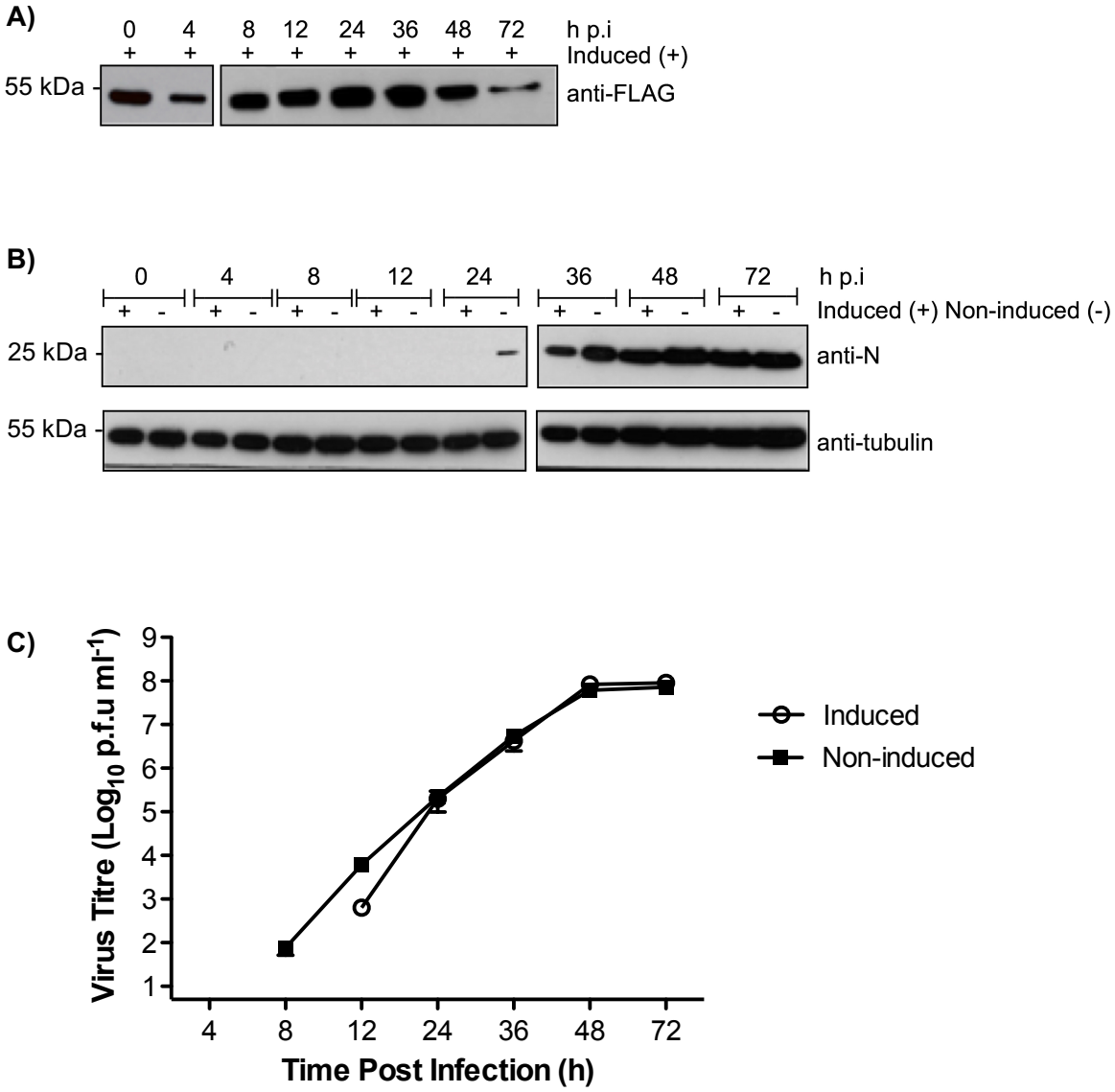


Figure 8-4: Low MOI with BUNV in Cells Over-Expressing IFI44

T-REx IFI44 cells were induced for 48 hours (h) with 1μg ml^{-1} tetracycline or an equal volume of PBS and infected with BUNV at multiplicity of infection (MOI) 0.01. At specified h post infection (h p.i.) cell monolayers and cell supernatants were collected for analysis of protein levels and virus release, respectively. **A)** Cell lysates were probed with anti-FLAG antibody to confirm the expression of FLAG-tagged IFI44 throughout the time course. **B)** Cell lysates were probed with an antibody targeting BUNV N (anti-N) to monitor virus protein production, whilst anti-tubulin antibody was used to detect cellular tubulin as a loading control for each sample. **C)** Virus release at specified time points was quantified by plaque assay on clarified culture supernatants, mean ± standard deviation of triplicate infections are shown.

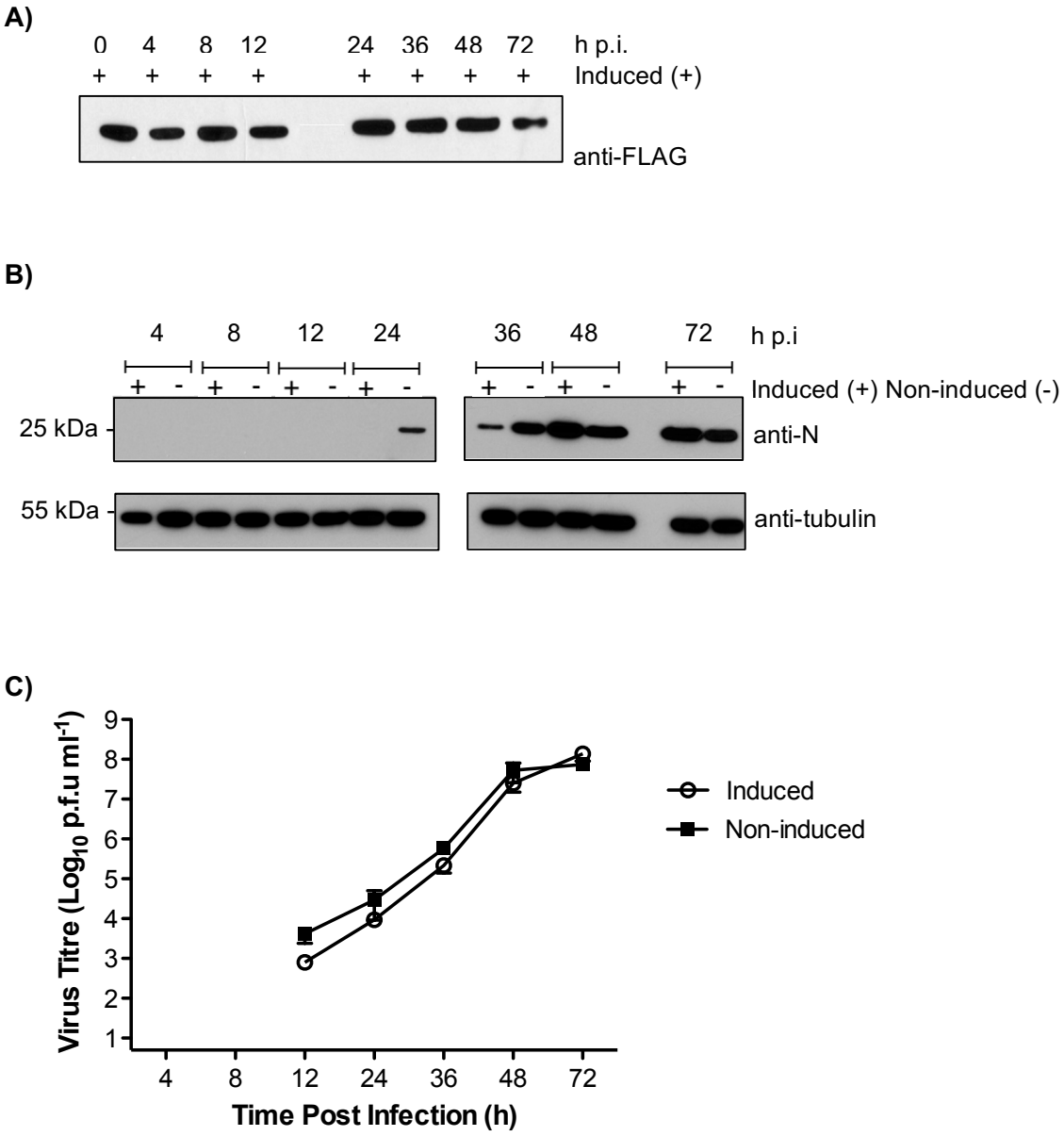


Figure 8-5: Low MOI with rBUNdelNSs2 in Cells Over-Expressing IFI44

T-REx IFI44 cells were induced for 48 hours (h) with 1µg ml^{-1} tetracycline or an equal volume of PBS and infected with rBUNdelNSs2 at multiplicity of infection (MOI) 0.01. At specified h post infection (h p.i.) cell monolayers and cell supernatants were collected for analysis of protein levels and virus release, respectively. **A)** Cell lysates were probed with anti-FLAG antibody to confirm the expression of FLAG-tagged IFI44 throughout the time course. **B)** Cell lysates were probed with an antibody targeting BUNV N (anti-N) to monitor virus protein production, whilst anti-tubulin antibody was used to detect cellular tubulin as a loading control for each sample. **C)** Virus release at specified time points was quantified by plaque assay on clarified culture supernatants, mean ± standard deviation of triplicate infections are shown.

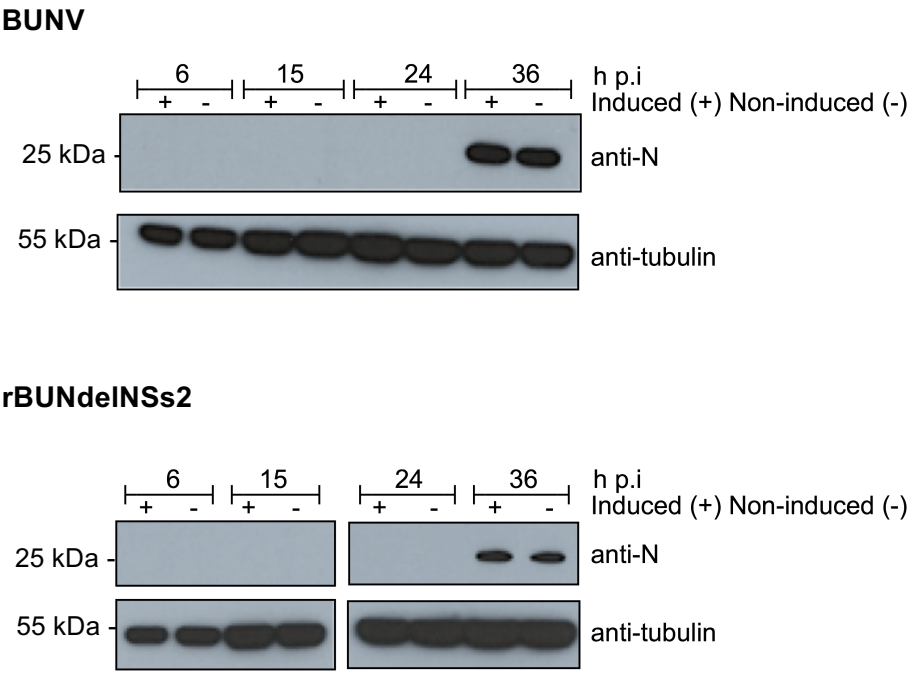


Figure 8-6: BUNV and rBUNdelNSS2 Low MOI in T-Rex CAT Control Cells
T-REx CAT cells were induced for 48 hours (h) with 1µg ml^{-1} tetracycline or an equal volume of PBS and infected at multiplicity of infection (MOI) 0.01 with either wild type Bunyamwera virus (BUNV) or the recombinant virus rBUNdelNSs2. At specified time points cell monolayers were collected for analysis of protein levels by western blot analysis. Antibodies targeting the virus N protein (anti-N) and cellular tubulin (anti-tubulin) were used.

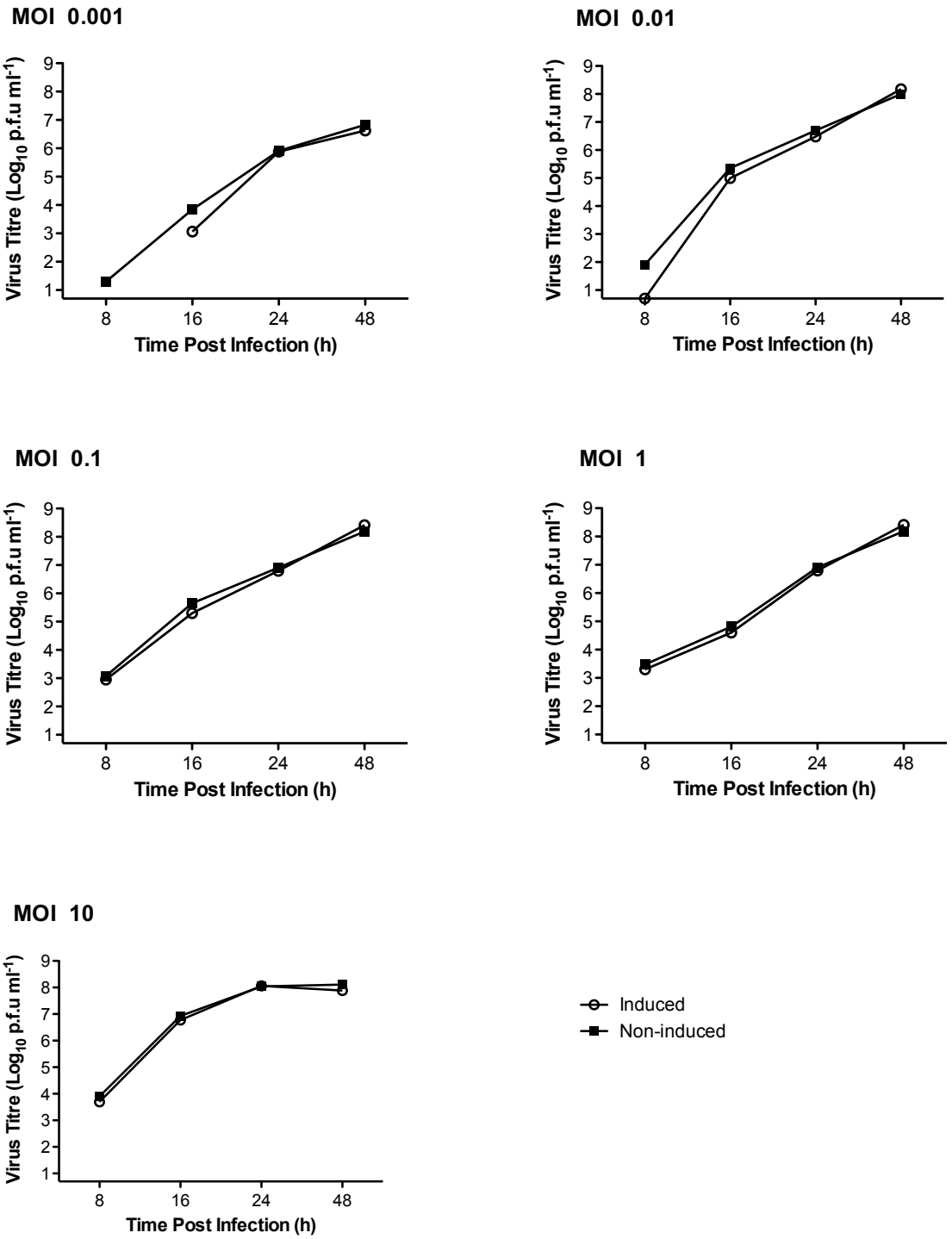


Figure 8-7: BUNV MOI Screen in T-REx IFI44 Cells, Virus Release Assay

T-REx IFI44 cells were induced for 48 hours (h) with 1µg ml^{-1} tetracycline or an equal volume of PBS and infected with wild type Bunyamwera virus (BUNV) at the specified multiplicity of infection (MOI). Cell supernatants were collected at specified time points and virus release measured by plaque assay. Cell lysates were also collected at 0, 24 and 48 h post infection (Figure 8-8).

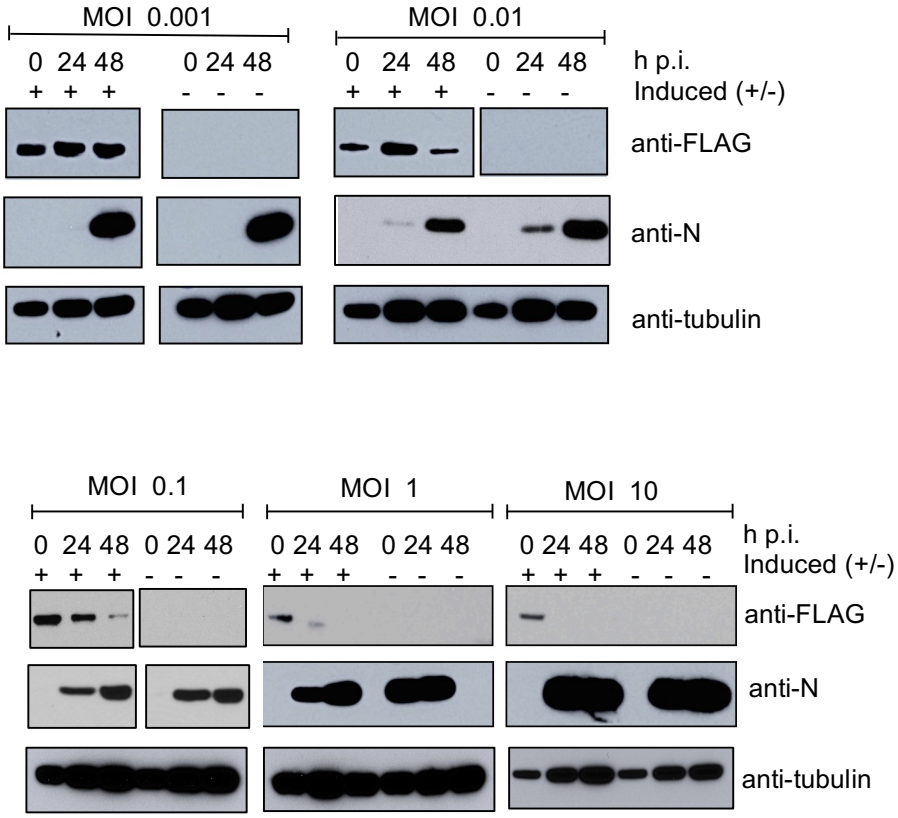


Figure 8-8: BUNV MOI Screen in T-REx IFI44, Cells Protein Expression

T-REx IFI44 cells were induced for 48 hours (h) with 1µg ml^{-1} tetracycline or an equal volume of PBS and infected with BUNV at specified multiplicity of infection (MOI). At specified time points cell supernatants (Figure 8-7) and cell monolayers were collected for analysis of virus release and protein levels, respectively. Cell lysates were probed with antibodies targeting FLAG-tagged IFI44 (anti-FLAG), virus N and NSs proteins (anti-N, anti-NSs) and, as a loading control, tubulin (anti-tubulin).

To ensure the selection of IFI44 inducible cells T-REx IFI44 cells were passaged with increased concentrations of selective antibiotics (20 μg ml^{-1} blasticidin and 1000 μg ml^{-1} hygromycin versus 5 μg ml^{-1} and 250 μg ml^{-1} respectively). This did not noticeably enhance antiviral activity as no significant decrease in virus release was detected in induced cells at > 12 h p.i. in BUNV or rBUNdelNSs2 infected cells (MOI 0.01 and 0.001 tested, data not shown). Incubating cells at 33°C following tetracycline induction and infection with BUNV at an MOI of 0.01 also failed to elicit a significant decrease in virus yield when compared to non-induced cells (data not shown).

To investigate the loss of detectable FLAG-tagged IFI44 observed during high MOI experiments, parallel high MOI experiments with BUNV and rBUNdelNSs2 were carried out. It was confirmed, that in contrast to low MOI experiments, there was no clear difference in the level of viral N protein for either BUNV or rBUNdelNSs2 in induced and non-induced cells. Once again, there was a reduction in the level of detectable IFI44 (Figure 8-9). Reduction in IFI44 levels occurred faster during BUNV infections than rBUNdelNSs2 infections, where it remained clearly detectable at 24 h p.i., with a faint band at 36 h p.i. in comparison to no detectable bands at either 24 h p.i. or 36 h p.i. in BUNV infected cells (Figure 8-9). Treating induced T-Rex IFI44 cells with the proteasome inhibitor, MG132, increased IFI44 levels during high MOI with BUNV and rBUNdelNSs2 virus (Figure 8-10). MG132 treatment also increased detection of BUNV NSs protein, whilst having no detectable affect on levels of BUNV N protein (Figure 8-10).

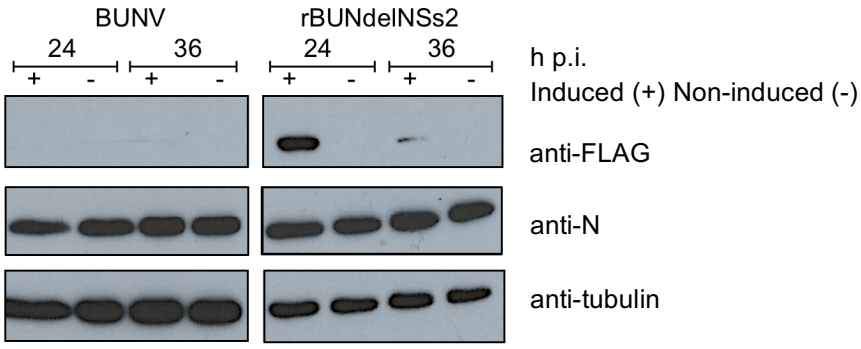


Figure 8-9: Expression of IFI44 and Virus N Protein in High MOI

T-REx IFI44 cells were induced with 1 μg ml^{-1} tetracycline or an equal volume of PBS for 48 hours (h) prior to infection with Bunyamwera virus (BUNV) or the recombinant rBUNdelNSs2 virus at multiplicity of infection 3. Cell lysates were collected at specified h post infection (h p.i.) and analysed by western blotting for expression of FLAG-tagged IFI44 (anti-FLAG), virus N protein (anti-N), and tubulin (anti-tubulin).

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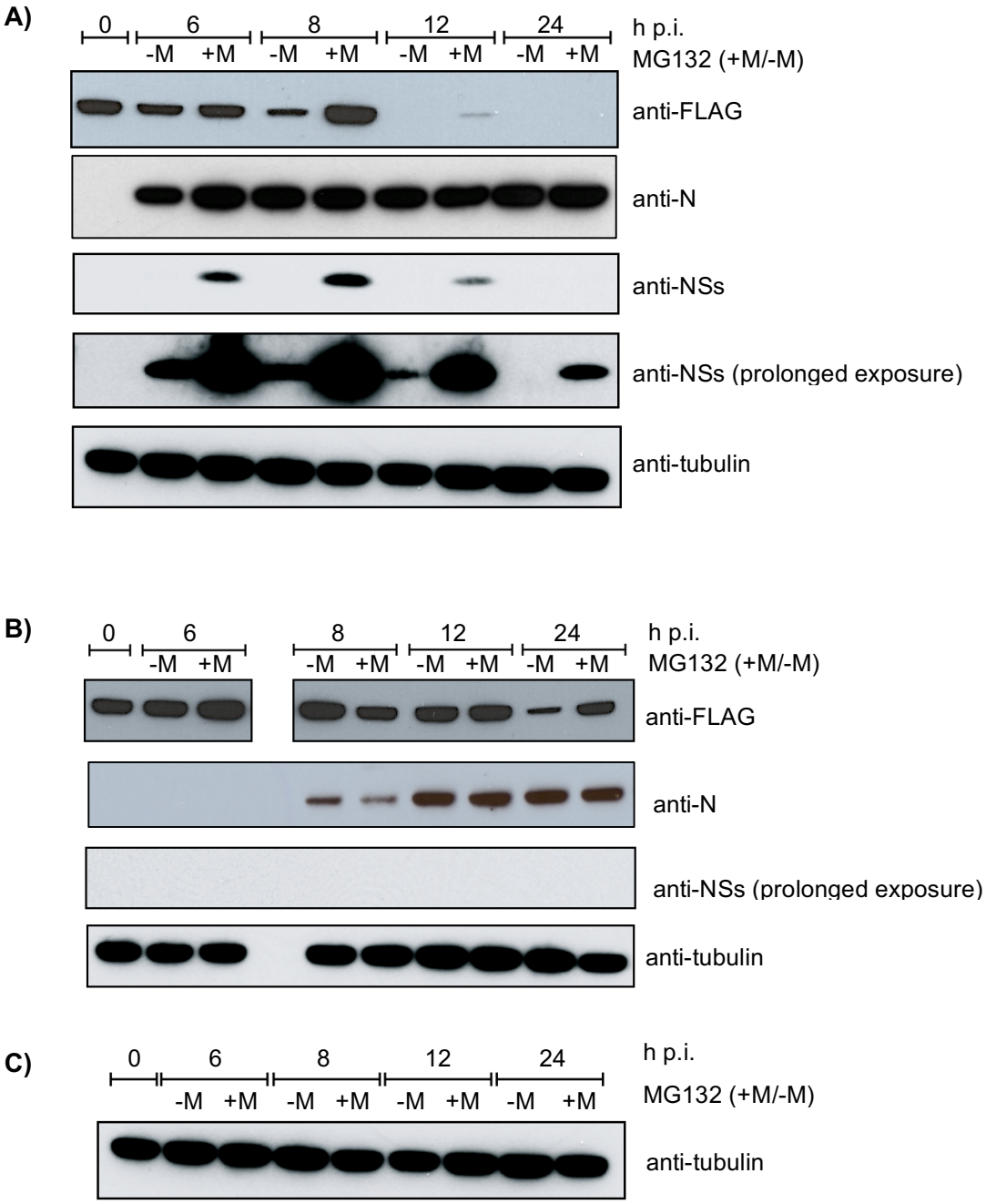


Figure 8-10: Inhibition of the Proteasome in T-Rex IFI44 Cells

A, B) T-REx IFI44 cells were induced with 1μg ml-[1] tetracycline for 48 hours (h) prior to infection with Bunyamwera virus (BUNV) (A) or the recombinant virus rBUNdelNSs2 (B). Cells were infected at multiplicity of infection 3 and the proteasome inhibitor MG132 added at a final concentration of 10 μM at 5 h post infection (h p.i.). Cell lysates were harvested at specified time points and probed with antibodies targeting FLAG-tagged IFI44 (anti-FLAG), virus NSs protein (anti-NSs), virus N protein (anti-N) and cellular tubulin (anti-tubulin). **C)** Cells were mock infected with PBS supplemented with 2% (v/v) FCS and cell lysates probed for tubulin to monitor MG132 toxicity levels.

8.3.4 Anti-Orthobunyavirus Activity of Membrane Bound ISGs

To enable screening for anti-orthobunyavirus activity A549 cell lines constitutively over-expressing TagRFP and either IFITM1, IFITM2, or IFITM3 were constructed by lentivirus transduction (Figure 8-11). An empty vector control cell line, A549-SCRPSY, over-expressing TagRFP alone was also created (Figure 8-11). The optimal concentration of the selective agent, puromycin, was determined by killing assay on non-transduced A549 cells to be 2 $\mu g\ ml^{-1}$ (data not shown). The level of transduction, as measured by image based cytometry of TagRFP expression, was > 98% for all cell lines (Figure 8-11A). Over-expression of IFITM2 and IFITM3, but not IFITM1, was also confirmed by western blot analysis (Figure 8-11C). It should be noted that IFITM2 and IFITM3 proteins are highly homologous and both proteins were detected by anti-IFITM2 (data not shown) and anti-IFITM3 antibodies. The IFITM3 antibody gave slightly better signal for both proteins and was therefore used for all future work.

To study the effect of IFITM over-expression on virus replication infections were carried out with rBUNGc-eGFP and rBUNdelNSs-GcGFP, recombinant viruses with GFP-tagged glycoproteins (Shi *et al.*, 2010). Cells were then analysed using image based cytometry, a technique that gives quantitative measurements of GFP and RFP expression in cells, allowing the percentage of infected and transduced cells to be counted. Over-expression of IFITM1 ($p < 0.005$) and IFITM3 ($p < 0.0005$) were found to significantly inhibit rBUNGc-eGFP virus replication (Figure 8-12). At 24 h p.i. and 48 h p.i. only 32.8% and 38.0% of A549-IFITM3 cells were positive for rBUNGc-eGFP, in comparison to 78.4% and 82.8% of empty vector control cells (Figure 8-12). In A549-IFITM1 cells 67.0% and 69.2% were positive for rBUNGc-eGFP at 24 h p.i. and 48 h p.i., respectively (Figure 8-12). A similar pattern was observed in rBUNdelNSs-GcGFP infections, with significant ($p<0.005$) reductions in GFP detection in A549-IFITM1 and -IFITM3 cell lines at 24 h p.i. and 48 h p.i. compared with the empty vector control cells (Figure 8-12). However, overall rates of infection were greatly reduced in comparison to rBUNGc-eGFP (Figure 8-12).

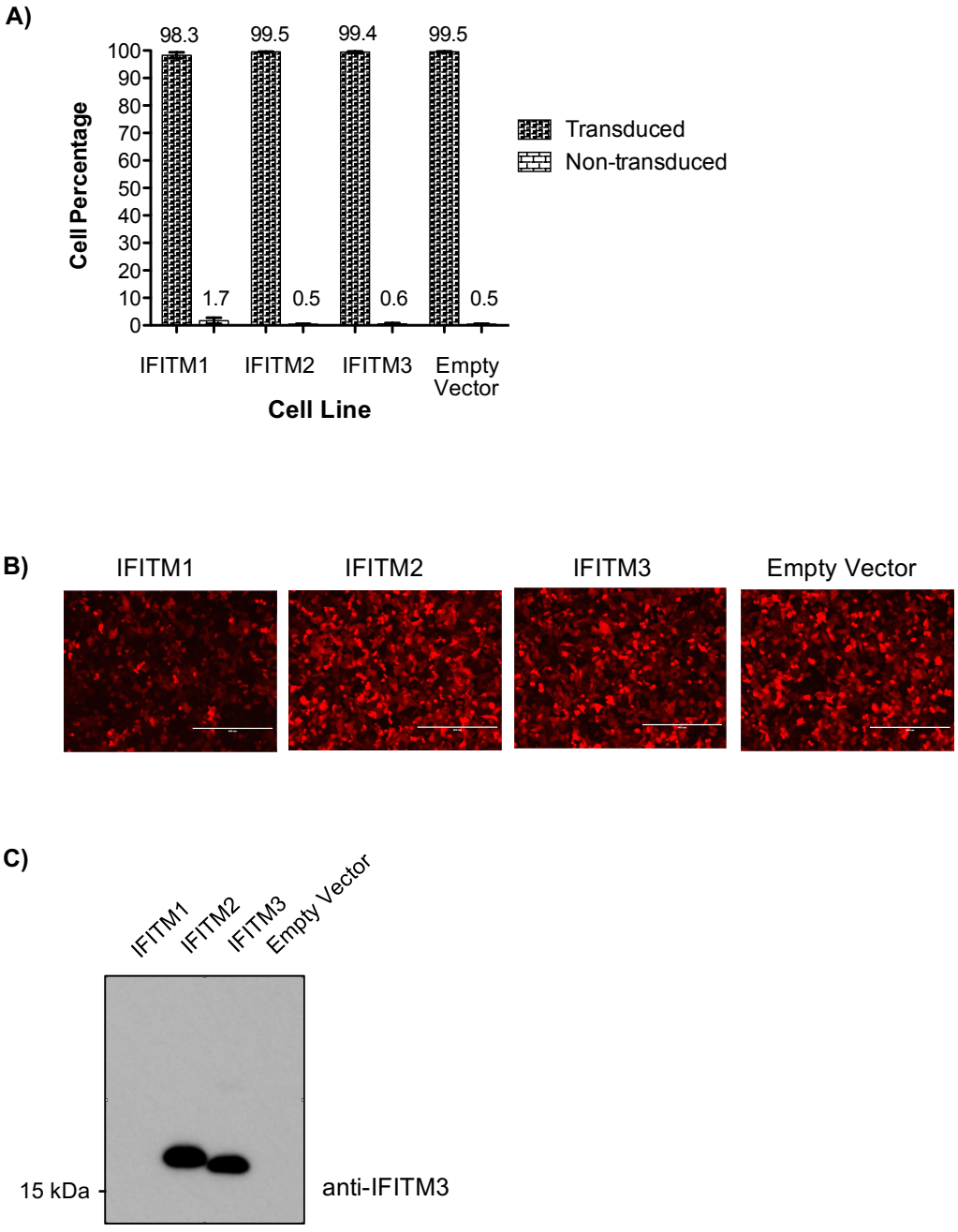


Figure 8-11: A549 Cells Constitutively Expressing IFITM Proteins

A549 cells were transduced with lentivirus vectors encoding TagRFP (empty vector) or TagRFP and IFITM1, IFITM2, or IFITM3. **A)** Passage (p) 2 cells were treated with trypsin to form single cell suspensions, which were then analysed on the Tali® Image Cytometer (Life Technologies) to determine the proportion of transduced cells. **B)** Fluorescent microscopy on adherent p2 cell monolayers showing expression of TagRFP. Scale bars represent 400 μm. **C)** Western blot analysis of p2 cell lysates with anti-IFITM3 antibody; due to high homology between proteins IFITM2 is recognised by the anti-IFITM3 antibody.

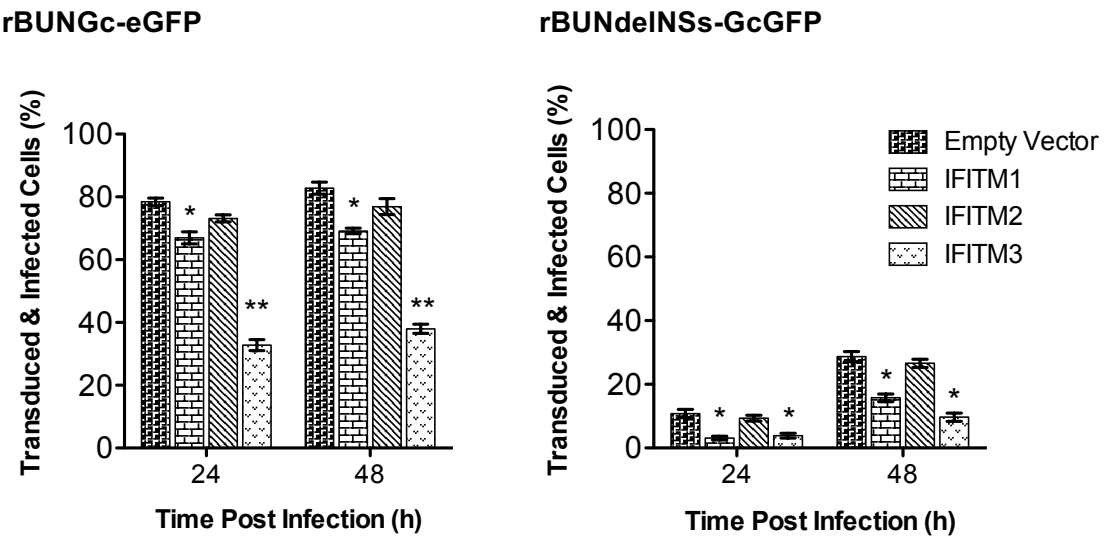


Figure 8-12: BUNGc-eGFP rBUNdelNSs-GcGFP

A549-IFITM cell lines and empty vector control, A549-SCRPSY, were infected at multiplicity of infection 1 with green fluorescent viruses rBUNGc-eGFP and rBUNdelNSs-GcGFP. Cell monolayers were harvested at 24 h post infection (h p.i.) and 48 h p.i. and analysed for GFP and RFP fluorescence by image based cytometry on the Tali® Image Cytometer (Life Technologies). Mean ± standard deviation of triplicate samples is shown, with significant differences from empty vector cells indicated by asterisks: *, $p<0.005$; **, $p<0.0005$.

To further investigate virus antagonism fluorescent microscopy was carried out. At 7 h p.i. rBUNGc-eGFP virus replication was clearly visualised in empty vector and A549-IFITM2 cells, with the aggregation of GFP signal in the perinuclear region (Figure 8-13). However, in A549-IFITM1 and -IFITM3 cells only isolated fluorescent spots were seen, indicating that virus replication and the trafficking of GFP-tagged glycoproteins to the Golgi has not yet occurred (Figure 8-13).

In addition to fluorescence-based studies with GFP-tagged viruses, antagonism of virus replication was investigated using wild type BUNV and rBUdelNSs2 virus. In a virus release assay over-expression of IFITM1 and IFITM3 was, once again, associated with significant inhibition of virus replication. In A549-IFITM3 cells antagonism was most evident early in infection, with a significant reduction in BUNV titre at 8 h p.i ($p < 0.005$) and 12 h p.i ($p < 0.01$) (Figure 8-14A). Virus titres remained suppressed at 24 h p.i. and 36 h p.i., although not to significant levels. In A549-IFITM1 cells the titre of BUNV was also significantly lower than control cells at 8 h p.i. ($p<0.05$) and 12 h p.i. ($p<0.01$), although the level of antagonism was lower than that observed in cells over-expressing IFITM3 (Figure 8-14A). The reductions in virus titre were also supported by decreased detection of BUNV N protein by western blot analysis at 24 h p.i. in both A549-IFITM1 and -IFITM3 cells (Figure 8-14B).

rBUNdelNSs2 virus replication was also antagonised in A549-IFITM1 and IFITM3 cells. In A549-IFITM3 cells there were small but significant reductions in rBUNdelNSs2 virus titre at 8 h p.i. ($p<0.01$), 12 h p.i. ($p<0.05$) and 24 h p.i. ($p<0.05$) (Figure 8-15A). This was coupled with a reduction in the detection of virus N protein by western blot analysis in A549-IFITM3 cells at 24 h p.i. (Figure 8-15B). In A549-IFITM1 cells the reduction in rBUNdelNSs2 virus was only significant at 8 h p.i. ($p<0.05$) and there was no apparent antagonism of viral N protein levels (Figure 8-15).

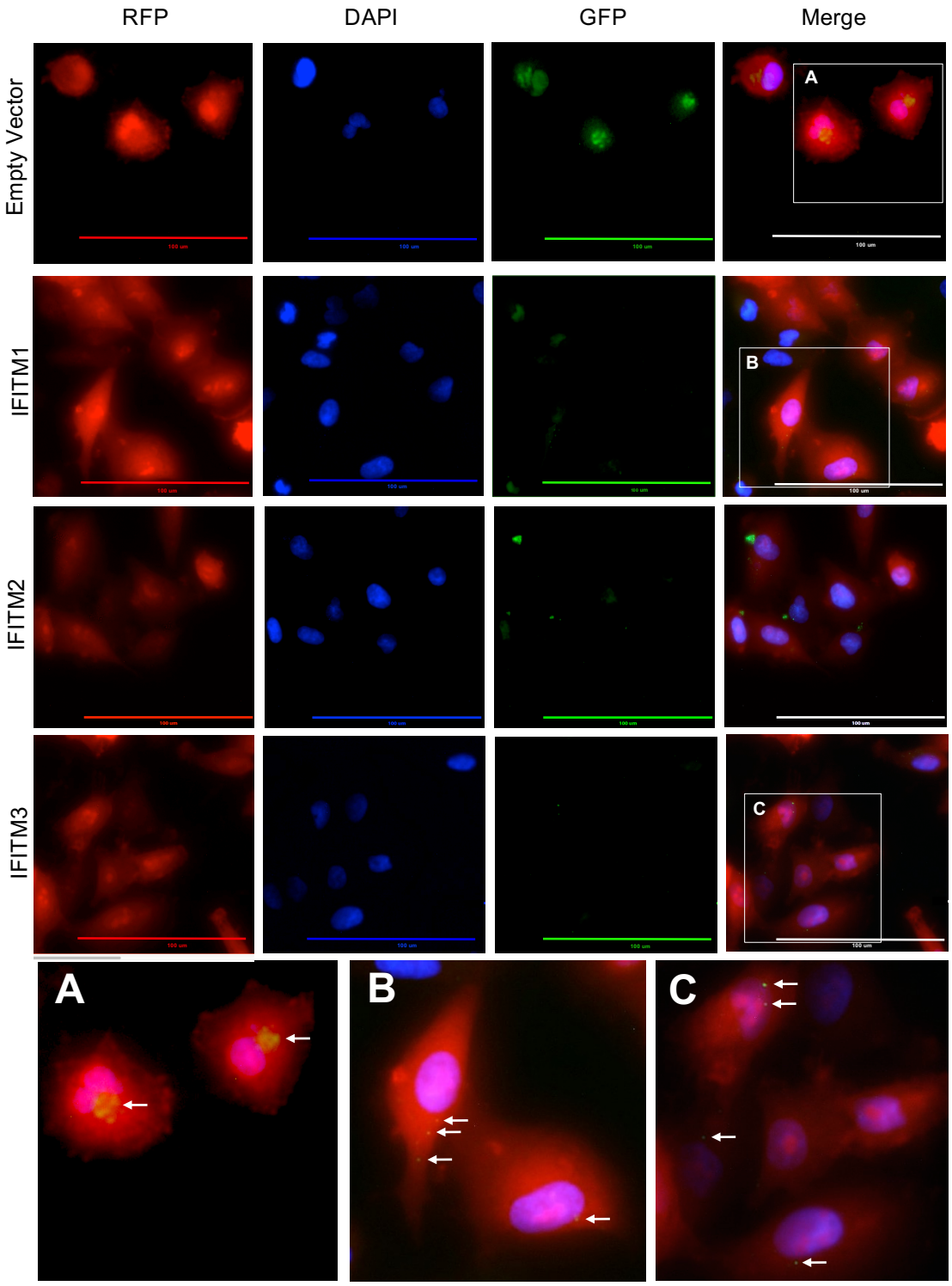


Figure 8-13: Fluorescent Microscopy of rBUNGc-eGFP in Cells Over-Expressing IFITM Proteins

A549-IFITM cell lines and empty vector control, A549-SCRPSY, were infected at multiplicity of infection 5 with the green fluorescent virus rBUNGc-eGFP. Cells were fixed 7 hours post infection and coverslips mounted in Mowiol supplemented with DAPI to stain cell nuclei. TagRFP, eGFP and DAPI fluorescence were captured by fluorescent microscopy (EVOS® FL, Life Technologies), scale bars represent 100 µm. A, B and C show enlarged images of selected regions, white arrows highlight regions with GFP signal.

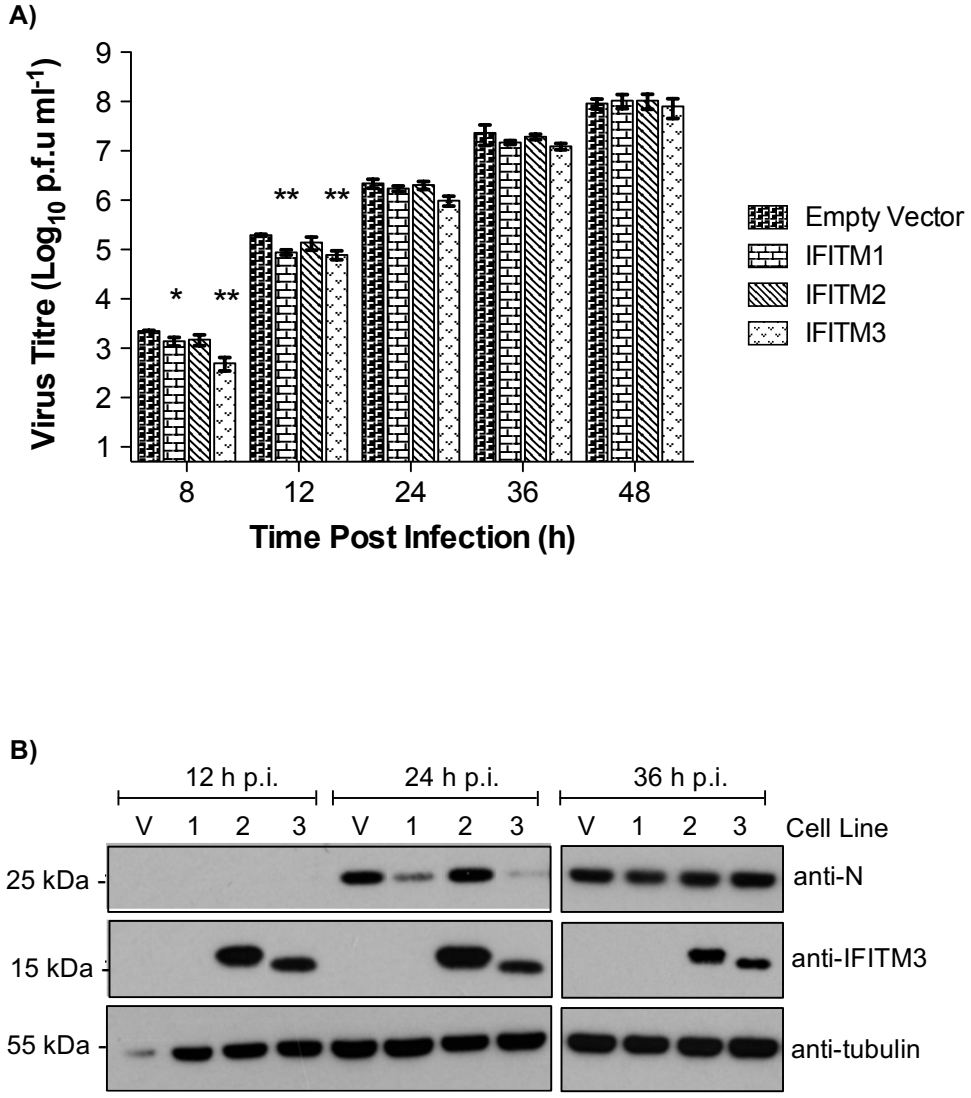


Figure 8-14: BUNV Replication in A549-IFITM Cells

A549-SCRPSY and A549-IFITM cell lines were infected, in triplicate, with Bunyamwera virus (BUNV) at multiplicity of infection 0.01 and cell supernatants and cell lysates collected at specified hours post infection (h p.i.). **A)** Virus release was quantified by plaque assay of clarified culture supernatants on Vero E6 cells. Mean values ± standard deviation of triplicate infections are shown, with significant differences versus empty vector control cells, A549-SCRPSY, indicated by asterisks: *, $p<0.05$; **, $p<0.01$. **B)** Cell lysates from A549-SCRPSY(V), -IFITM1 (1), -IFTIM2 (2), -IFITM3 (3) were analysed by western blotting. Antibodies targeting BUNV N protein (anti-N), IFITM2 and IFITM3 proteins (anti-IFITM3) and tubulin (anti-tubulin) were used.

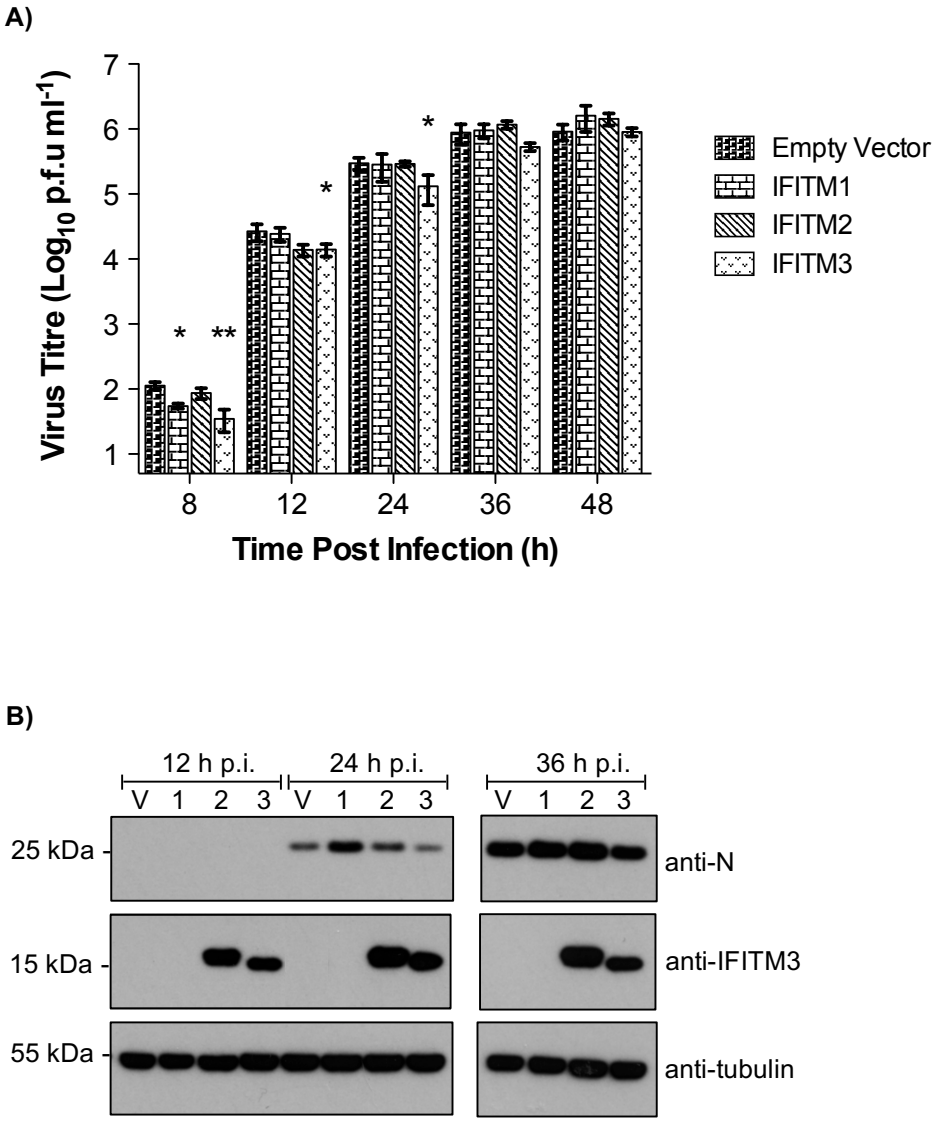


Figure 8-15: rBUNdelNSs2 in A549-IFITM Cells

A549-SCRPSY and A549-IFITM cell lines were infected, in triplicate, with recombinant virus rBUNdelNSs2 at multiplicity of infection 0.01 and cell supernatants and cell lysates collected at specified hours post infection (h p.i.). **A)** Virus release was quantified by plaque assay of clarified culture supernatants on Vero E6 cells. Infections were carried out in triplicate and graphs depict mean values ± standard deviation. Significant differences versus empty vector control cells, A549-SCRPSY, are indicated by asterisks: *, $p<0.05$; **, $p<0.01$. **B)** Cell lysates from A549-SCRPSY(V), -IFITM1 (1), -IFTIM2 (2), -IFITM3 (3) were analysed by western blotting. Antibodies targeting BUNV N protein (anti-N), IFITM2 and IFITM3 proteins (anti-IFITM3) and tubulin (anti-tubulin) were used.

To explore the spectrum of anti-orthobunyavirus activity A549-IFITM cell lines were tested with viruses from the Anopheles A serogroup (ANAV and TCMV), Anopheles B serogroup (BORV) and Tete serogroup (BMAV). Antibodies against these viruses were not available, therefore only virus release was measured. In contrast to BUNV, ANAV virus yield was significantly reduced ($p < 0.05$) in A549-IFITM1, -IFITM2, and -IFITM3 cells at 8 h p.i. (Figure 8-16). Virus yields remained significantly lower in A549-IFITM2 and -IFITM3 cell lines at 12 h p.i. ($p<0.01$, $p<0.01$) and 24 h p.i. ($p<0.05$, $p<0.01$) (Figure 8-16). In TCMV infected cells a significant ($p<0.01$) reduction in virus titre occurred only at 12 h p.i. in A549-IFITM3 cells, with no evidence of antagonism at other time points or in A549-IFITM1 or A549-IFITM2 cell lines (Figure 8-16).

The Anopheles B serogroup virus, BORV, grew to significantly higher titres than the Anopheles A serogroup viruses, ANAV and TCMV, at early time points with titres > 3 $\log_{10}$ p.f.u ml^{-1} at 8 h p.i. in A549-SCRPSY cells (Figure 8-16). Despite this, BORV replication was still significantly lower in A549-IFITM1, -IFITM2, and -IFITM3 cells at 8 h p.i. ($p<0.05$) and A549-IFITM2 and IFITM3 cells at 12 h p.i. ($p<0.05$) (Figure 8-16).

Lastly, replication of BMAV, a member of the Tete serogroup, was antagonised by IFITM2 and IFITM3 over-expression, with significantly ($p<0.05$) lower virus titres at 8 h p.i., 12 h p.i. and 24 h p.i. in A549-IFITM2 and -IFITM3 cells with respect to A549-SCRPSY control cells (Figure 8-16).

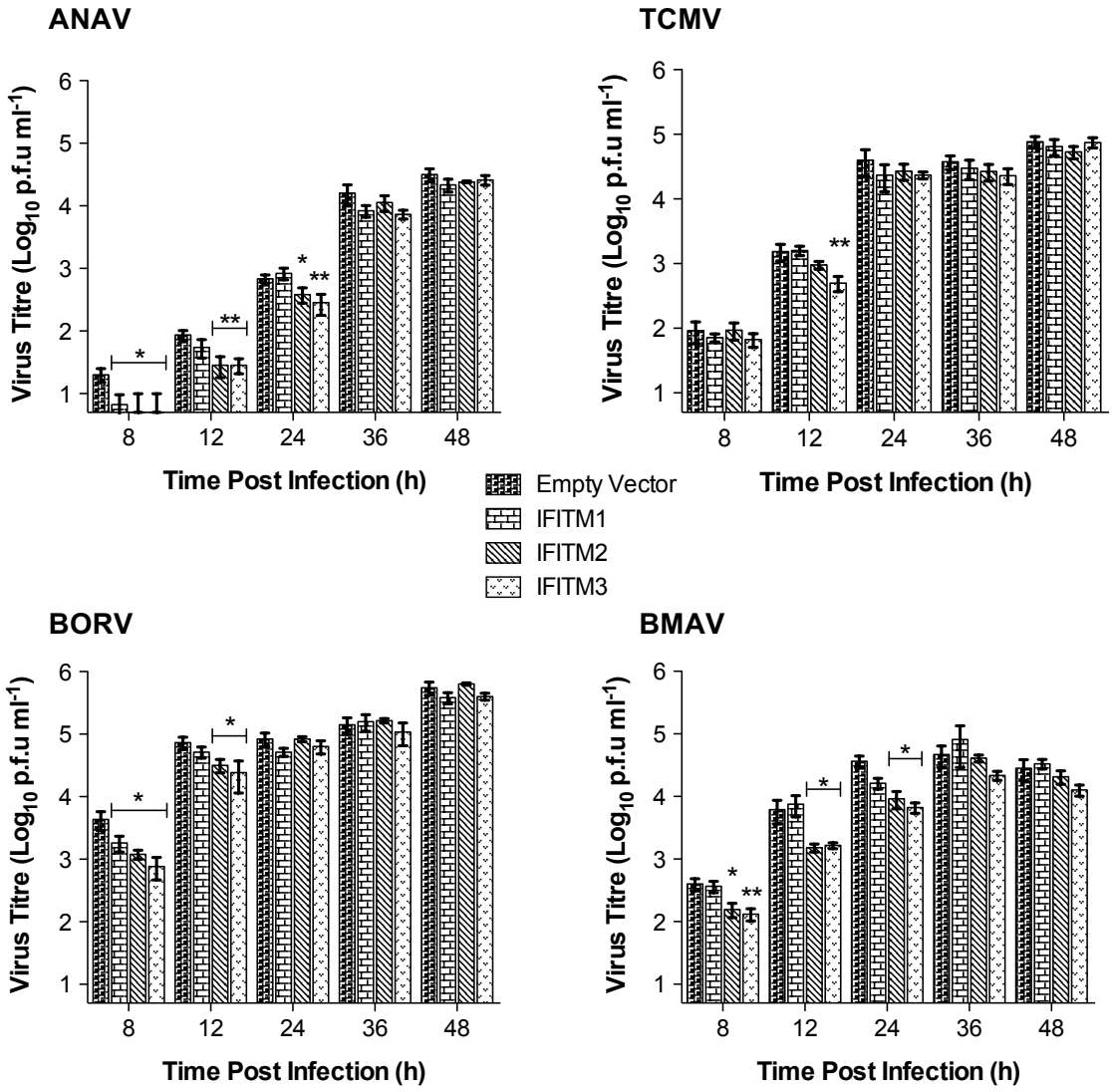


Figure 8-16: Replication of Anopheles A, Anopheles B and Tete Serogroup Viruses in A549-IFITM Cell Lines

A549-SCRPSY and A549-IFITM cell lines were infected with Anopheles A serogroup viruses, Anopheles A virus (ANAV) and Tacaiuma virus (TCMV), the Anopheles B serogroup virus Boraceia virus (BORV) and Batama virus (BMAV), a member of the Tete serogroup. Infections were carried out in triplicate at multiplicity of infection 0.01 and cell supernatants collected at specified hours (h) post infection for quantification by plaque assay on Vero E6 cells. Mean values ± standard deviation are shown, with significant differences versus empty vector control cells, A549-SCRPSY indicated by asterisks: *, $p<0.05$; **, $p<0.01$.

In addition to antagonising orthobunyavirus replication at low MOI it was noted that levels of detectable IFITM2 and IFITM3 proteins decreased during high MOI with BUNV and, to a lesser extent, with rBUNdelNSs2 (Figure 8-17A). To further investigate this observation naive A549 cells were infected with BUNV and rBUNdelNSs2 at MOI 10 and cell lysates tested for IFN-induced IFITM2 and IFITM3 expression. Neither IFITM2 nor IFITM3 was detected in BUNV or rBUNdelNSS2 infections at 24 h p.i. (Figure 8-17B). In contrast IFITM3 was clearly detected in cells infected with BMAV or BORV at 24 h p.i. (Figure 8-17B). As BUNV antagonises the type I IFN system A549 cells were treated with exogenous IFN to induce expression of the IFITM proteins. However, even in the presence of exogenous IFN IFITM2 and IFITM3 proteins were not detected in BUNV or rBUNdelNSs2 cell lysates, despite the detection of MxA, another ISG product (Figure 8-17C).

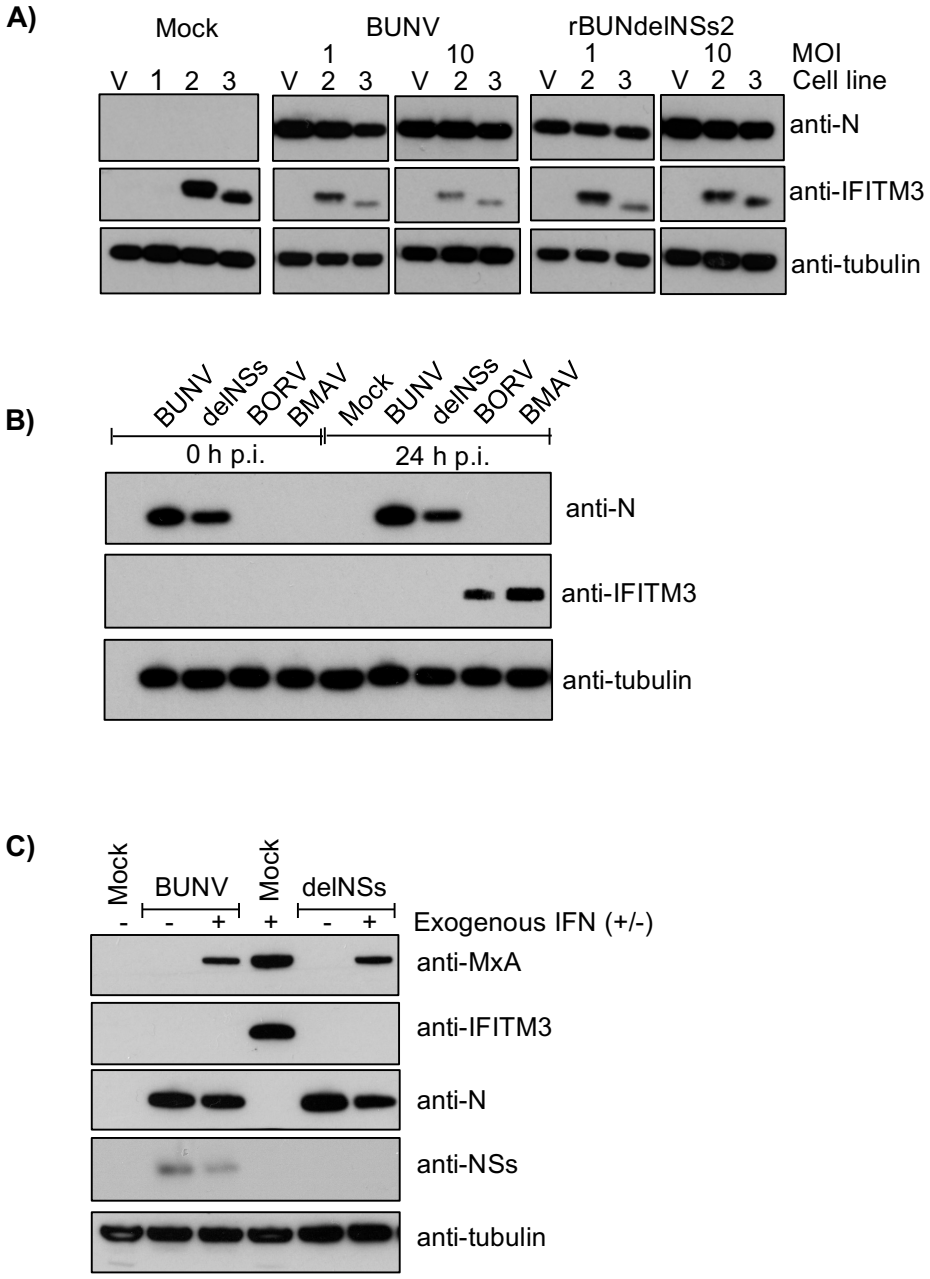


Figure 8-17: Expression of IFITM2 and IFITM3 in high MOI Virus Infection

A) A549 cells constitutively over-expressing IFITM2 or IFITM3 were infected at multiplicity of infection (MOI) 1 and MOI 10 with Bunyamwera virus (BUNV) and rBUNdelNSs2 virus (delNSs). Cell monolayers were harvested 24 hours post infection (h p.i.) and probed with antibodies targeting virus N protein (anti-N), IFITM2 and IFITM3 proteins (anti-IFITM3) and tubulin (anti-tubulin). V, 1, 2 and 3 refer to empty vector (A549-SCRPSY), A549-IFITM1, A549-IFITM2 and A549-IFITM3 cell lines, respectively. **B)** A549 cells were infected at MOI 10 with BUNV, rBUNdelNSs2 (delNSs), Boraceia virus (BORV) or Batama virus (BMAV). Cell monolayers were collected at 12 h p.i. and 24 h p.i. and probed with antibodies targeting BUNV-N protein, IFITM3 and tubulin. No antibodies for BORV or BMAV N were available, but virus infection was confirmed by titration (data not shown). **C)** A549 cells were stimulated with 1000 U ml^{-1} IFN (+) , or an equal volume of PBS (-) and infected with BUNV or rBUNdelNSs2 (delNSs) at MOI 10. Cell lysates were collected 24 h p.i. and probed with antibodies targeting MxA (anti-MxA), virus N protein (anti-N), IFITM2 and IFITM3 proteins (anti-IFITM3) and tubulin (anti-tubulin).

In contrast to IFITM proteins, which act early in the virus life cycle, BST2 (tetherin, CD317) inhibits virus replication by tethering newly produced virus particles to the cell surface, antagonising virus release. To study the effect of BST2 over-expression on orthobunyavirus replication 293T cells constitutively over-expressing human (h) or ovine (o) BST2 proteins were kindly provided by Dr Mariana Varela, University of Glasgow. BUNV did not appear to be strongly affected by the over-expression of hBST2 or oBST2 proteins, with no large differences in virus titre at any of the time points tested (Figure 8-18A). There was however, a 24-fold reduction in the virus titre of rBUNdelNSs2 in cells over-expressing hBST2 at 24 h p.i. in comparison to the empty vector control (Figure 8-19A). The decrease in viral titre, although not statistically significant, was supported by a drop in viral N protein levels, with detection of a fainter band at 36 h p.i. than in the control cell sample, despite no notable difference in the level of tubulin (Figure 8-19B). It should also be noted, that in both BUNV and rBUNdelNSs2 infections there was a decrease in detection of virus N protein in cells over-expressing oBST2A, despite no clear antagonism of virus titre (Figure 8-18, Figure 8-19).

Viruses from the Anopheles A serogroup (ANAV and TCMV), Anopheles B serogroup (BORV) and BMAV, a member of the Tete serogroup, were also screened using the BST2 cell lines. As no antibodies against these viruses were available, only virus release was measured. There was slight suppression of ANAV virus release at early time points, with 3.7-fold – 9.6-fold reductions in virus titre at 12 h p.i. in cells over-expressing hBST2, oBST2A or oBST2B (Figure 8-20). This was followed by a 6.3-fold reduction in oBST2A cells at 24 h p.i., and titres from oBST2A and oBST2B remained slightly lower than empty vector controls or hBST2 at 36 h p.i. and 48 h p.i. (Figure 8-20). TCMV replication did not appear to be antagonised by over-expression of either human or ovine BST2 proteins. There was a slight reduction in virus titre at 36 h p.i. in cells over-expressing hBST2, but there were no clear differences at any other time point (Figure 8-20).

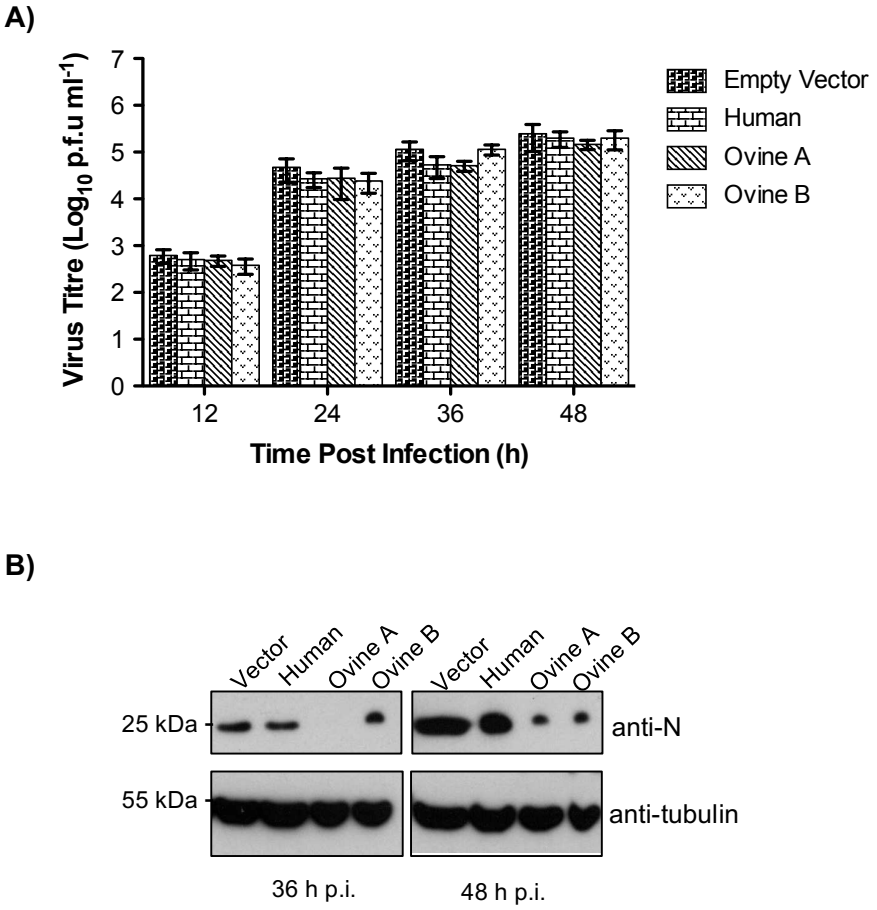


Figure 8-18: BUNV Replication in Cells Over-Expressing BST2

293T cells over-expressing human, ovine A or ovine B BST2 proteins were infected at multiplicity of infection 0.01 with wild type Bunyamwera virus (BUNV). **A)** Clarified culture supernatants were collected at specified hours post infection (h p.i.) and virus release quantified by plaque assay. Infections were carried out in duplicate and mean ± range are shown. **B)** Cell lysates were collected and analysed by western blotting, samples were probed with antibodies targeting BUNV N protein (anti-N) and tubulin (anti-tubulin).

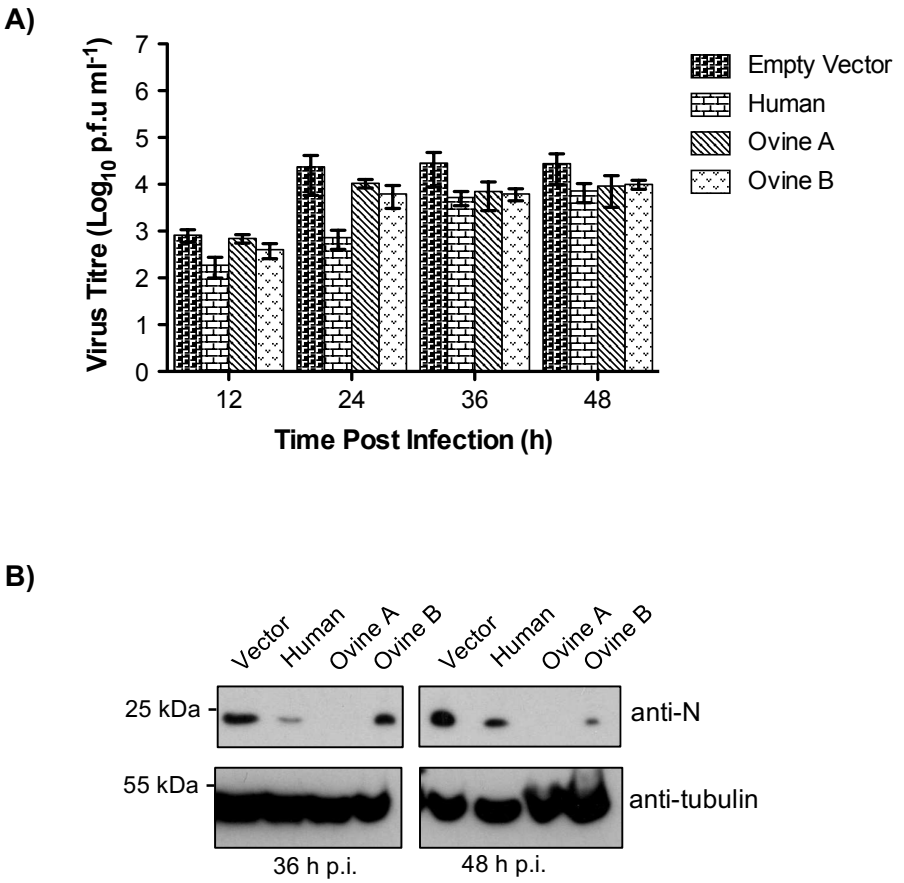


Figure 8-19: rBUNdelNSs2 Replication in Cells Over-Expressing BST2

293T cells over-expressing human, ovine A or ovine B BST2 proteins were infected at multiplicity of infection 0.01 with the recombinant rBUNdelNSs2 virus. **A)** Clarified culture supernatants were collected at specified hours post infection (h p.i.) and virus release quantified by plaque assay. Infections were carried out in duplicate and mean ± range are shown. **B)** Cell lysates were collected and analysed by western blotting, samples were probed with antibodies targeting BUNV N protein (anti-N) and tubulin (anti-tubulin).

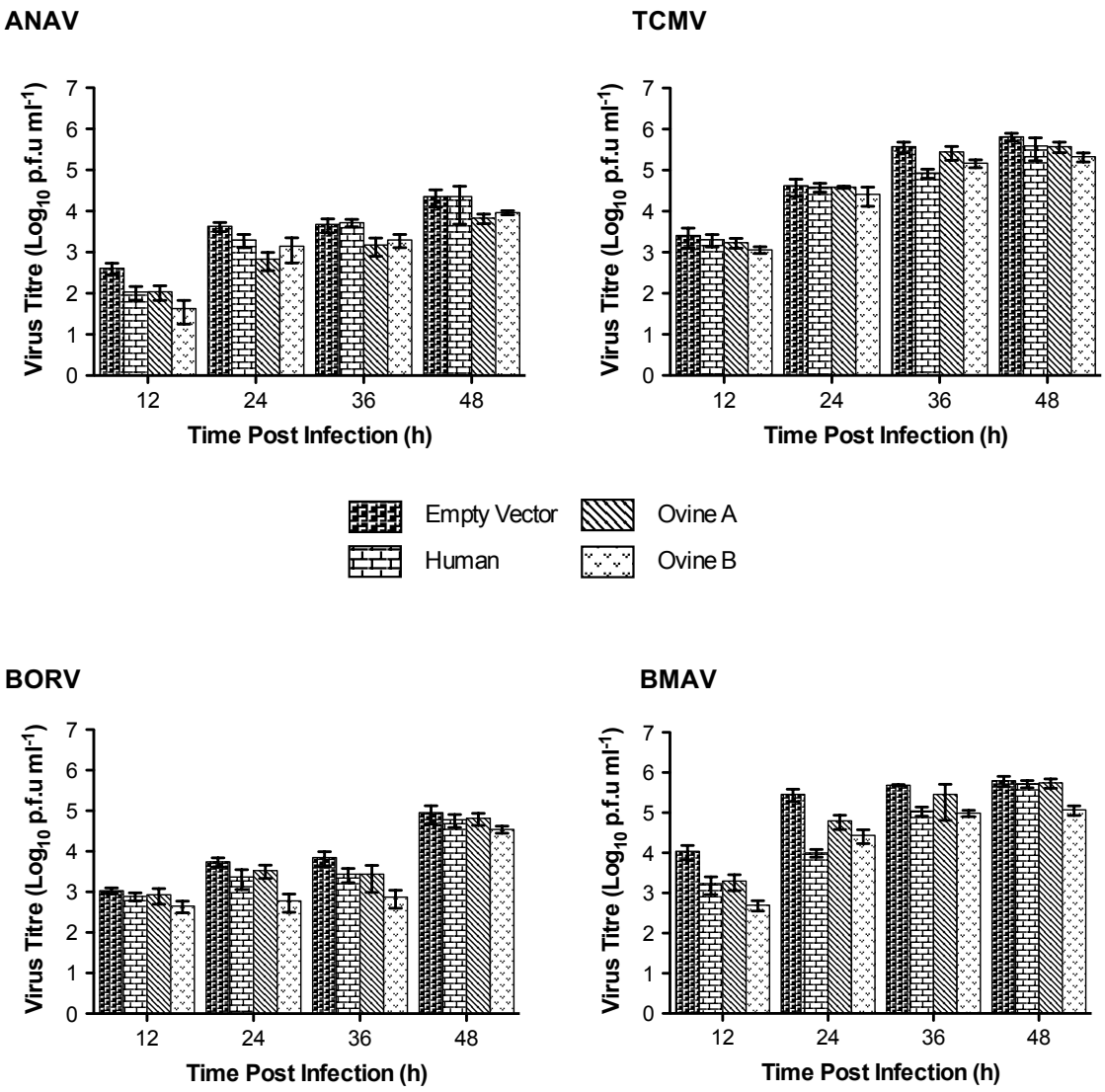


Figure 8-20: Replication of Anopheles A, Anopheles B and Tete Serogroup Viruses in Cells Over-Expressing BST2

293T cells over-expressing human or ovine BST2 proteins were infected at multiplicity of infection 0.01 with Anopheles A virus (ANAV), Tacaiuma virus (TCMV), Boraceia virus (BORV) or Batama virus (BMAV). Clarified culture supernatants were collected at specified hours (h) post infection and virus release quantified by plaque assay on Vero E6 cells. Infections were carried out in duplicate and mean ± range are shown.

Over-expression of hBST2 or oBST2A proteins also appeared to have no effect on BORV replication. However, over-expression of oBST2B did appear to stall virus release in BORV infections with reductions in virus titre at 24 h p.i. and 36 h p.i. (Figure 8-20). BMAV, a member of the Tete serogroup, showed greater evidence of antagonism by BST2, with lower virus titres in hBST2, oBST2A and oBST2B cell lines at 12 h p.i.. This was followed by a 28-fold and 10-fold reduction in virus titres at 24 h p.i. in hBST2 and oBST2B cell lines, respectively (Figure 8-20).

8.4 Discussion

8.4.1 IFI44 and Bunyamwera virus Antagonism

This work confirmed tetracycline inducible expression of FLAG-tagged IFI44 in T-REx IFI44 cells (Figure 8-1A). However, in concurrence with Hallen *et al.,* (2007) the only published paper citing use of an anti-IFI44 antibody, intermittent cross-reaction with a higher molecular weight protein occurred. Hallen *et al,* (2007) utilised an antibody raised in rabbits against a peptide conjugate of IFI44 (aa 271 - 285), which in addition to detecting IFI44 detected an unknown protein at approximately 70 kDa in both IFN-induced and non-induced cells. In this study an anti-FLAG and anti-IFI44 antibody raised against the complete IFI44 protein were used. Cross-reaction with an unidentified protein at approximately 115 kDa occurred in both tetracycline induced and non-induced cells with both anti-FLAG and anti-IFI44 antibodies (Figure 8-1A). Cross-reactive proteins are therefore unlikely to be IFI44 oligomers.

Carlton-Smith and Elliott (2012) reported a reduction in BUNV and rBUNdelNSs2 virus release in T-REx IFI44 cells over-expressing IFI44. More specifically an 18-fold and 6-fold reduction in BUNV virus titre at 36 h p.i. and 72 h p.i., respectively, and a 12-fold reduction in rBUNdelNSs2 virus titre at 72 h p.i. (Carlton-Smith, 2012; Carlton-Smith and Elliott, 2012). Despite replicating the published experimental conditions, the level of antagonism exerted by IFI44 over-expression was lower in this study. Initially, problems ensuring maintenance of an intact cell monolayer throughout the 72 h time course (the cell line is loosely adherent) prevented the acquisition of consistent or reliable results. This was resolved by seeding cells in 35 mm dishes rather

than 6-well plates and carrying out virus dilutions and washes with warmed Opti-MEM, not PBS. Furthermore, utilising a single dish for each time point in place of a single monolayer for the whole time course allowed both protein expression and virus release to be measured. Following these amendments, a significant ($p<0.05$) reduction in virus titre at 8 h p.i. and 12 h p.i. was detected at MOI 0.01 in BUNV infected cells. However, there was no evidence of inhibition of virus release at later time points (Figure 8-4C). Collecting cell monolayers allowed the presence of IFI44 during infection to be confirmed by western blot analysis (Figure 8-4A, Figure 8-5A). Failure to replicate previous findings was therefore not due to IFI44 degradation or failure of IFI44 induction. An additional stock of BUNV was also grown up from an alternative source of elite virus and tested at MOI of 0.01 and 0.001 (data not shown), reducing the likelihood that differences in virus preparations could account for the differing levels of virus antagonism by IFI44 over-expression. Despite failing to show significant inhibition of virus release at later time points (> 12 h p.i.), there was a reduction in viral N protein at 24 h p.i. and 36 h p.i in cells over-expressing FLAG-tagged IFI44 during low MOI experiments with both BUNV and rBUNdelNSs2 (Figure 8-4B, Figure 8-5B). This reduction was not due to differences in cell quantity as there were no clear differences in the level of the cell loading control, tubulin, when N and tubulin were stained for on the same gel (Figure 8-4B, Figure 8-5B). This reduction can also not be accounted for by the pre-treatment of cells with tetracycline as there was no reduction in the level of N protein in T-REx CAT control cells induced with tetracycline compared to those mock induced by the addition of PBS in place of the antibiotic (Figure 8-6). The reduction in virus N protein, coupled with significant reductions in virus titre early in infection supports the hypothesis that IFI44 has anti-bunyavirus activity (Carlton-Smith and Elliott, 2012).

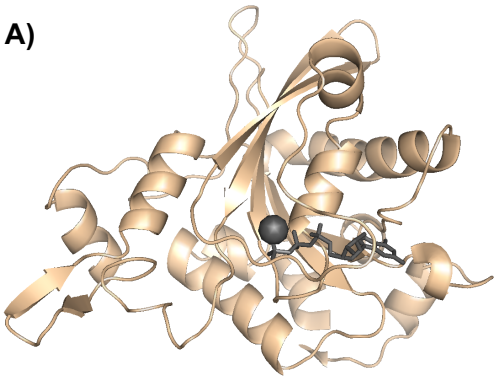
The inability to detect evidence of anti-bunyavirus activity during high MOI infections, with no reduction in viral titre or viral N protein, suggests that the anti-BUNV activity of IFI44 can be overcome by high levels of infection (Figure 8-7, Figure 8-8, Figure 8-9). There was, however, a reduction in the detection of IFI44 during high MOI experiments (Figure 8-2A Figure 8-8, Figure 8-9). This reduction was most apparent in BUNV infections, with no FLAG-tagged IFI44 detected 24 hp.i. in induced T-REx IFI44 cells infected with BUNV at MOI 3 (Figure 8-2A, Figure 8-9). The level of FLAG-tagged IFI44 during high MOI experiments was prolonged by inhibition of the

proteasome (Figure 8-10). BUNV NSs has been linked with degradation of host RNAPII and an attractive hypothesis would be that NSs mediates proteosomal degradation of IFI44 (van Knippenberg *et al.*, 2010). However, as a reduction in FLAG-tagged IFI44 was also detected in rBUNdelNSs2 infections, albeit at a later time point and to a lesser degree, it is likely that an alternative or additional NSs independent mechanism exists (Figure 8-9).

IFI44 is reportedly significantly upregulated in a number of clinical conditions and upon IFN treatment *in vitro* (Nzeusseu Toukap *et al.*, 2007; Wildenberg *et al.,* 2008; Bochkov *et al.*, 2010; Farina *et al.*, 2010). However, this study failed to detect IFI44 in 4 human cell lines (Huh7, A549, HeLa and HEK-293) following IFN treatment. Hallen *et al.,* (2007) found IFI44 to be upregulated in 2 cell lines (ME15 and D10) following treatment with IFNα. It was also reported that IFI44 was virtually uninducible in A549 cells supporting the results of this study. Unfortunately, Huh7, HeLa or HEK-293 cells were not included in the Hallen *et al.,* (2007) paper. However, it would be premature to classify these cell lines as not possessing IFN inducible IFI44 expression as the anti-IFI44 antibody was shown to lack sensitivity in western blots detecting FLAG-tagged IFI44 (Figure 8-1A). Screening cells by quantitative RT-PCR for significant upregulation of IFI44 mRNA may therefore be a more suitable methodology (Hallen *et al.,* 2007; Farina *et al.*, 2010; Gautam *et al.*, 2011).

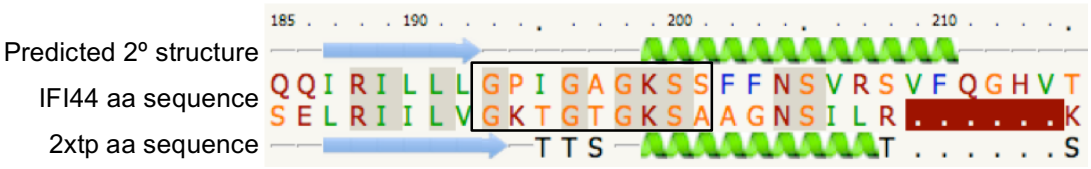
It has been proposed that IFI44 may induce an anti-proliferative state by binding and thereby reducing the availability of GTP, inhibiting the ERK pathway. Cellular fractionation, supported by IF, showed FLAG-tagged IFI44 localised in the cytoplasm, corroborating published data on IFI44 localisation (Figure 8-2) (Hallen *et al.*, 2007; Power *et al.*, 2015). Analysis of IFI44 sequences reveals a Walker A motif (GxxxxGKS) at residues 193 to 200. However, IFI44 has not yet been shown to bind GTP, and is said to lack homology to any known GTPases or G proteins (Hallen *et al.,* 2007). A structural model of IFI44 with 99.9% confidence is obtained when the sequence was entered into Phyre2 (Kelley and Sternberg, 2009). However, this model, which was based on the crystal structure of GIMAP2 (GTPase of immunity associated proteins 2, protein database identifier 2xtp), has only 15% amino acid (aa) sequence identity and 48% coverage, with only 213/444 aa residues aligned (Figure 8-21).

A)



Predicted Binding Site		
Residue		Mean distance (Å)
199	Lys	0.18
200	Ser	0.00
219	Leu	0.56
228	Ser	0.27
251	Asp	0.21

B)



C)

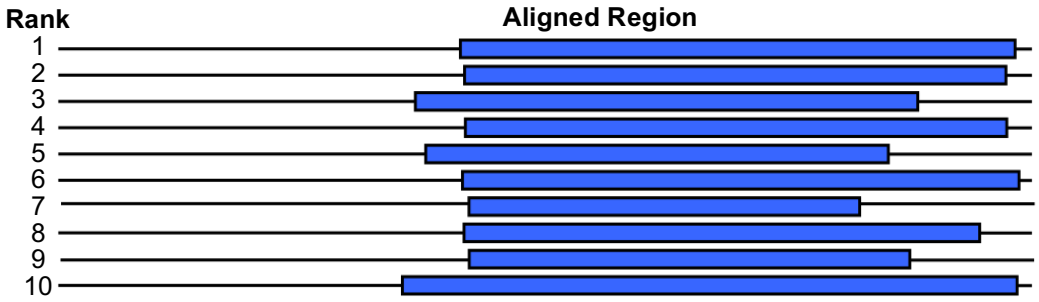


Figure 8-21: Structural Model of IFI44

Computational modelling of IFI44, accession number NP006408.3. **A)** The IFI44 amino acid sequence was analysed by Phyre2, the highest ranking model was based on the crystal structure of GIMAP2, a GTPase of immunity associated proteins 2 (PDB ID code 2xtp) (Kelley and Sternberg, 2009). Using this model, 3D ligand identified a possible binding site with GTP and magnesium acting as ligands. The Phyre2 and 3D ligand data were then combined in MacPyMOL to create the model shown. **B)** Phyre2 alignment data. The Walker A motif, highlighted in a black box, was predicted to be flanked by a β-sheet and α-helix. T=Hydrogen bond, S=bend. **C)** Aligned regions of the top 10 ranking structural models generated by Phyre2, against full length IFI44 are highlighted in blue. There was no coverage of the first 186 aa in any of the 499 hits generated by Phyre2.

Further processing of the Phyre2 IFI44 GIMAP2 model in 3D ligand predicts a binding site with GTP and magnesium (Figure 8-21A). The predicted binding site includes residues 199K and 200S, which form part of the Walker A motif. Furthermore, the Walker A motif is predicted to be preceded by a beta sheet and followed by an alpha helix, a structural feature that is conserved among nucleotide binding proteins (Figure 8-21B), supporting the hypothesis that IFI44 may contain a functional GTP binding site.

Cell proliferation assays coupled with targeted mutagenesis of the Walker A motif would aid in the exploration of this hypothesis. Furthermore, utilisation of IF and recombinant viruses, such as rBUNGc-eGFP which expresses GFP-tagged Gc, or rBUNL4V5 and rBUNLVL5 which express a V5-tagged L protein, would allow possible interactions between IFI44 and viral proteins to be studied (Shi *et al*., 2009; Shi *et al.*, 2010; Power *et al.*, 2015).

8.4.2 Anti-Orthobunyavirus Activity of the IFITM Proteins

Mudhasani et al., (2013) reported that the IFN-induced IFITM proteins have anti-bunyavirus activity, antagonising viruses from the phlebovirus, hantavirus and orthobunyavirus genera. However, the spectrum of activity varied; the phlebovirus RVFV was inhibited by only IFITM2 and IFIMT3, whilst the orthobunyavirus LACV was inhibited by IFITM1, IFITM2 and IFITM3 (Mudhasani *et al.*, 2013). To further investigate the spectrum of anti-bunyavirus activity and to determine if the antagonism of LACV, an orthobunyavirus in the California serogroup, was representative of pan-orthobunyavirus activity A549 cell lines constitutively over-expressing IFITM1, IFITM2 and IFITM3 were made. Over-expression of IFITM2 and IFITM3, but not IFITM1 was confirmed by western blot analysis (Figure 8-11C). Intensity of TagRFP expression was reduced in A549-IFITM1 cells in comparison to IFITM2, IFITM3 and empty vector cell lines (Figure 8-11B). However, lentivirus transduction of A549-IFITM1 cells was confirmed by image based cytometry with > 98% of cells positive for TagRFP (Figure 8-11A).

In this study 5 wild type orthobunyaviruses (BUNV, ANAV, TCMV, BORV and BMAV), and 1 recombinant orthobunyavirus (rBUNdelNSs2) were screened for antagonism by IFITM1, IFITM2 and IFITM3 proteins. In contrast to LACV, BUNV and rBUNdelNSs2 were restricted by over-expression of only IFITM1 and IFIMT3. This was evident by image based cytometry, fluorescent microscopy and virus release assays (Figure 8-12, Figure 8-13, Figure 8-14, Figure 8-15). There was also a reduction in BUNV N protein, detected by western blot analysis on cells over-expressing IFITM1 and IFITM3 (Figure 8-14). In further diversity the Anopheles A and Anopheles B serogroup viruses, ANAV and BORV, had significantly lower titres at 8 h p.i. in cells over-expressing IFITM1, IFITM2, and IFITM3 (Figure 8-16). However, at later time points antagonism only by IFITM2 and IFITM3 was maintained. BMAV, a member of the Tete serogroup, was also antagonised by over-expression of IFITM2 and IFITM3 but not IFITM1 (Figure 8-16). The Anopheles A serogroup virus TCMV was only inhibited by IFITM3 over-expression (Figure 8-16). This demonstrates that not only does the spectrum of activity vary between bunyavirus genera, it also varies within genera, and within orthobunyavirus serogroups, as Anopheles A serogroup viruses, ANAV and TCMV, display differing restriction profiles.

Orthobunyaviruses enter cells by clathrin-mediated endocytosis, and require endosomal acidification for entry into the host cell cytoplasm (Santos *et al.,* 2008; Hollidge *et al.*, 2012). Entry of LACV has also been shown to be Rab5-dependent, Rab7-independent, with acidification of early endosomes resulting in membrane fusion and virion release into the cytoplasm; residual virions are then released from late endosomes by a virus mediated mechanism (Hollidge *et al.*, 2012). Cells over-expressing IFITM1, IFITM2 or IFITM3 display changes in cholesterol homeostasis, with the formation of cholesterol laden endosomal compartments *(*Amini-Bavil-Olyaee *et al.,* 2013; Desai *et al.*, 2014). This has been attributed to the direct interaction of the IFITM proteins with VAPA and disruption of VAPA-OSBP interaction, which deregulates cholesterol homeostasis (Amini-Bavil-Olyaee *et al*., 2013). High levels of cholesterol in endosomal compartments has previously been linked with virus antagonism, inhibiting entry of IAV (Musiol *et al*., 2013; Sun and Whittaker, 2003). However, a general blockade on membrane fusion due to cholesterol deregulation does not explain the differing spectrum of anti-orthobunyavirus antagonism exerted by over-expression of IFITM1, IFITM2 or IFITM3, as observed in this study. Moreover, recent publications have

shown that IFITM3 antagonism of IAV and MERS-CoV is independent of cholesterol, although cholesterol deregulation does occur (Desai *et al.,* 2014; Wrensch *et al.*, 2014).

It is hypothesised that discrete differences in cellular localisation of IFITM proteins, coupled with the point at which viruses exit the endosomal pathway, may play a role in the level of antagonism. IFITM1 predominately localises at the plasma membrane whilst IFITM2 and IFITM3 localise to intracellular compartments (Weston *et al.,* 2014). IFITM3 co-localises with endosomal markers, including Rab7, LAMP1 and CD63; however, the exact localisation of IFITM2 has yet to be established (Feeley *et al.*, 2011; Amini-Bavil-Olyaee *et al.*, 2013; Weston *et al.,* 2014). Indeed there is a growing body of evidence that only viruses which fuse in late endosomes are susceptible to antagonism by IFITM3, whilst virions that exit the endosomal pathway at or before the development of late endosomes may circumvent the full force of IFITM3 (Weidner *et al*., 2010; Feeley *et al.,* 2011; Huang *et al.*, 2011; Anafu *et al.,* 2013; Mudhasani *et al.*, 2013). Exiting from both early and late endosomes, may therefore leave LACV susceptible to antagonism by both IFITM2 and IFITM3, whereas, orthobunyaviruses showing resistance to IFITM1 and IFITM2 antagonism, such as TCMV, may exit the endosomal pathway only at the late endosome (Figure 8-16). To date IFITM3 is the only IFITM protein to antagonise all orthobunyaviruses tested, inhibiting viruses from the Anopheles A, Anopheles B, Bunyamwera, California and Tete serogroups, and therefore may have pan-orthobunyavirus activity (Figure 8-15, Figure 8-15, Figure 8-16) (Mudhasani *et al.*, 2013).

In addition to by-passing IFITM antiviral activity by exiting the endosomal pathway at certain points orthobunyaviruses may directly antagonise the IFITM proteins. The primary virulence factor of orthobunyaviruses, the NSs protein, has been linked with host protein degradation and, during high MOI experiments, IFITM2 and IFITM3 proteins were not detected by western blot analysis (Figure 8-17). However, as expression of IFITM2 and IFITM3 was lost in both BUNV and rBUNdelNSs2 infections this phenomenon cannot be attributed to the NSs protein (Figure 8-17). Moreover, the NSs protein is not encapsidated in mature virus particles and would therefore not be present during initial virus entry, the point at which IFITM proteins act (Novoa *et al*., 2005). Furthermore, TCMV, a naturally occurring non-NSs orthobunyavirus, was resistant to over-expression of IFITM1 and IFITM2, supporting

the hypothesis that resistance to IFITM antagonism cannot be attributed to the NSs protein (Figure 8-16). Unfortunately, due to low virus titre high MOI experiments, and possible loss of IFITM 2 and IFITM3 expression, during TCMV infection could not be investigated. Receptor binding and membrane fusion of orthobunyaviruses is thought to be mediated by the Gc protein and a conserved fusion peptide domain located in the Gc protein, respectively (Sundin *et al.*, 1987; Pekosz *et al.*, 1995; Pekosz and González-Scarano, 1996; Hacker and Hardy, 1997; Shi *et al.*, 2007; Shi *et al.,* 2009). However, as virus replication is not upregulated in cells over-expressing IFITM proteins it is unlikely that the Gc proteins of resistant viruses are utilising IFITM proteins as virus entry co-factors, like HCoV-OC43 (Zhao *et al.,* 2014). It has been shown that IFITM1 binds HCV coreceptors CD81 and occludin, thus inhibiting virus entry, and it may be that subtle differences in entry co-factors account for the differing spectrum of IFITM antagonism seen in the orthobunyavirus genus (Narayana *et al.*, 2015; Wilkins *et al.*, 2013).

8.4.3 Anti-Orthobunyavirus Activity of BST2 Proteins

Orthobunyavirus assembly occurs at the Golgi where glycoproteins Gn and Gc accumulate and associate with viral RNPs. Virions with Golgi-derived envelopes are then transported via the exocytic pathway to the cell surface where membrane fusion between the plasma membrane and virion containing vacuole occurs, releasing virions into the extra-cellular environment (Novoa *et al.*, 2005; Fontana *et al.,* 2008). It is at this stage of the virus life cycle that the membrane bound ISG, BST2, is proposed to antagonise. BST2 proteins are found in the plasma membrane, trans-Golgi network and endocytic compartments, and their expression antagonises a wide range of enveloped viruses, 'tethering' virus particles to cellular membranes (Kupzig *et al.,* 2003; Neil *et al.*, 2008; Van Damme *et al.*, 2008; Jouvenet *et al.,* 2009; Sakuma *et al.*, 2009; Radoshitzky *et al.*, 2010; Weidner *et al.,* 2010).

It has been hypothesised that BST2 proteins may act as a host restriction factor for orthobunyaviruses as replication of SBV, a pathogen of ruminants, was antagonised by over-expression of hBST2, but not oBST2A or oBST2B (Personal communication, Dr Mariana Varela, The University of Glasgow). In agreement with this finding the virus

titre of BUNV, which is known to infect humans and domestic animals, was not inhibited by over-expression of hBST2, oBST2A or oBST2B (Figure 8-17). Moreover, the recombinant rBUNdelNSs2 virus, which lacks the primary virulence factor NSs, displayed 24-fold lower titres in cells over-expressing hBST2 at 24 h p.i., which was followed by reduced detection of N protein by western blot analysis at 36 h p.i. (Figure 8-18). In further support of the proposed host restriction role for BST2 proteins, the Anopheles A and Anopheles B serogroup viruses, TCMV and BORV, which are associated with human infection, were not antagonised by over-expression of hBST2 (Figure 8-18). Furthermore, there was a 28-fold reduction in the virus titre of BMAV, a member of the Tete serogroup not linked with human infection, at 24 h p.i. in cells over-expressing hBST2 (Figure 8-18). In contrast, there was minimal antagonism of ANAV, an Anopheles A serogroup not associated with human or ovine infection (da Rosa *et al.,* 1992). ANAV induces high levels of IFN during virus infection, and was sensitive to IFN pre-treatment in vitro (Figure 6-5, Figure 6-9). The 293T-tetherin cell lines are IFN-competent and as ANAV titres were low, $\leq 3 \times 10^4$ p.f.u ml^{-1} at 48 h p.i., sensitivity to a robust type I IFN response may be masking full antagonism of BST2 over-expression (Figure 8-18).

In testament to the anti-viral role BST2 proteins play, a number of BST2 antagonists have been identified, including HIV-1 Vpu, HIV-2 Env, Kaposi's sarcoma-associated herpesvirus (KSHV) K5 and the glycoproteins of Ebola virus and Sendai virus (Bampi *et al.,* 2013; Douglas *et al.*, 2009; Goffinet *et al.*, 2009; Le Tortorec and Neil 2009; Mansouri *et al.*, 2009; Iwabu *et al.,* 2009; Kaletsky *et al*., 2009; Habermann *et al.*, 2010; Hauser *et al.*, 2010; Lopez *et al.*, 2010; Kühl *et al*., 2011; McNatt *et al.*, 2013;). KSHV K5 ubiquinates hBST2, targeting the protein for degradation, whilst HIV-1 Vpu removes and displaces hBST2 from the plasma membrane and sites of virus assembly (Douglas *et al*., 2009; Goffinet *et al.,* 2009; Iwabu *et al*., 2009; Mansouri *et al.*, 2009; Habermann *et al.,* 2010; McNatt *et al.*, 2013). The exact mechanism of antagonism by the Ebola virus glycoprotein has yet to be elucidated although it has been shown to directly interact with BST2, which remains in the plasma membrane (Kaletsky *et al.*, 2009; Lopez *et al.*, 2010; Kühl *et al.*, 2011). Results presented in this chapter suggest that the NSs protein of BUNV may antagonise BST2, as the recombinant rBUNdelNSs2 virus which lacks a functional NSs protein displayed increased sensitivity to hBST2 in a virus release assay (Figure 8-19). BUNV NSs is a potent

antagonist of the type I IFN system; however, in this study hBST2 was constitutively over-expressed and not under control of the type I IFN signalling pathway. Moreover, NSs mediated inhibition of RNAPII would be expected to effect expression of hBST2, oBST2A and oBST2B to similar levels, but rBUNdelNSS2 only showed increased sensitivity to hBST2 suggesting targeted or species specific antagonism of hBST2. Furthermore, additional mechanisms of orthobunyavirus evasion or antagonism of BST2 proteins must exist as naturally occurring non-NSs viruses, TCMV and BORV, were also resistant to over-expression of hBST2 and oBST2A (Figure 8-20).

8.4.4 Concluding Remarks

The results presented in this chapter suggest that in addition to NSs mediated inhibition of the type I IFN response orthobunyaviruses possess uncharacterised mechanisms of ISG evasion and antagonism. BUNV and rBUNdelNSs2 viruses consistently down regulated the expression of ISGs (IFI44, IFITM3 and MxA) during high MOI experiments (Figure 8-2A, Figure 8-8, Figure 8-9, Figure 8-10, Figure 8-17). To lose detection of constitutively over-expressed proteins suggests ISG degradation, rather than general protein shut-off due to cap snatching by virus L protein as this would also occur in BMAV and BORV infections. Furthermore, the utilisation of divergent orthobunyaviruses questions the utility of extrapolating results from 'type' species, as the activity of ISGs varied not only within the orthobunyavirus genus but within virus serogroups. The availability of divergent viruses, including naturally occurring non-NSs viruses, may help elucidate ISG mechanisms of action and virus evasion strategies, as uncharacterised, non-NSs mechanisms of over coming the host immune response exist.

Chapter 9: Final Reflections

9.1 Fulfilment of Aims

The original, primary aim of my PhD was to investigate the anti-bunyavirus activity of IFI44. In brief, IFI44 was shown to antagonise BUNV and rBUNdelNSs2 virus replication during low MOI experiments, with a significant decrease in viral titre and N protein levels early in infection (Figure 8-4. Figure 8-5, Figure 8-7). However, the anti-viral activity of IFI44 was limited to early time points only (< 24 h p.i.) and there was no evidence of anti-viral activity at MOI ≥ 0.1 (Figure 8-4, Figure 8-5, Figure 8-7). The decision was therefore made to change the direction of my work to focus on orthobunyaviruses that were identified by Mohamed *et al.,* (2009) as lacking a functional NSs ORF.

The change in project direction coincided with reports of anti-bunyavirus activity of the membrane bound ISGs, IFITM and BST2 (Mudhasani *et al*., 2013; Personal communication, Dr Mariana Varela, University of Glasgow). To test for anti-orthobunyavirus activity cell lines over-expressing IFITM and BST2 proteins were utilised (Figure 8-11). Anti-orthobunyavirus activity was initially confirmed for BUNV and rBUNdelNSs2, with reduced viral titres during low MOI experiments (Figure 8-14, Figure 8-15, Figure 8-18, Figure 8-19). Anti-orthobunyavirus activity was further investigated by screening cell lines with viruses from the Anopheles A (ANAV and TCMV), Anopheles B (BORV) and Tete (BMAV) serogroups (Figure 8-16, Figure 8-20). The results provide evidence of non-NSs mediated mechanisms of ISG evasion and antagonism as detection of IFI44, IFITM3 and MxA were repeatedly reduced during high MOI experiments with rBUNdelNSs2 (Figure 8-8, Figure 8-9, Figure 8-10, Figure 8-17). Furthermore, TCMV, a naturally occurring non-NSs virus, was resistant to over-expression of IFITM and BST2 proteins (Figure 8-16, Figure 8-20). The results also support the hypothesis that BST2 proteins may play a role in host restriction (Figure 8-18, Figure 8-19, Figure 8-20).

To study the prevalence of non-NSs viruses within the orthobunyavirus genus a panel of 50 virus isolates belonging to 14 serogroups was obtained from The World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, USA. This ultimately resulted in a panel of 26 viruses that replicated in Vero E6 cells. In collaboration with Dr Thomas Briese, CII, New York, HTS was carried out

on 21/26 viruses during a 5 week placement at the CII. HTS data supplemented with RACE-PCR, RNA ligation and over-lapping PCR reactions resulted in complete genome sequences for 19 of these viruses (Table 5-2). For the remaining 2 viruses, PLSV and WELV, complete coding sequence and cRNA 5'UTRs and complete S segment sequences were obtained (Table 5-2). The sequencing data identified viruses in the Anopheles A, Anopheles B, Capim, Guama, Minatitlan, and Tete serogroups as non-NSs viruses. It has been postulated that loss of NSs occurs through truncations at the C terminus. Interestingly, WELV, a Tete serogroup virus that branches basally to the rest of the Tete serogroup was predicted to possess a full length NSs ORF, whilst other members of the serogroup contain truncated ORFs that would encode putative proteins of only 20 - 36 aa (Figure 5-6, Figure 5-9, Table 5-2). The sequencing data also gives further evidence of virus reassortment and challenges current classification methods (Figure 5-6, Figure 5-7, Figure 5-8, Figure 5-9, Table 5-4).

To supplement genome sequencing data, basic biological characterisation was carried out on all viruses with evidence of replication in Vero E6 cells. This revealed several interesting and novel findings. Firstly, the presence of a putative NSs ORF does not correlate with *in vitro* IFN antagonism as Gamboa serogroup viruses struggled to replicate at low MOI in IFN-competent A549 cells and induced high levels of IFN during virus infection (Figure 4-7, Figure 6-1, Figure 6-2). Secondly, MPOV, which is predicted to encode an NSs protein that truncates prior to the C terminal Med8 interaction domain, is associated with decreased IFN antagonism *in vitro* in comparison to BUNV, BWAV, NDV, or EREV (Figure 6-1, Figure 6-2). Thirdly, antagonism of the type I IFN system *in vitro* is not exhibited by all viruses associated with human infection as neutralising antibodies to BORV, a non-NSs virus that induced high levels of IFN *in vitro,* have been isolated from villagers in Brazil (Figure 6-5). However, IFN antagonism *in vitro* did still correlate with viruses associated with clinical illness in man. Lastly, metabolic labelling gave evidence of host cell shut-off in viruses lacking an NSs ORF (Figure 4-10, Figure 4-11, Figure 4-13).

Determining the complete genome sequence, and not just complete coding sequence, facilitated the development of a reverse genetics system for BMAV, a member of the Tete serogroup. This allowed mutagenesis of the BMAV N protein, and the role of the N protein in low IFN activation to be investigated. Recombinant viruses with mutations

proximal to the amino terminus, a region involved in RNA binding and N-N interactions in other virus N proteins, induced higher levels of IFN *in vitro*. It is postulated that the highly disordered amino terminal of BMAV N protein may bind RNA in an, as yet, uncharacterised conformation with RNP structure contributing to the low level of IFN activation observed *in vitro*.

9.2 The Bigger Picture

The work presented in this thesis contributes to 4 major lines of study: IFN induction and RNA encapsidation, pathogenesis and the NSs protein, ISG screening, and virus evolution and classification.

In 2013 a series of publications reported crystal structures for orthobunyavirus N proteins, shedding light on N-N interactions, RNA binding and RNP topology (Figure 1-4) (Ariza *et al.,* 2013; Reguera *et al.*, 2013; Niu *et al.*, 2013; Dong *et al.*, 2013b). The N protein structures were highly conserved, despite low sequence homology, with RNA binding occurring in a central cleft and N protomers interacting in a head-to-tail fashion to form ordered tetramers in the presence of RNA. However, the work presented in this thesis gives evidence of variation within the Tete serogroup N protein amino terminus, a key region in N-N interactions and RNA binding. Interestingly, Tete serogroup viruses were shown to only induce low levels of IFN *in vitro* despite lacking an NSs protein (Figure 6-6, Figure 6-7). Furthermore, recombinant viruses with mutations in the N terminus of the virus N protein induced higher levels of biological IFN and IFN-β promoter activity than wt virus (Figure 7-13). Determining the crystal structure of Tete serogroup N proteins will greatly aid in future investigations of RNA binding and RNP formation, and structural studies are ongoing. The amino termini of Tete serogroup N proteins are predicted to be highly disordered (Figure 7-2). This may complicate structural analysis by x-ray crystallography as electron density is not observed for disordered regions of protein sequence. In lieu of structural data measuring IFN induction by purified RNPs and assaying for resistance to RNase may help elucidate the role of the N protein in IFN induction and RNA binding.

A central dogma in bunyavirology is that the NSs protein, the primary virulence factor, antagonises the type I IFN system at the transcriptional level through inhibition of RNAPII. The work presented in this thesis challenges this conserved mechanism. Firstly, NSs encoding NDV antagonised IFN induction *in vitro* but did not cause host cell shut-off (Figure 6-1, Figure 6-2, Figure 6-4, Figure 4-12). Secondly, Gamboa serogroup viruses, which are predicted to encode an NSs protein of 130 aa, struggled to replicate in IFN-competent A549 cells at low MOI and induced high levels of IFN during virus infection. Thirdly, non-NSs viruses in the Anopheles A, Capim and Tete serogroups showed evidence of host-cell shut-off in metabolic labelling experiments (Figure 4-.10, Figure 4-11, Figure 4-13) Lastly, non-NSs viruses TCMV, GJAV, GMAV, BMAV, TETEV and TSUV induced lower levels of IFN *in vitro* than rBUNdelNSs2 (Figure 6-5). Together these data demonstrate that there is still much to learn about the underlying mechanisms of orthobunyavirus pathogenesis and broadening research to include non-type species will expedite this endeavour.

Testing ISG cell lines with divergent, non-type, virus species showed that the level of anti-virus activity varied within the orthobunyavirus genus, something that had already been demonstrated for MxA (Bridgen *et al.,* 2004). However, the work presented in this thesis shows not only that activity can vary within the genus, but also within a serogroup and within a virus species (Figure 8-16, Figure 8-20). This questions the utility of using type species in ISG screens as findings cannot be extrapolated to other viruses of interest. Furthermore, as IFITM proteins antagonise virus entry, trapping incoming virions within endocytosed vacuoles, studying the pattern of virus restriction may help elucidate mechanisms involved in virus entry.

At the start of my studies complete genome sequences were available only for viruses in the Bunyamwera, California and Simbu serogroups; now, with the work presented in this thesis and recently published reports, complete coding sequences are available for viruses in 15 out of 18 ICTV-recognised serogroups and the newly proposed Mapputta serogroup. Genome sequencing has revealed that non-NSs viruses are far more prevalent than first thought, and that non-NSs viruses from multiple serogroups are associated with human infection. This challenges our understanding of orthobunyavirus pathogenesis as the NSs protein was thought to be an essential virulence factor in virus zoonoses (Hart *et al.,* 2009). Understanding the mechanisms behind non-NSs virus

pathogenesis is vital as deletion of the NSs ORF is a key principal in recombinant vaccine design. Moreover, it has been documented that recombinant viruses genetically modified to lack an NSs ORF can recover virulence over virus passage (Personal communication, Dr Mariana Valera, The University of Glasgow).

Genome sequencing has also contributed to the debate surrounding orthobunyavirus classification. Orthobunyaviruses are traditionally classified by antigen cross-reactions in NTs and CFTs (Plyusnin *et al.*, 2012). This system struggles to classify reassortant viruses as NTs, which detect antibodies against the virus glycoproteins, may give conflicting results from CFTs, which detect anti-N antibodies. Genome sequencing, coupled with phylogenetic analysis, gives a detailed map of virus evolution and it has been hailed as a possible solution for virus classification. However, phylogenetic analysis is only as good as the sequence data it is based on, and a strategy for the classification of reassortant viruses would still need to be developed. Moreover, there are documented discrepancies between virus sequences published by different laboratories and new serogroups have been proposed on the basis of partial sequencing data, even when there were clear gaps in phylogenetic coverage (Nunes *et al.*, 2005; Bakonyi *et al.,* 2013; Forshey *et al.,* 2014; Groseth *et al.,* 2014; Hang *et al.*, 2014; Hontz *et al.*, 2015). Therefore, any classification system based on phylogenetic analysis will require stringent controls. This could involve a minimum requirement for the inclusion of specified reference sequences, although this will need continued monitoring and updating to reflect current knowledge.

Lastly, the advantages of carrying out basic biological characterisation along side HTS must be emphasised; otherwise it is impossible to unravel the link between genome characteristics and biological phenotype. Moreover, the importance of determining complete genome sequences, as opposed to complete coding sequence, should also be noted. Reverse genetics is a powerful tool that allows manipulation of the virus genome and the correlation between genotype and phenotype to be studied and robust reverse genetics systems have been developed for viruses in the Bunyamwera, Simbu, California and now Tete serogroups. They would not have been possible without complete genome sequencing.

The emergence and rapid spread of SBV in Europe in 2011 highlighted the continued threat that orthobunyaviruses pose and emphasised the ongoing threat of virus emergence and spread. There are no licensed treatments for orthobunyavirus infections with therapeutic options limited to broad spectrum antivirals, that have questionable benefit, and general supportive measures. There has been limited success in developing vaccines for livestock pathogens such as AKAV, however, most disease control programmes rely on vector control and vector avoidance as the primary intervention. Understanding the molecular basis of virus replication and pathogenesis will facilitate the development of effective therapies, whilst understanding virus evolution will facilitate public health planning.

Appendices

Appendix 1: Primer Sequences

A) Primers for RACE-PCR and UTR Sequencing

Primer Name	Sequence (5'- 3')	Description	Designed
Oligo d(T) AP	GACCACGCGTATCGATGTCGAC	Anchor primer used in all RACE reactions.	Roche
Oligo d(T) Seq Rv	GTCGACATCGATACGCG	Reverse sequencing primer targeting the anchor sequence of oligo d(T) primer.	This study
ANAV S 5'UTR	AATGGCATGATGATCTTTAGC	RACE-PCR and UTR sequencing primers for Anopheles A virus S, M and L cRNA as indicated.	This study
ANAV S 5'UTRseq	AATGTTAGGTCACTTGATG		
ANAV S 3'UTR	GCTAGAGTTAAGAAAGGAC		
ANAV S 3'UTRseq	GAGACAGAGATATGGAGG		
ANAV M 3'UTR	ATAGCCAAGACAACCAAG		
ANAV L 5'UTR	CGATTTGTGGCAATCCAT		
ANAV L 5'UTRseq	CTGATGCTGGATTTCATTG		
ANAV L 3'UTR	TTCAACTGCAGAAATGCTTG		
ANAV L 3'UTRseq	GCCGTGTGCATTCTTGT		
5 ColAn S 5'UTR	CTAACGGCATAACCAGAACTG	RACE-PCR and UTR sequencing primers for ColAn 57389 virus S, M and L cRNA as indicated.	This study
5 ColAn S 5'UTRseq	GGACCTCTCTGTTCATGG		
5 ColAn S 3'UTR	ATCTTGGATTTGCACCTGG		
5 ColAn S 3'UTRseq	TGGTAAAGAGTGCCGTTG		
5 ColAn M 5'UTR	CGGATTTTCACATGCTCAC		
5 ColAn M 5'UTRseq	TCCTCCAGTCTTCCACAG		
5 ColAn M 3'UTR	ACCAATGCTGGAAATCAAC		
5 ColAn M 3'UTRseq	GCAGGGTTGGAGAAGAG		
5 ColAn L 5'UTR	CAATCGGGTTAGGTATGTGC		
5 ColAn L 5'UTRseq	AGGATGTCAACAAGAGGG		
5 ColAn L 3'UTR	GACCCTATTTACCACACATTTG		
5 ColAn L 3'UTRseq	TCTACTGTGCTTGCCAG		
6 LMV S 5'UTR	AGCTAGCGGCATAATCAGAAC	RACE-PCR and UTR sequencing primers for Las Maloyas virus S, M and L cRNA as indicated.	This study
6 LMV S 5'UTRseq	CCTGGCATCAACTGAAC		
6 LMV S 3'UTR	GGGGTAGTTAGAGTTAAAAAGGG		
6 LMV 3'UTRseq	GAATTGCTCCAGCCGAC		
6 LMV M 5'UTR	AAGTTATTGTTGTGCCGCTG		
6 LMV M 5'UTRseq	CCTCCCAGCATCTTCTC		
6 LMV M 3'UTR	TGAAAATTGAAGCTGGGCAC		
6 LMV M 3'UTRseq	GCATGCTCCTGTATTGGT		
6 LMV L 3'UTR	CCATCAAGCAAGCACTTCAC		
6 LMV L 3'UTRseq	ACCACACATTTGATATGCC		
6 LMV L 5'UTR	GTTCTTGGGTTGATTTGGAG		
6 LMV L 5'UTRseq	TTCATTCACAGCCCGAG		
9 TRMV S 5'UTR	TAACACCAGCCTTTTCAGCC	RACE-PCR and UTR sequencing primers for Trombetas virus S, M and L cRNA as indicated.	This study
9 TRMV S 5'UTRseq	CCTGGCATTAACTGAACC		
9 TRMV S 3'UTR	AGGAGTGGTCAGAGTTAAGAAAG		
9 TRMV S 3'UTRseq	ATGGTTAAGAGTGCAGTTG		
9 TRMV M 5'UTR	AGAGAGACGCTAGTTGAAGT		
9 TRMV M5'UTRseq	TAAAGGACCTCCGACCA		
9 TRMV M 3'UTR	CAACACCGATGCCCAAATACC		
9 TRMV M 3'UTRseq	ATTCCTGCACTACCTGG		
9 TRMV L 5'UTR	TCTTGTTCTCGGGTTGATCT		
9 TRMV L 5'UTRseq	CGGGTTAGGTATATGCAC		
9 TRMV L 3'UTR	AAACAAGCACTCCACAATGAG		
9 TRMV L 3'UTRseq	CGATGTGGTCGGAAATG		
24 TCMV S 5'UTR	CGAGCCAGCATTGTATATATCC	RACE-PCR and UTR sequencing primers for Tacaiuma virus BeAn73 S, M and L cRNA as indicated.	This study
24 TCMV S 5'UTRseq	AAATGCCCTGTTATGCG		
24 TCMV S 3'UTR	AATATGACACCTGGAGAATGGA		
24 TCMV S 3'UTRseq	ATTCCCGTCCTCATGTTG		
24 TCMV M 5UTR	CACCAACTTGCTTTTCTGGAC		
24 TCMV M 5'UTRseq	GCTTGTGGAGATTTGGAC		

24 TCMV M 3'UTR	GGAGAGAGATGACAAATGCAG		
24 TCMV M 3'UTRseq	CGGGTTCTTGTTCAGTC		
24 TCMV M 4267fw	TAAGAAAGCAGCAGGCAG		
24 TCMV L 5UTR	TTTGATAGTCATGTGGACGG		
24 TCMV L 5'UTRseq	CAGGGGTAATGTTTGGC		
24 TCMV L 3'UTR	GAATGCCTAACACCCCAAGA		
24 TCMV L 3'UTRseq	GTGCTTTTGACTTTTCTGG		
24 TCMV L 3'UTRseq 2	ACAGATTCACTTGTGCT		
31 ANBV S 5'UTR	CCTCCCGGTAAAGTTCAAGA	RACE-PCR and UTR sequencing primers for Anopheles B virus S, M and L cRNA as indicated.	This study
31 ANBV S 5'UTRseq	ACGTTTTCATCTGCTGC		
31 ANBV S 3'UTR	TGTAAAGAAAGGGCAAATGCAG		
31 ANBV S 3'UTRseq	CGTCTAGGCCTCACATTG		
31 ANBV M 5'UTR	CTGAGTGTATGATTCCTGTGTC		
31 ANBV M 5'UTRseq	CTGCAGAGCTTCCAATC		
31 ANBV L 5'UTR	CTTAACTTCTCTGAAACATCCC		
31 ANBC L 5'UTRseq	ACGTCGTTCCTGTACTC		
31 ANBV L 3'UTR	GTAAAATATGAGGTGAACTGGC		
31 ANBV L 3'UTRseq	CTTGATTGAACTGGCTCC		
25 BORV S 5'UTR	AGAGGCATCACAAATTGGT	RACE-PCR and UTR sequencing primers for Boraceia virus S, M and L cRNA as indicated.	This study
25 BORV S 5'UTRseq	TAAGGTCAGTAGGCTGC		
25 BORV S 3'UTR	GATGCAACCCGAATTCTCAG		
25 BORV S 3'UTRseq	AGCTGAATGGATGACCAC		
25 BORV L 5'UTR	GTTTCACGGGCAACTTTCAT		
25 BORV L 5'UTRseq	TGGGTCAAAATCATCAATACAG		
25 BORV L 3'UTR	GGCATTTTATCTGTCCTGGTGA		
25 BORV L 3'UTRseq	GCTTCCAAAGGTGTTCTTCA		
25 BORV Mm 3'UTR	GGCACACCAGGAAGAACTCC		
25 BORV Mgh 3'UTR	GCTACCAGACGTCCAGTTTCA		
25 BORV Mgh 5'UTRsq	CTGGCAGCTCAAATGATTCT		
25 BORV Mm 5'UTR	TCATAATCGGGCCACCAATC		
25 BORV Mgh 3'UTRsq	CCCAAATTTCATTTCCATTG		
25 BORV Mgh 5'UTR	ATTGAGGCGTTCCATGTCCT		
25 BORV Mm 5'UTRsq	ACCTGTTGGTGGGTTTTTCT		
25 BORV Mm 5'UTRsq	TTTTTCTGGACGACATTTCC		
29 NDV S 5'UTR	AACATTTCTGTGCCTGGG	RACE-PCR and UTR sequencing primers for Nyando virus S, M and L cRNA as indicated.	This study
29 NDV S 5'UTRseq	TATTGACCACTTCCACCT		
29 NDV S 3'UTR	TCCCAGGCACAGAAATG		
29 NDV S 3'UTRseq	AATTCCTGGGCCGATTC		
29 NDV M 5'UTR	GTGTGTTTGAAGAACCTGA		
29 NDV M 5'UTRseq	AATGTGAGGTCTTCGTG		
29 NDV M 3'UTR	TGGTTAGGTGAAGCAGGT		
29 NDV M 3'UTRseq	ACTAGACACATTCAAGAGC		
29 NDV L 5'UTR	TGATTGACACCTCCTGAG		
29 NDV L 5'UTRseq	ACTGGTAGTTGTCTGGAG		
29 NDV L 3'UTR	GCTAGCAAATGACTTCGAC		
29 NDV L 3'UTRseq	TCTGGTGGATGAGAAAGTG		
29 NDV S 3'UTRseq2	TGACACCAAAAGACCTTC		
34 M 4304 Fw	GGCCTACTGATTTCAATTCT	RACE-PCR and UTR sequencing primers for Eretmapodites virus M and L cRNA as indicated.	This study
34 M 219 Rv	ATATCCCGCTAGTGTTGC		
34 L 112 Rv	GCCTTGCAATGAGTATGTC		
34 L 6799 Fw	ATGGGTAGGAGCATGTTTGA		
34 L 370Rv	AGTAGTGGGCATATAGTTGA		
28 TET S 5'UTR	GCCCCTTTCTTTCCAGGT	RACE-PCR and UTR sequencing primers for Tete virus S, M and L cRNA as indicated.	This study
28 TET S 5'UTRseq	CTCAACAGCCAGGTGACG		
28 TET S 3'UTR	TGGCTCATGCTATTTTGCTG		
28 TET S 3'UTRseq	GTTCTTCCCGCTTGCAGT		
28 TET M 5'UTR	TCAGATCGGCATGAAAATGA		
28 TET M 5'UTRseq	GATTGCATTTGTGCCAGTTT		
28 TET M 3'UTR	GTCTGCGGGCATAACATACC		
28 TET M 3'UTRseq	TGCAAAGTGAAAGAGGAAGG		
28 TET L 5'UTR	CCACTCATGGGGTTTGCAC		
28 TET L 5'UTRseq	AAACATCTTGTCGCCAACTC		
28 TET L 3'UTR	ATAGGGCTGGGCTAGACGAC		
28 TET L 3'UTRseq	GATGAAAACAAACCGGACAG		

27 BMV S 5'UTR	GCATGGGCTTCTTCAGGTATTC	RACE-PCR and UTR sequencing primers for Batama virus S, M and L cRNA as indicated.	This study
27 BMV S 5'UTRseq	GCATCCTTGCTTTTAGGC		
27 BMV S 3'UTR	TACCTGGAAGGAGGGAGCAA		
27 BMV S 3'UTRseq	CTCAAGTCTGGCTCGGTG		
27 BMV M 5'UTR	CGGCATGAAAACGATTTAGGT		
27 BMV M 5'UTRseq	CTTGCTCTCATTGCGTGTT		
27 BMV M 3'UTR	TGTCCCCAAGATCAAACAA		
27 BMV M 3'UTRseq	GCACCAATTGATCAGACACAC		
27 BMV L 5'UTR	CATTTTGTTCCCGACCTTGAC		
27 BMV L 5'UTRseq	CAAAGCAGAACTCCCTAGCA		
27 BMV L 3'UTR	TGTATGCACAGAGCCGGATT		
27 BMV L 3'UTRseq	TTGGGGTGAAATCTTTGCT		
20 MTRV S 5'UTRseq	CTTTTGCCTCCTTAGCC	RACE-PCR and UTR sequencing primers for Matruh virus S, M and L cRNA as indicated.	This study
20 MTRV S 5'UTR	GATTTGCTGGGTGAAGGGTG		
20 MTRV S 3'UTRseq	GCAGCGCATACAAGGTC		
20 MTRV S 3'UTR	ATAAGGAGAAAATGGACACGG		
20 S 865Fw	ATTTTTATACCAACCCCGAC		
20 MTRV M 5'UTRseq	TCTCGACTGGATTGCAC		
20 MTRV M 5'UTR	GTTCCTTCTTCCTTGTCAACT		
20 MTRV M 3'UTRseq	GACCTACGATGCACAAC		
20 MTRV M 3'UTR	GTTGCTAAAAATGAAGTCCTGG		
20 M 4287 Fw	AGACCACCTAAAACTACAGC		
20 MTRV L 5'UTRseq	CGCCAATGTAAAAGTCAG		
20 MTRV L 5'UTR	TGCCCTTACTATTACTACCTCA		
20 MTRV L 3'UTRseq	ACATTGTTGAGCAAGCAG		
20 MTRV L 3'UTR	ATGTACAGAGAAGGAATGGATG		
21 TSUV S 5'UTR	TATGACGGTTTGCAGGATGATG	RACE-PCR and UTR sequencing primers for Tsuruse-like virus S, M and L cRNA as indicated.	This study
21 TSUV S 5'UTRseq	CATCATCTCCTTTGCCTC		
21 TSUV S 3'UTR	GGCTGTTGGATTGGCTAGATG		
21 TSUV S 3'UTRseq	GATGCTGACAACTGGTG		
21 TSUV M 5'UTR	GTTCCTTCCTCTTTGTCTATCG		
21 TSUV M 5'UTRseq	CCCTCCAGTTGTTTCTAC		
21 TSUV M 3'UTR	GGAAATGCACAGAAGAAAGTTG		
21 TSUV M 3'UTRseq	GTGAAGGAGGAAGGATTG		
21 TSUV L 5'UTR	GTCAGCAAAAGCATTCATGT		
21 TSUV L 5'UTRseq	GCTCGTGTCATCTGTTG		
21 TSUV L 3'UTR	GTGGAACAAGCAGAGTATGAG		
21 TSUV L 3'UTRseq	CACACTTCAAACGTAAAGC		
22 WELV S 5'UTR	TCTTTCTATCTCTTCCTTGTGC	RACE-PCR and UTR sequencing primers for Weldona virus S and L cRNA as indicated.	This study
22 WELV S 5'UTRseq	GTGACCTCCCAAGAACC		
22 WELV S 3'UTR	AGCACAAGGAAGAGATAGAAAG		
22 WELV S 3'UTRseq	AAGTTCTACCCACTGGC		
22 S 120Rv	ATCCTCTAAACCTGCACCTG		
22 S 796Fw	AACATCAATTGATTTAGCTGTCCTTTC		
22 WELV L 5'UTR	ATGGTCATTCAATTCAGGAG		
22 WELV L 5'UTRseq	TTCGCAAGTCAAAGAACC		
22 WELV L 3'UTRseq	CCCCTGCTTTCGCTAAC		
22 WELV L 3'UTR	GGCCATCATACCAAAGG		
33 S 206Rv	AAGCCCTCAAGAAAAAGAC	RACE-PCR and UTR sequencing primers for Guama virus S, M and L cRNA as indicated.	This study
33 S 718 Fw	CTGGACAAGAGCTGGATTG		
33 S 967Fw	AATTAGCCTTGATTGGCAC		
33 M 5'UTR	AGCAAATGTGTGTGGTATC		
33 M 4442 Fw	TTGTCTGTGAGGCGTTGA		
33 M 5'UTRseq	TCTGCAATCCCATGATCC		
33 L 226 Rv	CACAATATCCAGAGCAGG		
33 L 6731Fw	AAATGGAAGGTACATCATGG		
36 S 188 Rv	TTTGCTGGAGTAAGCTGG	RACE-PCR and UTR sequencing primers for Gamboa virus S, M and L cRNA as indicated.	This study
36 S 761 Fw	GTTCTTCACTGCTCTTGG		
36 M 241 Rv	TCACAGTCCATTTCCTCCA		
36 M 4825Fw	ATAGAACACACGAGGCAAG		
36 L 205 Rv	ACCAGCTTCACATCATTCC		
36 L 3'UTR	GGCGATGATTTTGATTCAC		
36 L 3'UTRseq	ATTAGCACGAGAAGACCT		

41 Pal S 5'UTR	CTGAAGATTGCAATGGCC	RACE-PCR and UTR sequencing primers for Palestina virus S, M and L cRNA as indicated.	This study
41 Pal S 5'UTRseq	CCAATTGTCCATTCCAG		
41 S 864fw	AGTTAATGGTAGGTGCGCAC		
41 Pal S 3'UTR	CAGAGTCCAACAGAAGC		
41 Pal S 3'UTRseq	TTCTAAAGAAACACCTGCG		
41 Pal S 3'UTRseq2	AGTTAATGGTAGGTGCG		
41 M 144 Rv	CCCCATCTGTGAAGCATC		
41 Pal M 5'UTR	CAGGCATGTAGCTTTTGTTC		
41 Pal M 5'UTRseq	GCCTAATGAGTTCGATCC		
41 Pal M 3'UTR	AAAGACATTCGATGTGGG		
41 Pal M 3'UTRseq	CAGTGCTTGGTGTGTTAG		
41 Pal L 5'UTR	GCTAAAGTCCAGATCCAG		
41 Pal L 5'UTRseq	TGGTTGTGGGGTCCATC		
41 Pal L 3'UTR	TGGACTAACCGAAATGGAG		
41 Pal L 3'UTRseq	ATTGGGGACAAATGGTAG		
26 CAPV S 5'UTR	TCTTAGATGCAGTCTCGTCC	RACE-PCR and UTR sequencing primers for Capim virus S, M and L cRNA as indicated. Additional primers (M2, L2) were required to reach termini after initial sequencing with primers based on HTS data.	This study
26 CAPV S 5'UTRseq	GCTTCAACCTCAATATTGG		
26 CAPV S 3'UTR	TCTCTCATTCTTCCCTGGC		
26 CAPV S 3'UTRseq	CAGTGGATGTCAGAAAGG		
26 CAPV M 5'UTR	GACGTTGTCCCTTGAAACCA		
26 CAPV M 5'UTRseq	GAGACAAGCATTTTCCGTTG		
26 CAPV M 3'UTR	TGCACCAAAGGATTGGATTG		
26 CAPV M 3'UTRseq	GGACGTGGCTGTGTAAAGTC		
26 CAPV L 5'UTR	CGTCATGAAATGCTATTGTGGAG		
26 CAPV L 5'UTRseq	CTGAAAGTATTATGGCGGTTG		
26 CAPV L 3'UTR	AACGAATGGTCCACAGTAATGC		
26 CAPV L 3'UTRseq	ACTGGGTCAAGGCTAAGGAG		
26 CAPV M2 3'UTR	GTGTAAAGTCAGTGAGCAAGG		
26 CAPV M2 3'UTRseq	ACGAGAGAGAGATGTATGGG		
26 CAPV L2 5'UTR	TGGGGAAAAGTTGAAGGAAGTTG		
26 CAPV L2 5'UTRseq	TAGACTCATCGCTCACCG		
18 MORV S 5'UTR	CGATGGCAAGAGGTAAAAGC	RACE-PCR and UTR sequencing primers for Moriche virus S, M and L cRNA as indicated.	This study
18 MORV S 5'UTRseq	AGTTCTCAGCATCGCTCC		
18 MORV S 3'UTR	GGATGACAACAAGGAAGGAGGA		
18 MORV S 3'UTRseq	AAGGCTGGACTATCGGCA		
18 MORV M 5'UTR	GCATGGAAACTTAGGGATGTAG		
18 MORV M 5'UTRseq	ATTTGCTGTTGTTGTTCCC		
18 MORV M 3'UTR	TCGCCCTTCCTGAATCTATTTG		
18 MORV M 3'UTRseq	GTTGTGTTGGGAACTGTAG		
18 M 4591Fw	GGGAGTAAAGTTAAGCTAGATAGG		
18 M 4673Fw	ATCAGCGAGATCAACAGG		
18 MORV L 5'UTR	GTCAGAAGATATGTGTAGATGC		
18 MORV L 5'UTRseq	ATCCTGGCCTAATAGCTTG		
18 MORV L 3'UTR	ATAGGGGAAAAGGTAGACTGG		
18 MORV L 3'UTRseq	ATGGAAGGTGCAAGTTGG		
17 JD S 5'UTR	GTCTTCTGGCTCCACTTCTATG	RACE-PCR and UTR sequencing primers for Juan Diaz virus S, M and L cRNA as indicated.	This study
17 JD S 5'UTRseq	TCTCTAGCAGGGGTTGTC		
17 JD S 3'UTR	ATACAGAGTCCAACAAAAGCAG		
17 JD S 3'UTRseq	AGAGGATGTGAGGAATGC		
17 JD M 5'UTR	TCTGTTTGCTGTGGTTGTTCC		
17 JD M 5'UTRseq	ATACCGTGATCCATCTGC		
17 JD M 3'UTR	CTGTAAGGAAGAAGTGAATGCC		
17 JD M 3'UTRseq	TGTTTCTCCCTATGTGCG		
17 JD L 5'UTR	CTGATGAGATATGCAAGTGC		
17 JD L 5'UTRseq	AGATTAGTCGCCTCGCAG		
17 JD L 3'UTR	AGACGAATCAAATCACTGGGG		
17 JD L 3'UTRseq	TAGAAGGGGCTAGTTGGG		
48 S 185Rv	TCGTATTCCTCTCCTGGG	RACE-PCR and UTR sequencing primers for M'Poko virus S, M and L cRNA as indicated.	This study
48 S 774 Fw	ACTCTCACTGGACTTGCT		
48 M 151 rv	ATTTTCGATCCACCTGTGA		
48 M 4394Fw	AAAGCCCAAGGCAAAATG		
48 L 175rv	ATAATCGTGTCTCCTGCTC		
48 L 6825 Fw	AGAGTACAAACTTCGCGG		

B) Primers for Sequence Confirmation (PCR products, pGEM-T easy)

Primer Name	Sequence (5'- 3')	Description	Designed
M13-FP	TGTAAAACGACGGCCAGT	Primers for sequencing pGEM-T easy constructs.	GATC
M13-RP	CAGGAAACAGCTATGACC		
BUNCAL2	CCCCTACCACCCACCC	Primer targeting conserved region in California serogroup orthobunyavirus S segments.	Bowen *et al.,*2001
S25F	GTCACAGTAGTGTACTCCACWDNAA	Primers targeting conserved terminal sequences in orthobunyavirus S segments.	Mohamed *et al*., 2009
S13+	GTCACAGTAGTGTACYCC		
S13-	CTGACAGTAGTGTGCYCC		
BUN S-	AGTAGTGTGCTCCAC	Primers targeting conserved terminal sequences in orthobunyavirus S segments.	Dunn *et al.,* 1994
BUN S+	AGTAGTGTACTCCAC		
23 M 51rv	GCAGCATTTTAGTTAACATGG	Primers used for sequence confirmation of Anopheles A virus N segment cRNA.	This study
23 M 155Rv	TGGGGGAGGCCATAATC		
23 M 1693fw	TGAGAACCATTACAAGCAAG		
23 M 2595rv	GCATCTTCATTTCCATAGG		
23 M 3097fw	TTGTAATGATGCTGTCGAG		
23 M 4541fw	AAACCAAGAGGGTGATTGA		
23 L 570fw	GCTGAAGATTGCACATGG	Primers used for sequence confirmation of Anopheles A virus L segment cRNA.	This study
23 L 1460rv	ATGTGGCTACTCTAAAAGTG		
23 L 2172fw	AAATAGAGATTGAGCAGCG		
23 L 2824rv	CCATTGCAATTAACCAGAAG		
23 L 4827fw	GCTCATGATTTTTGCAGTG		
23 L 5734fw	GCAAGCTGTTATGAGGAGAG		
23 L 5797rv	ATTGATGTCAGGACCATGGA		
23 L 6294Rv	CAAGAATGCACACGGCA		
6 LMV M 915 Fw+	TTCTGGGTTATGCTCTGG	Primers used for sequence confirmation of Las Maloyas virus M segment cRNA.	This study
6 LMV M 1782 Fw	GATGGAATTGAACTCATGTG		
6 LMV 1864 FwSeq	TTGAGCATGAGGACAGC		
6 LMV M 2823 Fw+	GCCAGCAATTAAACCCAC		
6 LMV M 2498Rv	TTTTTCAACCATTCTGCAGC		
9 L 2394 fw	GAACCTGAACCTCAAACAG	Primers used for sequence confirmation of Trombetas virus L segment cRNA.	This study
9 L 2953 Rv	CAGCGTTCTTTGGCTAATC		
31 M 122 Rv	ACTTGCAACTTCACCTCC	Primers used for sequence confirmation of Anopheles B virus M segment cRNA.	This study
31M 3239 Fw	TAATACGACTCACTATAGGGTTGCTTATTTGGTCAATG		
31 M 3485Fw	GGGAAAGGAGAGCCAAAG		
31 M 3740Fw	GCTGGGTGCTTAGATTG		
31 M 3852RV	ATTTAGCTCACAGTCCATCC		
31 M 4066Rv	TCTGGTCACTATTTCCGAC		
31 M 4050 fw	CGGAAATAGTGACCAGAC		
31M 4156Rv	GTTCACTTTTCCATTAGGCAAG		
31 M 4184fw	TGGAGACTTGCTTCTTGAG		
31M 4307Rv	AGGCCTTCCTCTGAGAC		
31 M 4322rv	AAGAAGCCCGTTATAGGAG		
31 M 4340Rv	AAGAAGCCCGTTATAGGAG		
31 M 4348Rv	CTTTGGTAAAGAAGCCCG		
31 L 2936 fw	AATGGGAGACAAAATGCTG	Primers used for sequence confirmation of Anopheles B virus L segment cRNA.	This study
31 L 3471 Rv	TCTTTGAAAACAGCCATTGC		
31 L 3451 Fw	TGTGCAATGGCTGTTTTC		
31 L 4277 Rv	CTTTTCATCGAACCACTTG		
36 M 862fw	GACACTACTGAAAGAATGAG	Primers used for sequence confirmation of Gamboa virus M segment cRNA.	This study
36 M 1373rv	GTTCCGCATGTACATTTCC		
36 M 2863fw	GGTGTCTTCTGCTTTGGA		
36 M 3837fw	AGAAATAGAAGCAACGGTG		
36 M 3854rv	ACCGTTGCTTCTATTTCTG		
36 M 4805rv	AAATGCCTATGTTCTGGAC		
41 S 3'term rv	AGTAGTGTGCCCCACTGCC	Primers used for sequence confirmations of	This study

41 S 3 term rv seq	AGTAGTGTGCCCCACTG	Palestina S segment cRNA.	
41M 344fw	GCCGAATGATGAGTTGATG	Primers used for sequence confirmation of Palestina virus M segment cRNA.	This study
41 M 1209 fw	AAAGGGAAACTCGAATCG		
41 M 1430 Fw	ACCAGGGAAATATCAAGCC		
41M 1448rv	GGCTTGATATTTCCCTGGT		
41 M 1880 rv	TGCCGCTTTCACTTGAAC		
41 M 1982fw	GCGACAGTACCAACAAAG		
41 M 2231 Rv	CTGTGCAACTGAGCTTATATC		
41 M 2470 Fw	TTGCTGACCCCTTCTGTG		
41 M 2500 fw	TATTGCTTTGGGAGGGAC		
41 M 2511 rv	CCCAAAGCAATAAGTCCC		
41 M 2983 fw	GGCTATCGTTCAGCAAAG		
41 M 3069 Rv	ACAAAAACAAGCAACCCTC		
41 M 3225Rv	CGCGCCTGCTGATATTAG		
41 M 3563fw	TTTGGGTGAGATAAGGGTG		
41 M 3885 rv	GAGGGCAAACGGTGTCA		
41 M 4276 rv	ATATCCTTTAGCCGCCC		
41 M 4311 Fw	GGGCGGCTAAAGGATATTC		
41 M 4435_Fw	AGAGGGCTATTTGCTAGTG		
41 M 4486 Fw	ATTGCATATTCAAATAAAGGTCC		
41M 4310 Fw PmX	TGGGCGGCTAAAGGATATTC	Primers for primer extension experiments on Palestina virus S and M segments.	This study
41S 784 Fw PmX	ATTCGGCATCAGGGTTTAAG		
41 L 346fw	AAAGTATCGGTTAGTGACG	Primers used for sequence confirmation of Palestina virus L segment cRNA.	This study
41 L 11261rv	TGAATTGTTGCTCCCAAG		
41 L 1417 fw	AGACACAGTCGTATAATGGT		
41 L 1879Rv	TCACTAGCCTTTGACACC		
41 L 2093 fw	ACCAAGACGCTTTTTTCAG		
41 L 2263rv	ACCCTGGGAACCATATAG		
41 L 2851 rv	TATTGCGGGTTTGTCGT		
41 L 3205 fw	GAGTGCTCAGGATGTTTTC		
41 L 3778 rv	ATCTACTCGCCATATCTTC		
41 L 3793 fw	ACGGCAATAAAGCATGG		
41 L 4699rv	CCAATTCTTTGCTCGTG		
26 M 2977 fw	AAAACAGGGGTGTCTACAG	Primers used for sequence confirmation of Capim virus M segment cRNA.	This study
26 M 3611 Rv	TACATCCTTCCTTCTTGCAG		

C) Primers for Sequence Confirmation (pTvT7 clones)

Primer Name	Sequence (5'- 3')	Description	Designed
pTvT7 Lin+	GGGTCGGCATGGCATCTC	Primers for linearising pTvT7 plasmids.	This study
pTvT7 Lin-	CTATAGTGAGTCGTATTAATTTCGCG		
34 pTvT7 S+	TAATACGACTCACTATAGAGTAGTGTACTCCACAATTGAATTC	Primers for restriction free cloning of Eretmapodites virus S, M and L cRNA into pTvT7 backbone.	This study
34 pTvT7 S-	ATGCCATGCCGACCCAGTAGTGTGCTCCACAATG		
34 pTvT7 M+	TAATACGACTCACTATAGAGTAGTGTACTACCAAGTGAC		
34 pTvT7 M-	ATGCCATGCCGACCCAGTAGTGTGCTACCAAGGA		
34 pTvT7 L-	ATGCCATGCCGACCCAGTAGTGTGCTCCTACATTAG		
34 pTvT7 L+	TAATACGACTCACTATAGAGTAGTGTACTCCTACATTAAAAATC		
6 pTvT7 S+	TAATACGACTCACTATAGAGTAGTGAACTCCATATAAAGTCAAAAG	Primers for restriction free cloning of Las Maloyas virus S, M and L cRNA into pTvT7 backbone.	This study
6 pTvT7 S-	GATGCCATGCCGACCCAGTAGTGTGCTCCATATCAAGTAC		
6 pTvT7 M+	TAATACGACTCACTATAGAGTAGTGAACTACTCAAAAGAGAAATC		
6 pTvT7 M-	GATGCCATGCCGACCCAGTAGTGTGCTACTCAAAGAGAA		
6 pTvT7 L+	TAATACGACTCACTATAGAGTAGTGTACTCCTATAATAAAAACCAGTAG		
6 pTvT7 L-	GATGCCATGCCGACCCAGTAGTGTGCTCCTATAATAAAGATACTAA		
5 pTvT7 S+	TAATACGACTCACTATAGaGTAGTGAACTCCATATAAAGTCAAAAG	Primers for restriction	This study

5 pTvT7 S-	GATGCCATGCCGACCCAGTAGTGTGCTCCATATAAAGTACATAA	free cloning of ColAn 57389 virus S, M and L cRNA into pTvT7 backbone.	
5 pTvT7 M+	TAATACGACTCACTATAGAGTAGTGAACTACTTGCAAAAAAAC		
5 pTvT7 M-	GATGCCATGCCGACCCAGTAGTGTGCTACTTGCAAAAAC		
5 pTvT7 L+	TAATACGACTCACTATAGAGTAGTGTACTCCTATAATAAAAATCAATAAAATGG		
5 pTvT7 L-	GATGCCATGCCGACCCAGTAGTGTGCTCCTATAATAAAAATACT		
23 pTvT7 S+	TAATACGACTCACTATAGAGTAGTGTACTCCATATAAAGTCAAAC	Primers for restriction free cloning of Anopheles A virus S segment cRNA into pTvT7 backbone.	This study
23 pTvT7 S-	GATGCCATGCCGACCCAGTAGTGTGCTCCATATAAAGTAC		
24 pTvT7 S+	TAATACGACTCACTATAGAGTAGTGTACCCCACTAGAAG	Primers for restriction free cloning of Tacaiuma virus S, M and L cRNA into pTvT7 backbone.	This study
24 pTvT7 S-	ATGCCATGCCGACCCAGTAGTGTGCTCCGCTAG		
24 M pTvT7+	TAATACGACTCACTATAGAGTAGTGTACTACTCAGTGGAATAAC		
24 M pTvT7 -	GATGCCATGCCGACCCAGTAGTGTGCTACTCAGTAGAAC		
24 pTvT7 L-	ATGCCATGCCGACCCAGTAGTGTGCTCCTATCAAG		
24 pTvT7 L+	TAATACGACTCACTATAGAGTAGTGTACTCCCTATCAAAAG		
26 S pTvT7 Fw	AGTAGTGAACTTCGTAGGAAGTTAAAATC	Primers for restriction free cloning of Capim virus S segment cRNA into pTvT7 backbone.	This study
26 S pTvT7 Rv	GATGCCATGCCGACCCAGTAGTGCTCTTCGTAGAAAGTATTTAAAAC		
pTvT717 JD S+	TTAATACGACTCACTATAGAGTAGTGAACTTCGTAGAAAAGTTC	Primers for restriction free cloning of Juan Diaz virus S, M and L cRNA into pTvT7 backbone.	This study
pTvT717 JD S-	GATGCCATGCCGACCCAGTAGTGTTCTTCGTAGAAAGTATTTAC		
pTvT717 JD M+	TTAATACGACTCACTATAGAGTAGTGTACCGCTGTTAGTAAAAATACC		
pTvT7 17 JD M-	GATGCCATGCCGACCCAGTAGTGTTCCGCTGTTTGTAAAATAC		
pTvT7 17 JD L+	TAATACGACTCACTATAGAGTAGTGTACCCTTGACATAATATTTAAAAC		
pTvT7 17 JD L-	GATGCCATGCCGACCCaGTAGTGTGCCCTTGACATAG		
20 pTvT7 S Fw+	TAATACGACTCACTATAGAGTAGTGTACTCCACAAAATACAAAATCG	Primers for restriction free cloning of Matruh virus S, M and L cRNA into pTvT7 backbone.	This study
20 pTvT7 S Rv-	ATGCCATGCCGACCCAGTAGTGTGCTCCACAAATAC		
20 pTvT7 M Fw+	TAATACGACTCACTATAGAGTAGTGTACTACCATTTAAGAAAACG		
20 pTvT7 M Rv-	ATGCCATGCCGACCCAGTAGTGTGCTACCATCTAAGAATTATC		
20 pTvT7 L Fw+	TAATACGACTCACTATAGAGTAGTGTACCCCTGGG		
20 pTvT7 L Rv-	ATGCCATGCCGACCCAGTAGTGTGCCCCTGG		
21 pTvT7 S-	ATGCCATGCCGACCCAGTAGTGTGCTCCACAAATAC	Primers for restriction free cloning of Tsuruse-like virus S, M and L cRNA into pTvT7 backbone.	This study
21 pTvT7 S+	TAATACGACTCACTATAGAGTAGTGTACCCCACAAAATAC		
21 pTvT7 M+	TAATACGACTCACTATAGAGTAGTGTACTACCATTTAAGAAAAC		
21 pTvT7 M-	ATGCCATGCCGACCCAGTAGTGTGCTACCATATAAGAATTATC		
21 pTvT7 L+	TAATACGACTCACTATAGAGTAGTGTACCCCTGGGTTTC		
21 pTvT7 L-	ATGCCATGCCGACCCAGTAGTGTGCCCCTGGA		
22 pTvT7 S Fw+	TAATACGACTCACTATAGAGTAGTGTATTCCGTAAAAAACAAAAC	Primers for restriction free cloning of Weldona virus S segment cRNA into pTvT7 backbone	This study
22 pTvT7 S Rv-	ATGCCATGCCGACCCAGTAGTGTGCTCCGTAATAC		
31 M pTvT7 Fw	GATGCCATGCCGACCCAGTAGTGTACTACTTGGTAAAGAAAATTC	Primers for restriction free cloning of Anopheles B virus S, M and L cRNA into pTvT7 backbone.	This study
31M pTvT7 Rv	GATGCCATGCCGACCCAGTAGTGTGCTACTTGGTATAGAAAATTAC		
31 M pTvT7 Rv55	GATGCCATGCCGACCCAGTAGTGTGCTACTTGGTATAG		
31 M pTvT7 Fw 54	TAATACGACTCACTATAGAGTAGTGTACTACTTGGTAAAGA		

D) Primers for BMAV Reverse Genetics and Structural Studies

Primer Name	Sequence (5'- 3')	Description	Designed
pTvT7 Lin+	GGGTCGGCATGGCATCTC	Primers for linearising pTvT7 backbone.	This study
pTvT7 Lin-	CTATAGTGAGTCGTATTAATTTCGCG		
pUC118-F	AGCGCCCAATACGCAAAC	Forward and reverse primers for sequencing pTvT7 inserts.	Designed by Dr Xiaohong Shi, UoG
pUC-R	TGCCTGCAGGTCGACTCTAG		
pTvT7 BM S+	TAATACGACTCACTATAGAGTAGTGTACCCCA CAAAATAC	Primers for restriction free cloning of wt Batama virus (BMAV) into pTvT7 linearised vector.	This study
pTvT7 BM S-	ATGCCATGCCGACCCAGTAGTGTGCTCCAC		
pTvT7 BM M-	ATGCCATGCCGACCCAGTAGTGTGCTACCA		
pTvT7 BM M+	TACGACTCACTATAGAGTAGTGTACCACCATT TAAGAAAACG		
pTvT7 BM L+	AATACGACTCACTATAGAGTAGTGTACCCCTG GGTTTCG		
pTvT7 BM L-	ATGCCATGCCGACCCAGTAGTGTGCCCCTGGA		
BMAV Sdel+	ATGATTGAGATTGATTACCATTTCTCAG	Primers for removing N terminal 25 aa from pTvT7 BMAV S construct, and adding the first 2 N protein aa from Bunyamwera virus.	This study
BMAV Sdel-	TTTGTATGGTTTGCTATATGCAATC		
BM Sdel QkC+	GCATATAGCAAACCATACAAAATGATTGAGAT TGATTACC		
BM Sdel QkC-	GGTAATCAATCTCAATCATTTTGTATGGTTTG CTATATGC		
BMAV Sdel29+	AACATGTAAATAATTCTCTTTTAAGAGATAGG GGTTG	Primers to replace BMAV N protein termini (N and C) with those of Bunyamwera virus	This study
BMAV Sdel29-	GATTCCGAATTCTTTAAGGAACTCCC		
27 delWN Bu ins Fw	GCAAACCATACAAAATGTCAAAGGCAAAGTCA CC	Primers for adding Weldona virus N protein termini to pTvT7 BMAV S construct.	This study
27 delWN Bu ins Rv	CTTAAAAGAGAATTATTCAATTGATGTTGAAT TCTTTAAGGAACTC		
27 delwNWu ins Fw	GCAGGTTTAGAGGATGATGAGATTGATTACCA TTTCTCAGC	Primers for adding Weldona virus N protein termini and UTRs to pTvT7 BMAV S construct.	This study
27 delwNwU ins Rv	CTAAATCAATTGATGTTGAATTCTTTAAGGAA CTCC		
wNwU Cr-	TAAGGAACTCCCTAGCAGCCTCAGAGAAAC		
wNwU Cr+	GGAGTTCCTTAAAGAATTCAACATCAATTGAT TTAGCTG		
BM_N D24A Fw	CCGCATTTGCTGAAGCTGAGATTGATTACC	Quickchange primers for single alanine substitutions in pTvT7 BMAV S construct, aa residues numbered as for wtBMAV.	This study
BM_N D24A Rv	GGTAATCAATCTCAGCTTCAGCAAATGCGG		
BM_N S18A Fw	CAATCAGCGTTGCAGCTGCTGCCGCATTTGCT GAAG		
BM_N S18A Rv	CTTCAGCAAATGCGGCAGCAGCTGCAACGCTG ATTG		
BM_N S12A Fw	GGTGAGTCAGAACCAGCTATCAGCGTTGCAGC		
BM_N S12A Rv	GCTGCAACGCTGATAGCTGGTTCTGACTCACC		
BM_N R6A Fw	GTCCAAAGTAAAAGCTGGTGAGTCAGAACC		
BM_N R6A Rv	GGTTCTGACTCACCAGCTTTTACTTTGGAC		
BM_N I13del Fw	GAGTCAGAACCATCAAGCGTTGCAGCTAG	Quickchange primers for single point deletions in pTvT7 BMAV S construct, aa residues numbered as for wtBMAV.	This study
BM_N I13del Rv	CTAGCTGCAACGCTTGATGGTTCTGACTC		
BM_N P11del Fw	GAGGTGAGTCAGAATCAATCAGCGTTGCAG		
BM_N P11del Rv	CTGCAACGCTGATTGATTCTGACTCACCTC		
BM_N A19del Fw	GCGTTGCAGCTAGTGCATTTGCTGAAGATG		
BM_N A19del Rv	CATCTTCAGCAAATGCACTAGCTGCAACGC		
BM_N D24del Fw	CCGCATTTGCTGAAGAGATTGATTACCATTTC		
BM_N D24del Rv	GAAATGGTAATCAATCTCTTCAGCAAATGCGG		
BM_N R6S12A Fw	CAAAATGTCCAAAGTAAAAAGAGGTGAGTCAG AACCATCAATCAGCGTTGCAGCTAG	Additional quickchange primers for multiple point deletions or alanine substitutions, as indicated, in pTvT7 BMAV S constructs, aa residues numbered as for wtBMAV.	This study
BM_N R6S12A Rv	CTAGCTGCAACGCTGATTGATGGTTCTGACTC ACCTCTTTTTACTTTGGACATTTTG		
BM_N P11I13del-	GCACTAGCTGCAACGCTTGATTCTGACTCACC TCTTTTTAC		
BM_N P11I13del+	GTAAAAAGAGGTGAGTCAGAATCAAGCGTTGC AGCTAGTGC		
BM_N A19D24 del+	CAAGCGTTGCAGCTAGTGCATTTGCTGAAGAG		
BM_N A19D24 del-	CTCTTCAGCAAATGCACTAGCTGCAACGCTTG		
pTM1 BM S+	GAAAAACACGATAATACCATGTCCAAAGTAAA AAGAG	Primers for restriction free cloning of wt Batama virus (BMAV) into pTM1	This study
pTM1 BM S-	AATTAGGCCTCTCGAGTTAGTTTCTGTCCTG		
pTM1 BM M+	AAAACACGATAATACCATGAGAATTTTTATAT		

	TCTTCTCAAC	linearised vector	
pTM1 BM M-	TAATTAGGCCTCTCGAGTTATCTGTCTTTATA TATTTGGTG		
pTM1 BM L+	AAACACGATAATACCATGGATCAAGAAATGAT TAAACG		
pTM1 BM L-	ATTAGGCCTCTCGAGCTATGCCTTGAAGTG		
pTM-R	CAACTCAGCTTCCTTTCGGGC	Primers for sequencing pTM1 clones.	Designed by Dr Xiaohong Shi, UoG
pTM-F	GGTGCACATGCTTTACATGTG		
pHT Lin Fw	AATTCGAGCTCCGTCGAC	Primers for linerasing pEHISTEV.	This study
pHT Lin Rv	GCCCTGAAAATACAGGTTTTCG		
pHT BMAV N Fw+	CCTGTATTTTCAGGGCATGTCCAAAGTAAAAA GAGGTGAG	Primers for cloning BMAV N protein ORF into pEHISTEV.	This study
pHT BMAV N Rv-	GTGGTGGTGGTGTTATTAGTTTCTGTCCTGGT CTAATCC		
pHT BM inRv	CGACGGAGCTCGAATTTTATTAGTTTCTGTCC TGGTCTAATCC		
pHT BM inFw	CCTGTATTTTCAGGGCTCCAAAGTAAAAAGAG GTGAG		
pHT RvSeq	AGGGGTTATGCTAGTTATTGC	Reverse primers for sequencing pEHISTEV clones.	This study
pHT RevSeq2	ACTCAGCTTCCTTTCGGG		

UoG; University of Glasgow.

E) Primers for Cloning Virus ORFs into pCMV-empty

Primer Name	Sequence (5'- 3')	Description	Designed
CMV-F	CGCAAATGGGCGGTAGGCGTG	Primer for sequencing pCMV	GATC
BUNV N pCMV+Fw	CTATAGGCTAGCCACCATGATTGAGTTGGAATTTCACG	Primers for cloning BUNV ORFs into pCMV backbone.	This study
BUNV N pCMV-Rv	GCGGCCGCTCTAGAATTACATGTTGATTCCGAATTTAGC		
BUNV NSs pCMV-	GCGGCCGCTCTAGAATCAGCATCTTCTCAAGTAGG		
BUNV NSs pCMV+	CTATAGGCTAGCCACCATGATGTCGCTGCTAACACC		
BUNV M pCMV+Fw	CTATAGGCTAGCCACCATGAGAATTCTAATACTGCTTTTAGC		
BUNV M pCMV-Rv	GCGGCCGCTCTAGAATCACTTTTGCTTAATTTCTTGC		
BUNV L pCMV+Fw	CTATAGGCTAGCCACCATGGAGGACCAAGCTTATG		
BUNV L pCMV-Rv	GCGGCCGCTCTAGAATCAGAAAAAGGTAAATAGAGACTTTCC		
BMAV N pCMV-Rv	GCGGCCGCTCTAGAATTAGTTTCTGTCCTGGTCTAATCC	Primers for cloning BMAV ORFs into pCMV backbone.	This study
BMAV N pCMV+Fw	CTATAGGCTAGCCACCATGTCCAAAGTAAAAAGAGGTGAGT		
BMAV M pCMV+Fw	CTATAGGCTAGCCACCATGAGAATTTTTATATTCTTCTCAACA ATC		
BMAV M pCMV-Rv	GCGGCCGCTCTAGAATTATCTGTCTTTATATATTTGGTGGTC		
BMAV L pCMV+Fw	CTATAGGCTAGCCACCATGGATCAAGAAATGATTAAACG		
BMAV L pCMV-Rv	GCGGCCGCTCTAGAACTATGCCTTGAAGTGGTAC		
GAMV NSs pCMV+Fw	CTATAGGCTAGCCACCATGATTCGACGGCAAGC	Primers for cloning specified virus ORFs into pCMV backbone.	This study
GAMV pCMV-Rv	GCGGCCGCTCTAGAATCATTGCTCCATGCTGCC		
WELV N pCMV-Rv	GCGGCCGCTCTAGAATCAATTGATGTTGAATTCTTTCAAG		
WELV N pCMV+Fw	CTATAGGCTAGCCACCATGTCAAAGGCAAAGTCAC		
WELV NSs pCMV+Fw	CTATAGGCTAGCCACCATGATTTGGTCTTTTCTAGTTCAAC		
WELV NSs pCMV-Rv	GCGGCCGCTCTAGAACTATCTCTTCCTTGTGCTTAGG		
MTRV NSs pCMV+Fw	CTATAGGCTAGCCACCATGAGAAGCAGAAGGCTAAG		
MTRV NSs pCMV-Rv	GCGGCCGCTCTAGAATCAGATATTCCGTGTCCATTTTC		
NDV NSs pCMV+Fw	CTATAGGCTAGCCACCATGATGTCGAGCCAACTG		
NDV NSs pCMV-Rv	GCGGCCGCTCTAGAACTATATATCCTGAGAGTCTATGCATTG		
EREV NSs pCMV-Rv	GCGGCCGCTCTAGAACTATATATCCTGACAGTCTGTGG		
EREV NSs pCMV+Fw	CTATAGGCTAGCCACCATGATGTCCAGTCTACTGCC		
MPOV NSs pCMV+Fw	CTATAGGCTAGCCACCATGTCAGTCGAAGTACTTATAACC		
MPOV NSs pCMV-Rv	GCGGCCGCTCTAGAATTAAGTCAGAGTCCGCAAC		

GATC; GATC Biotech, DNA Sequencing and Bioinformatics service.

Appendix 2: Virus Database Excerpt

For publication purposes only, information on published sequences included in phylogenetic trees is shown. Information has also been condensed, with vector information given at the genus level, and geographical location and host range summarised. It should also be noted that only locations with positive virus isolations are included, areas positive for neutralising antibodies have been omitted. (N/A) Not available, (NR) Not reported (negative serology), (NTAb) Neutralising antibodies. The information included in this appendix was sourced from the International Catalog of Arboviruses (Berge, 1975) and the publications referenced in Chapter 1 and Chapter 5 of this thesis.

Virus name	Serogroup	Accession Numbers			Geographic Location	Vector	Natural Host Range	Human Infection
		S	M	L				
Abbey lake virus	Bunyamwera	KJ710424	KJ710423	KJ710425	China	*Culex*	N/A	NR
Aino virus	Simbu	NC018460	NC018459	NC018465	Japan, Australia	*Culex, Culicoides*	Cattle, horses	NR
Akabane viru	Simbu	NC009896	NC009895	NC009894	Japan, Australia, Kenya	*Culex, Culicoides, Aedes*	Cattle	NR
Alajuela virus	Gamboa	KM272188	KM272187	KM272186	Argentina	N/A	N/A	NR
Anhembi virus	Bunyamwera	JN572064	JN572063	JN572062	Brazil	*Wyeomyia*	Rodents	NTAb
Batai virus	Bunyamwera	JX846604	JX846605	JX846606	India, SE Asia, Russia, E Europe	*Culex, Anopheles, Aedes*	Birds, rodents, ruminants	NTAb
Brazoran virus	Unclassified	NC022037	NC022038	NC022039	North America	*Culex.*	N/A	NR
Bruconha virus	Group C	KM280937	KM280924	KM280929	N/A	N/A	N/A	N/A
Buffalo Creek virus	Mapputta	KJ481927	KJ481928	KJ481929	N/A	N/A	N/A	N/A
Bunyamwera virus	Bunyamwera	NC001927	NC001926	NC001925	Africa	*Aedes, Mansonia, Culex.*	Non-human primates, rodents, domestic animals, birds	Clinical cases
Buttonwillow virus	Simbu	KF697162	KF697161	KF697160	North America, Africa	N/A	Non-human primates, guinea pigs	Clinical cases
Bwamba virus	Bwamba	EU564827	KJ867183	KJ867184	Africa	*Anopheles*	N/A	Clinical cases
Cache Valley virus	Bunyamwera	GU018037	KC436107	KC436106	North America	*Anopheles, Culisetini*	Rodents, deer, fox, raccoon, horses, monkeys	NTAb
Cachoeira Porteira virus	Bunyamwera	JN968592	JN968591	JN968590	Brazil	N/A	N/A	N/A
Calchaqui virus	Gamboa	KM272185	KM272184	KM272183	Argentina	*Aedes*	Horses	NR
Calovo virus	Bunyamwera	KJ542624	KJ542625	KJ542626	Russia, E Europe, Sri Lanka	*Aedes, Anopheles*	N/A	NR
Caraparu virus	Group C	KF254778	KF254777	KF254776	N/A	*Culex, Wyeomyia*	Rodents	Clinical cases
Cat Que virus		NC024075	NC024074	NC024076	N/A	N/A	N/A	N/A

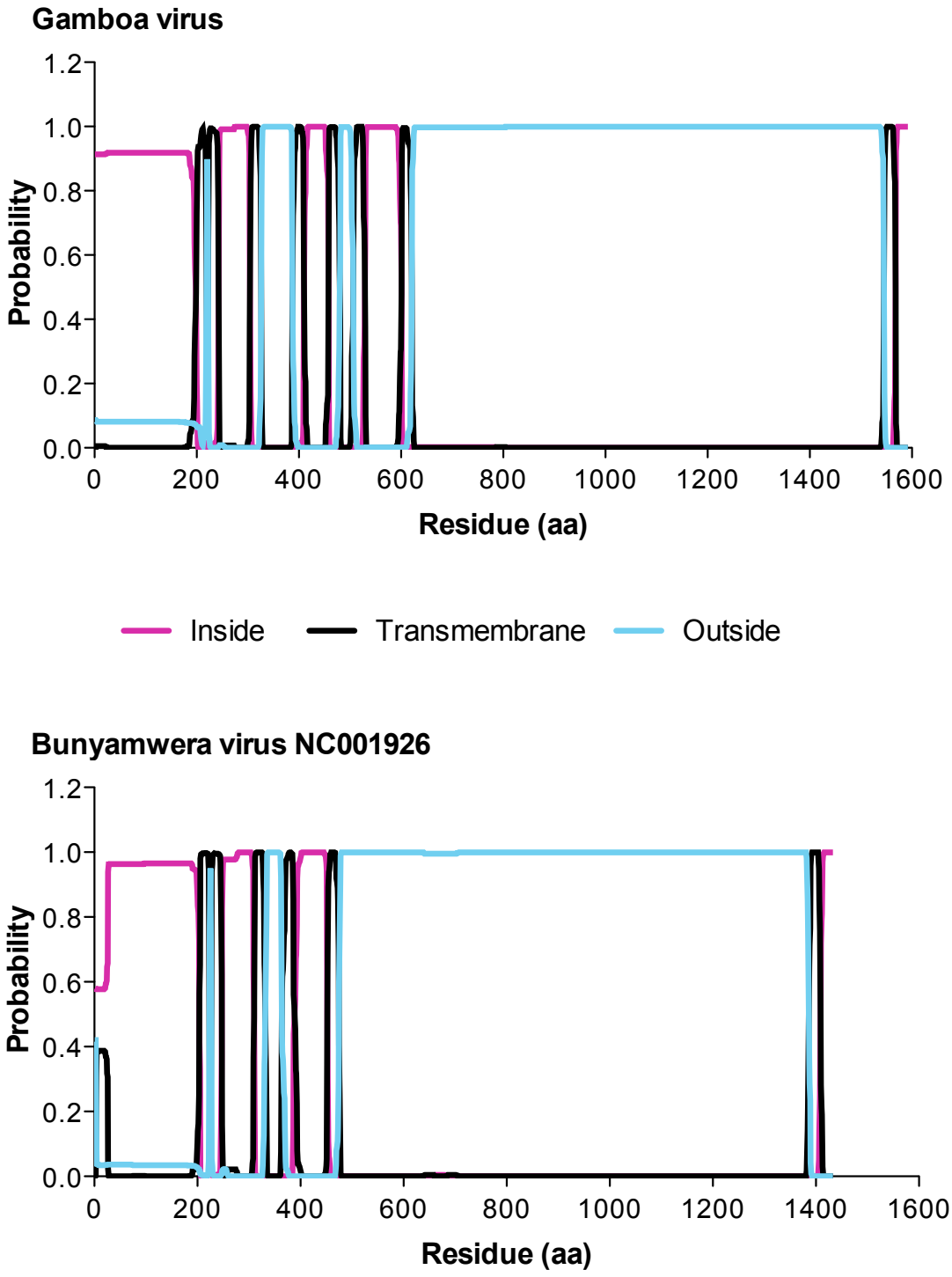
Chatanga virus	California	KF719221	KF719222	KF719223	N/A	N/A	N/A	N/A
Douglas virus	Simbu	HE795092	AB698478	HE795090	Australia	*Culicoides*	Cattle, sheep, horses, goats, deer, buffalo	NR
Facey's Paddock virus	Simbu	KF697136	KF697137	KF697138	N/A	N/A	N/A	N/A
Gamboa virus	Gamboa	KM272176	KM272175	KM272174	South America	*Aedeomyia*	N/A	N/A
Gamboa virus	Gamboa	KM272182	KM272181	KM272180	N/A	N/A	N/A	N/A
Gamboa virus	Gamboa	KM27217*	KM272178	KM272177	N/A	N/A	N/A	N/A
Guaroa virus	California	KM245522	KM245523	KM245524	Colombia, Brazil, Panama	*Anopheles*	N/A	Clinical cases
I612045 virus	Unclassified	HM627180	HM627181	HM627179	N/A	N/A	N/A	N/A
Iaco virus	Bunyamwera	JN572067	JN572066	JN572065	N/A	*Wyeomyia*	N/A	N/A
Ilesha virus	Bunyamwera	KF234073	KF234074	KF234075	Africa	*Anopheles*	N/A	Clinical cases
Ingwavuma virus	Simbu	KF697141	KF697140	KF697139	Africa, India, Thailand, Cyprus	*Aedes, Culex*	Pigs, dogs, ducks, chickens, buffalo, cattle, birds	NTAb
Inkoo virus	California	U47137	U88059	EU789573	Finland	Mosquitoes unspeciated	Cattle, reindeer, snow hare, red fox, moose, hazel grouse	NTAb
Iquitos virus	Simbu	KF697144	KF697143	KF697142		N/A	N/A	
Jamestown Canyon	California	HM007350	HM007351	HM007352	North America	*Aedes, Hybomitra, Chrysops*	White-tailed deer, moose, elk	NTAb
Jatobal virus	Simbu	JQ675601	JQ675602	JQ675603	N/A	N/A	N/A	N/A
Kaeng Khoi virus	Unclassified	KJ867203	KJ867204	KJ867205	N/A	N/A	N/A	N/A
Khurdun virus	Unclassified	KF981635	KF981634	KF981633	N/A	N/A	Birds	N/A
La Crosse virus	Gamboa	NC004110	NC004109	NC004108	North America	*Aedes, Culex*	Chipmunk, sentinel grey squirrels, sentinel rabbits	Clinical cases
Leanyer virus	Simbu	HM627177	HM627176	HM627178	N/A	N/A	N/A	N/A
M'Poko virus	Turlock	AM711133	N/A	N/A	Central African Republic	*Culex*		NTAb
Macaua virus	Bunyamwera	JN572070	JN572069	JN572068	Brazil	*Wyeomyia*	Birds	NTAb
Madre de Dios virus	Simbu	KF697146	KF697145	KF697147	N/A	N/A	N/A	N/A
Madrid virus	Group C	KF254781	KF254780	KF254779	Republic of Panama	Culex	Sentinel rodents	Clinical case
Manzanilla virus	Simbu	KF697148	KF697149	KF697150	Trinidad	N/A	Monkey.	NTAb
Mapputta virus	Mapputta	KJ481921	KJ481922	KJ481923	Australia	Anopheles	Cattle, horses, pigs, marsupials, rodents	NTAb
Maprik virus	Mapputta	NC026282	NC026283	NC026281	New Guinea	*Aedes, Anopheles, Culicidae*	N/A	NR

Marituba virus	Group C	KF254772	KF254771	KF254770	N/A	N/A	N/A	N/A
Mermet virus	Simbu	KF697152	KF697151	KF697153	North America	*Culex*	Birds	NR
Mojui dos Campos virus	Unclassified	KH867200	KH867201	KH867202	N/A	N/A	N/A	N/A
Murrumbidgee virus	Mapputta	NC022597	NC022596	NC022595	N/A	N/A	N/A	N/A
Ngari virus	Bunyamwera	KC608154	KC608153	KC608152	Africa	*Aedes, Anopheles*	N/A	NR
Nyando ERET147	Nyando	KJ867194	KJ867195	KJ867196	Africa	*Anopheles*	N/A	N/A
Nyando UgAr1712	Nyando	KH867191	KJ867192	KJ867193	Africa	*Anopheles*	N/A	Clinical cases
Nyando virus MP401	Nyando	KJ867188	KJ837189	KJ867190	Africa	N/A	N/A	
Nyando virus ArB16055	Nyando	AM709781	N/A	N/A	Africa	N/A	N/A	
Nyando YM176-66	Nyando	KJ867197	KJ867198	KJ867199	Africa	N/A	N/A	
Oriboca virus	Group C	KF254775	KF254774	KF254773	South America	*Aedes, Culex*	Sentinel mice, sentinel monkey, rodents	Clinical cases
Oropouche virus	Simbu	KP052852	KP052851	KP052850	Brazil, Trinidad	*Aedes, Culex, Culicoide.*	Monkeys, sloth, birds	Clinical cases
Orthobunyavirus FSL2923	Simbu	KF254792	KF254791	KF254790	N/A	N/A	N/A	N/A
Oyo virus	Unclassified	HM639778	HM639779	HM639780	N/A	N/A	N/A	N/A
Peaton virus	Simbu	HE795095	HE795094	HE795093	Australia	*Culicoides*	Cattle	NR
Pongola virus	Bwamba	EU564828	KJ867177	KJ867178	Africa	*Aedes, Anopheles*	Goat, donkey	Clinical cases
Sango virus	Simbu	HE795101	HE795100	HE795099	Africa	*Culicoides*	Cattle (5/2,199)	N/A
Sathuperi virus	Simbu	NC018462	NC018466	NC018461	India, Nigeria	*Culex, Culicoides*	Cattle	NR
Schmallenberg virus	Simbu	JX853181	JX853180	JX853179	N/A	N/A	N/A	N/A
Shamonda virus	Simbu	NC018464	NC018467	NC018463	Nigeria	*Culicoides*	Cattle	NR
Simbu virus	Simbu	NC018477	NC018478	NC018476	South Africa	*Aedes, Coquillettidia*	N/A	NTAb
Snowshoe hare virus	California	EU294510	EU262553	EU203678	North America, China	*Aedes, Culiseta, Lethocerus*	Lemming, sentinel rabbit, horses, deer, moose	Clinical cases
Sororoca virus	Bunyamwera	JN572073	JN572072	JN572071	Brazil	Sabethini (Tribe)		N/A
Tahyna virus	California	GU390673	GQ386823	KF361881	Europe, Africa, Asia, USSR	*Aedes, Culiseta.*	Rabbit, hare, boar, cattle, horse, birds, deer, canis	Clinical cases
Taiassui virus	Wyeomyia	JN572076	JN572075	JN572074	N/A	N/A	N/A	N/A
Tensaw virus	Bunyamwera	FJ943505	FJ943508	FJ943510	North America	*Aedes, Anopheles, Culex, Mansonia, Psorophora.*	Dogs, raccoon, cattle, cotton rat, marsh rabbit, birds	Clinical cases
Tucunduba virus	Bunyamwera	JN572079	JN572078	JN572077	Brazil	*Wyeomyia*	N/A	N/A
Utinga virus	Simbu	KF697156	KF697155	KF697154	Brazil	N/A	Primates, sloths, birds, pigs, bat (1/947), marsupial (1/834), rodent	N/A

							(1/1692)	
Utive virus	Simbu	KF697158	KF697159	KF697157	N/A	N/A	N/A	N/A
Wyeomyia virus	Bunyamwera	JN572082	JN572081	JN572080	South America	*Aedes, Anopheles, Culex, Coquillettidia, Hemagogus, Trichoprosopon, Psorophora, Wyeomia*	N/A	Clinical cases
Zungarococha virus	Unclassified	KF254783	KF254782	JN157805	N/A	N/A	N/A	N/A

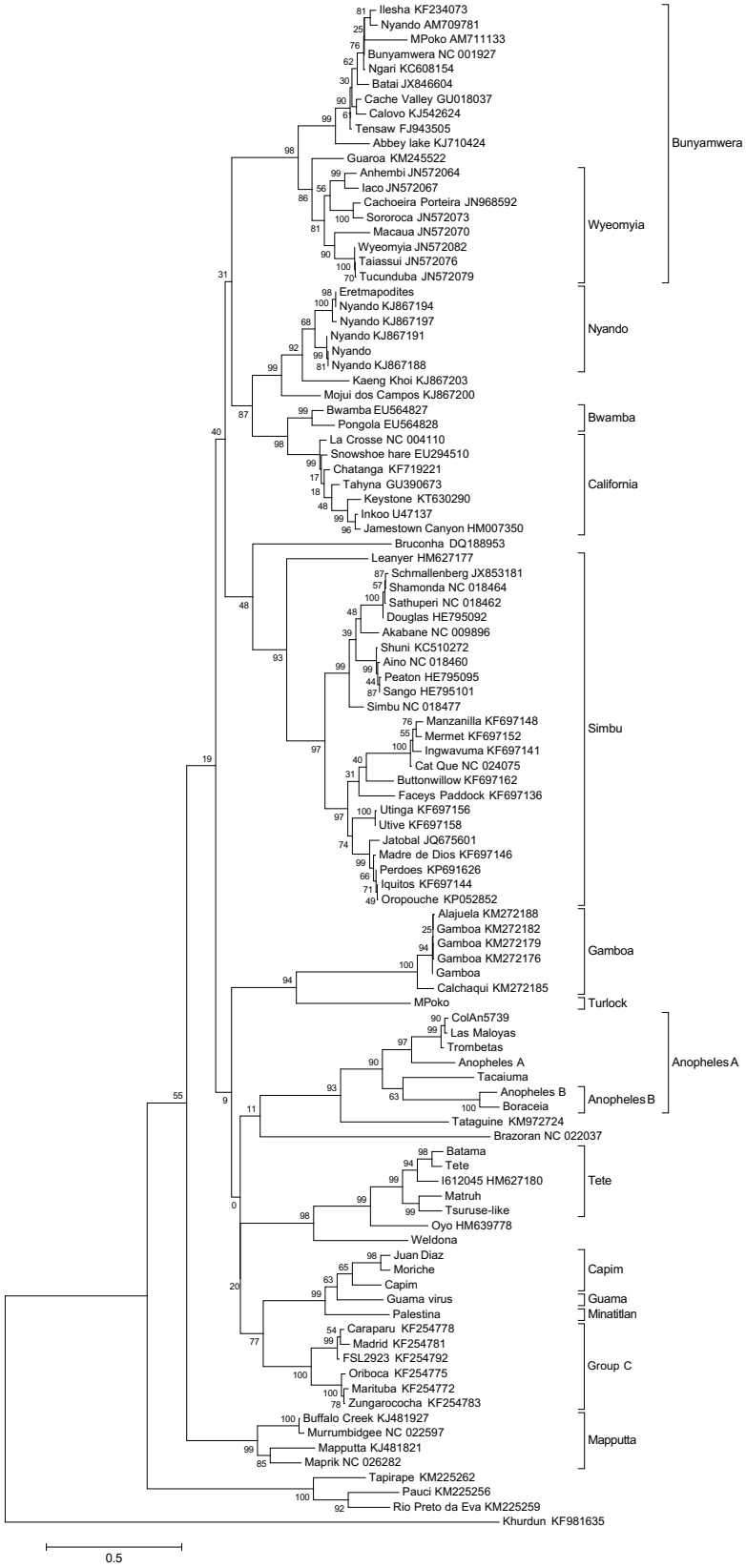
Appendix 3: Transmembrane Topology Predictions

TMHMM v2.0 predictions for Gamboa virus and Bunyamwera virus M polyproteins.

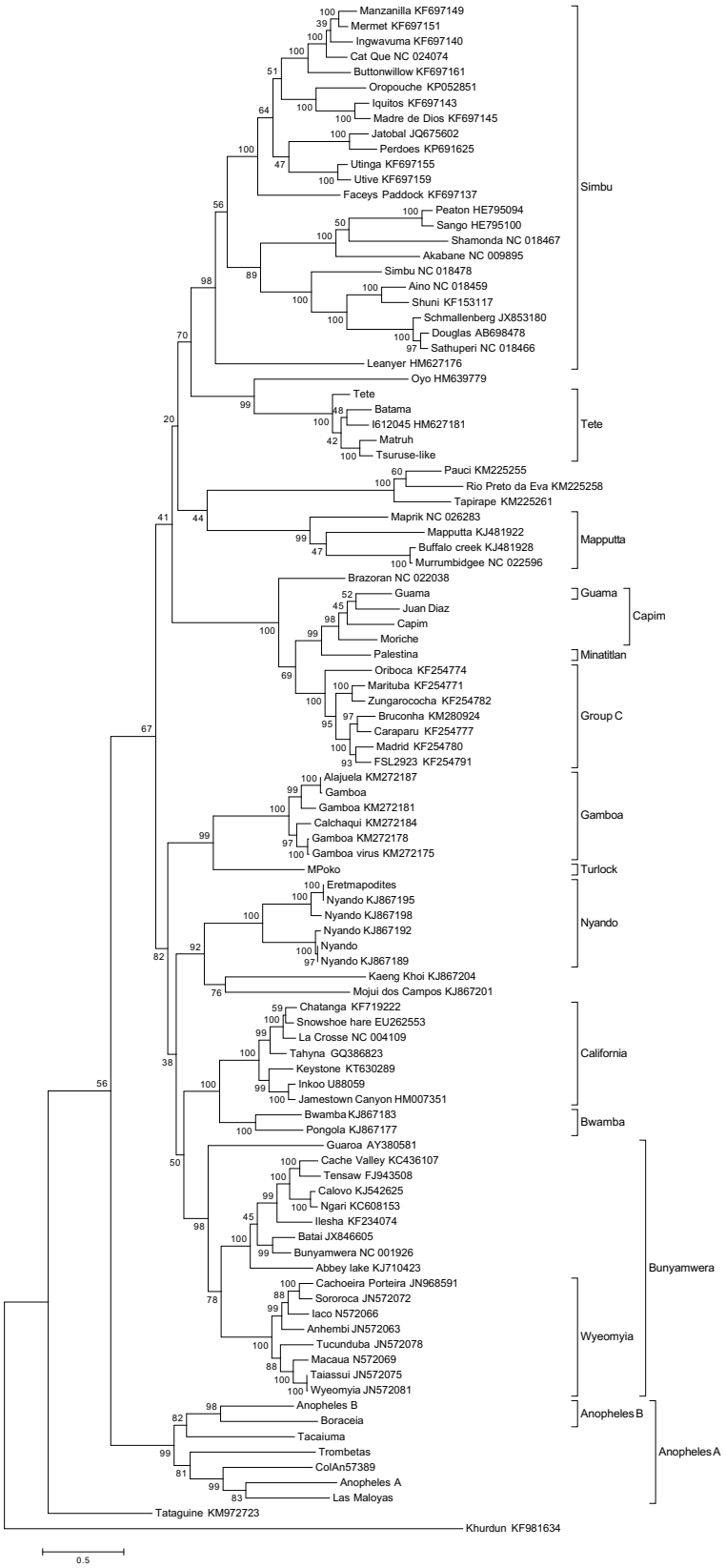


Appendix 4: Non-Compressed Maximum-likelihood Phylogenetic Trees

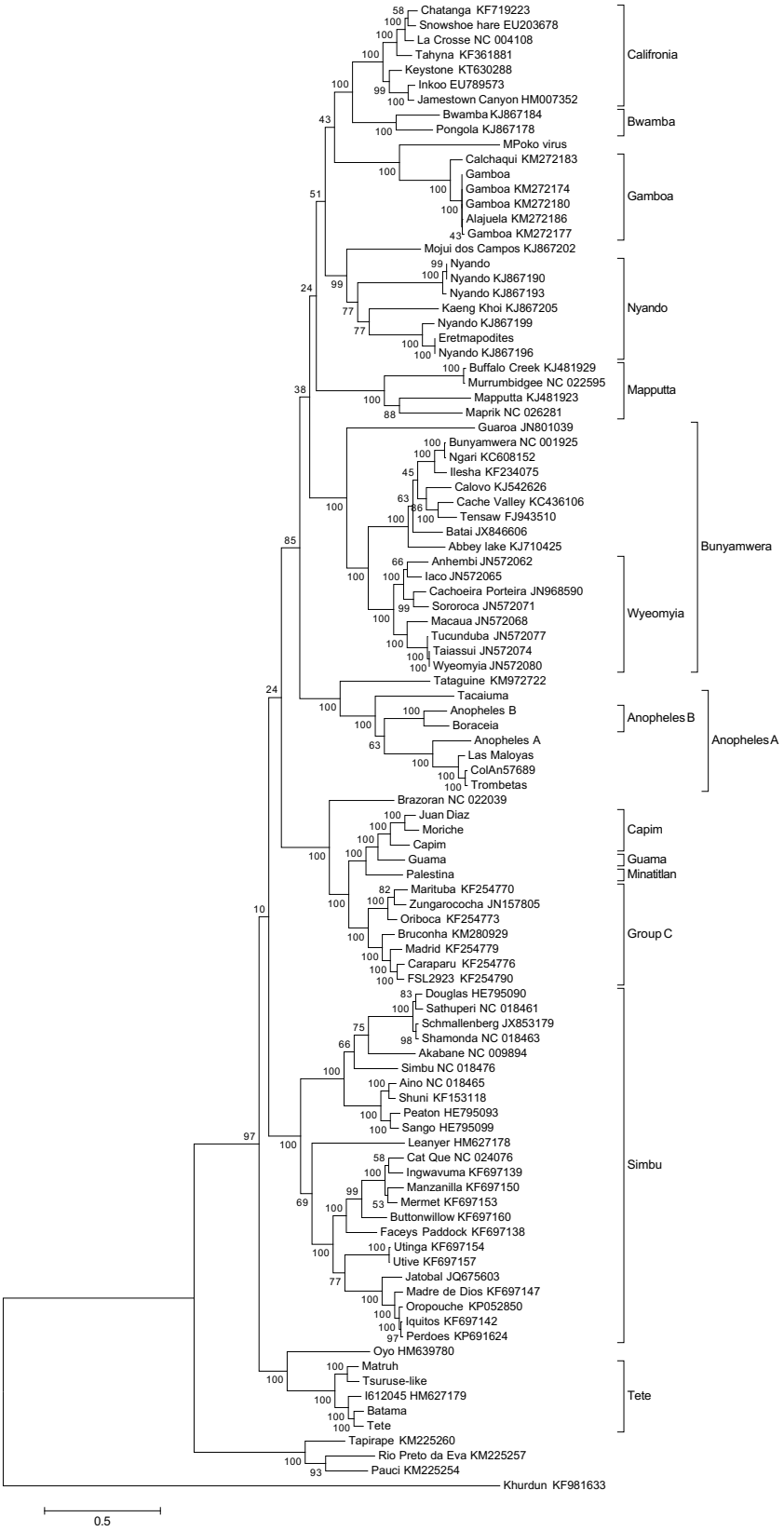
A) N ORF



B) M ORF

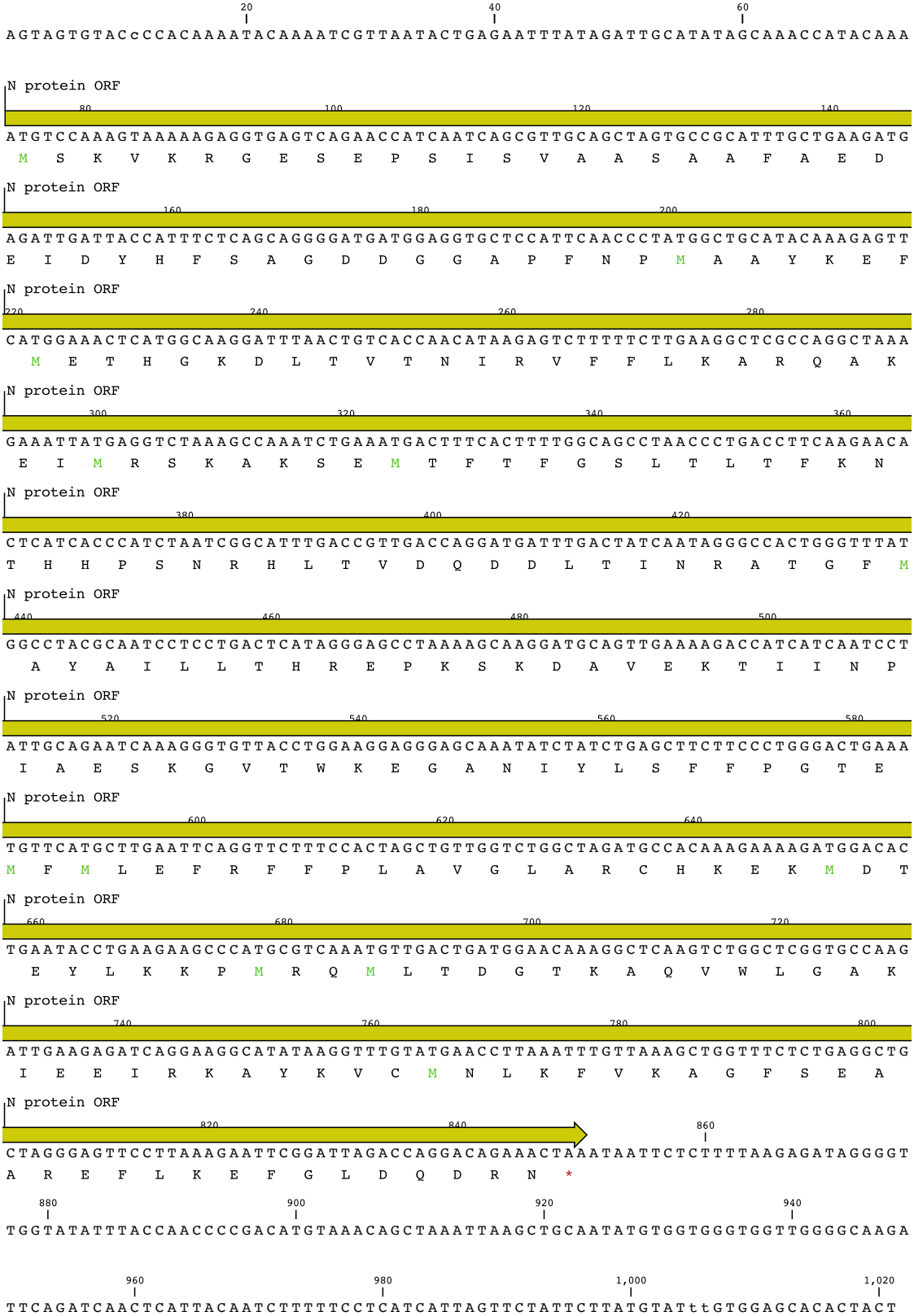

Manzanilla KF697149
Mermet KF697151
Ingwavuma KF697140
Cat Que NC 024074
Buttonwillow KF697161
Oropouche KP052851
Iquitos KF697143
Madre de Dios KF697145
Jatobal JQ675602
Perdoes KP691625
Utinga KF697155
Utive KF697159
Faceys Paddock KF697137
Peaton HE795094
Sango HE795100
Shamonda NC 018467
Akabane NC 009895
Simbu NC 018478
Aino NC 018459
Shuni KF153117
Schmallenberg JX853180
Douglas AB698478
Sathuperi NC 018466
Leanyer HM627176
Simbu
Oyo HM639779
Tete
Batama
I612045 HM627181
Matruh
Tsuruse-like
Tete
Pauci KM225255
Rio Preto da Eva KM225258
Tapirape KM225261
Maprik NC 026283
Mapputta KJ481922
Buffalo creek KJ481928
Murrumbidgee NC 022596
Mapputta
Brazoran NC 022038
Guama
Juan Diaz
Capim
Moriche
Guama
Capim
Palestina
Minatitlan
Oriboca KF254774
Marituba KF254771
Zungarococha KF254782
Bruconha KM280924
Caraparu KF254777
Madrid KF254780
FSL2923 KF254791
Group C
Alajuela KM272187
Gamboa
Gamboa KM272181
Calchaqui KM272184
Gamboa KM272178
Gamboa virus KM272175
Gamboa
MPoko
Turlock
Eretmapodites
Nyando KJ867195
Nyando KJ867198
Nyando KJ867192
Nyando
Nyando KJ867189
Nyando
Kaeng Khoi KJ867204
Mojui dos Campos KJ867201
Chatanga KF719222
Snowshoe hare EU262553
La Crosse NC 004109
Tahyna GQ386823
Keystone KT630289
Inkoo U88059
Jamestown Canyon HM007351
California
Bwamba KJ867183
Pongola KJ867177
Bwamba
Guaroa AY380581
Cache Valley KC436107
Tensaw FJ943508
Calovo KJ542625
Ngari KC608153
Ilesha KF234074
Batai JX846605
Bunyamwera NC 001926
Abbey lake KJ710423
Bunyamwera
Cachoeira Porteira JN968591
Sororoca JN572072
Iaco N572066
Anhembi JN572063
Tucunduba JN572078
Macaua N572069
Taiassui JN572075
Wyeomyia JN572081
Wyeomyia
Anopheles B
Boraceia
Anopheles B
Tacaiuma
Trombetas
ColAn57389
Anopheles A
Las Maloyas
Anopheles A
Tataguine KM972723
Khurdun KF981634
0.5

C) L ORF


Chatanga KF719223
Snowshoe hare EU203678
La Crosse NC 004108
Tahyna KF361881
Keystone KT630288
Inkoo EU789573
Jamestown Canyon HM007352
Californía
Bwamba KJ867184
Pongola KJ867178
Bwamba
MPoko virus
Calchaqui KM272183
Gamboa
Gamboa KM272174
Gamboa KM272180
Alajuela KM272186
Gamboa KM272177
Gamboa
Mojui dos Campos KJ867202
Nyando
Nyando KJ867190
Nyando KJ867193
Kaeng Khoi KJ867205
Nyando KJ867199
Eretmapodites
Nyando KJ867196
Nyando
Buffalo Creek KJ481929
Murrumbidgee NC 022595
Mapputta KJ481923
Maprik NC 026281
Mapputta
Guaroa JN801039
Bunyamwera NC 001925
Ngari KC608152
Ilesha KF234075
Calovo KJ542626
Cache Valley KC436106
Tensaw FJ943510
Batai JX846606
Abbey lake KJ710425
Anhembi JN572062
Iaco JN572065
Cachoeira Porteira JN968590
Sororoca JN572071
Macaua JN572068
Tucunduba JN572077
Taiassui JN572074
Wyeomyia JN572080
Wyeomyia
Bunyamwera
Tataguine KM972722
Tacaiuma
Anopheles B
Boraceia
Anopheles B
Anopheles A
Las Maloyas
ColAn57689
Trombetas
Anopheles A
Brazoran NC 022039
Juan Diaz
Moriche
Capim
Capim
Guama
Guama
Palestina
Minatitlan
Marituba KF254770
Zungarococha JN157805
Oriboca KF254773
Bruconha KM280929
Madrid KF254779
Caraparu KF254776
FSL2923 KF254790
Group C
Douglas HE795090
Sathuperi NC 018461
Schmallenberg JX853179
Shamonda NC 018463
Akabane NC 009894
Simbu NC 018476
Aino NC 018465
Shuni KF153118
Peaton HE795093
Sango HE795099
Leanyer HM627178
Cat Que NC 024076
Ingwavuma KF697139
Manzanilla KF697150
Mermet KF697153
Buttonwillow KF697160
Faceys Paddock KF697138
Utinga KF697154
Utive KF697157
Jatobal JQ675603
Madre de Dios KF697147
Oropouche KP052850
Iquitos KF697142
Perdoes KP691624
Simbu
Oyo HM639780
Matruh
Tsuruse-like
I612045 HM627179
Batama
Tete
Tete
Tapirape KM225260
Rio Preto da Eva KM225257
Pauci KM225254
Khurdun KF981633
0.5

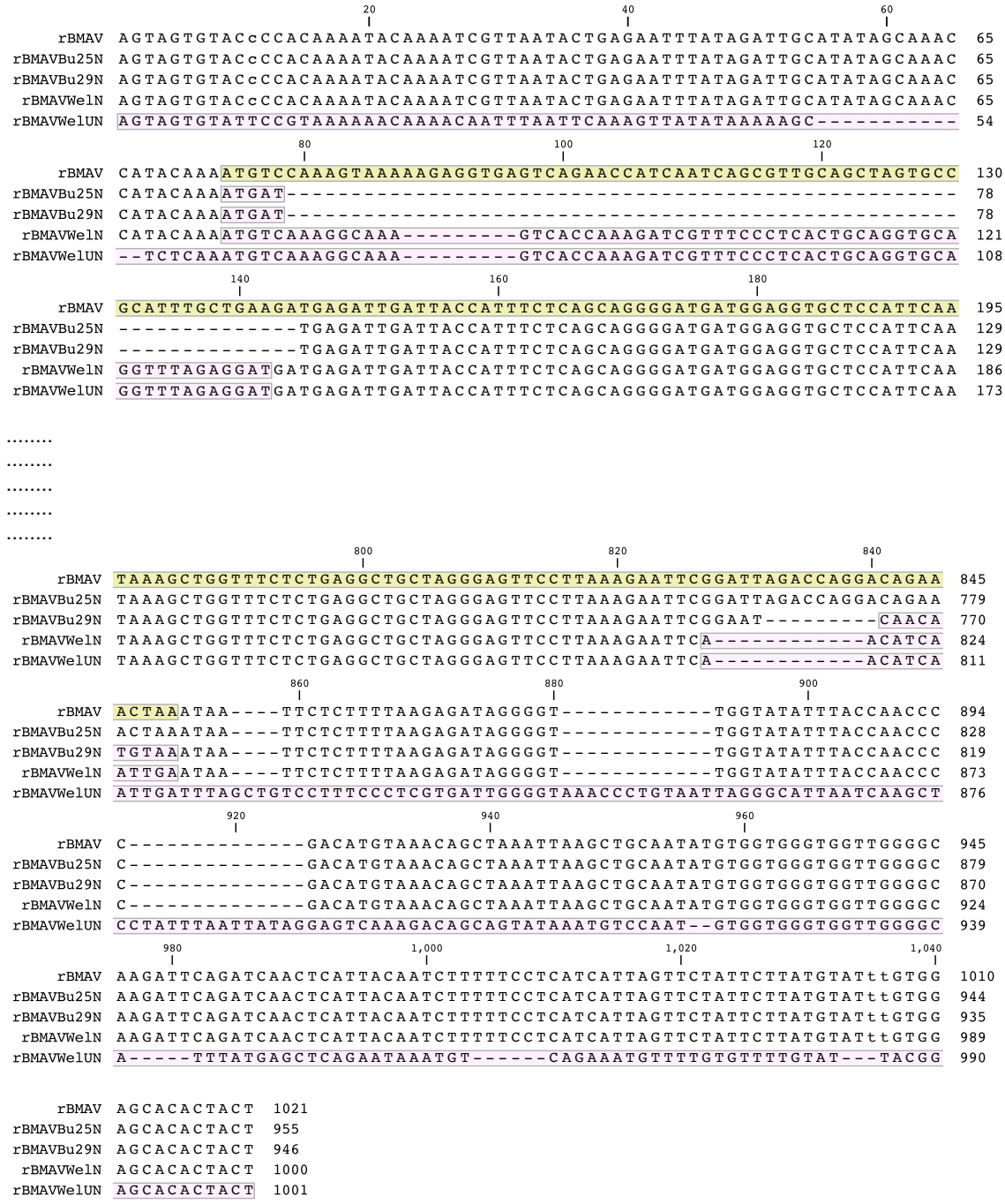
Appendix 5: rBMAV cRNA Sequences

A) wt rBMAV S segment cRNA



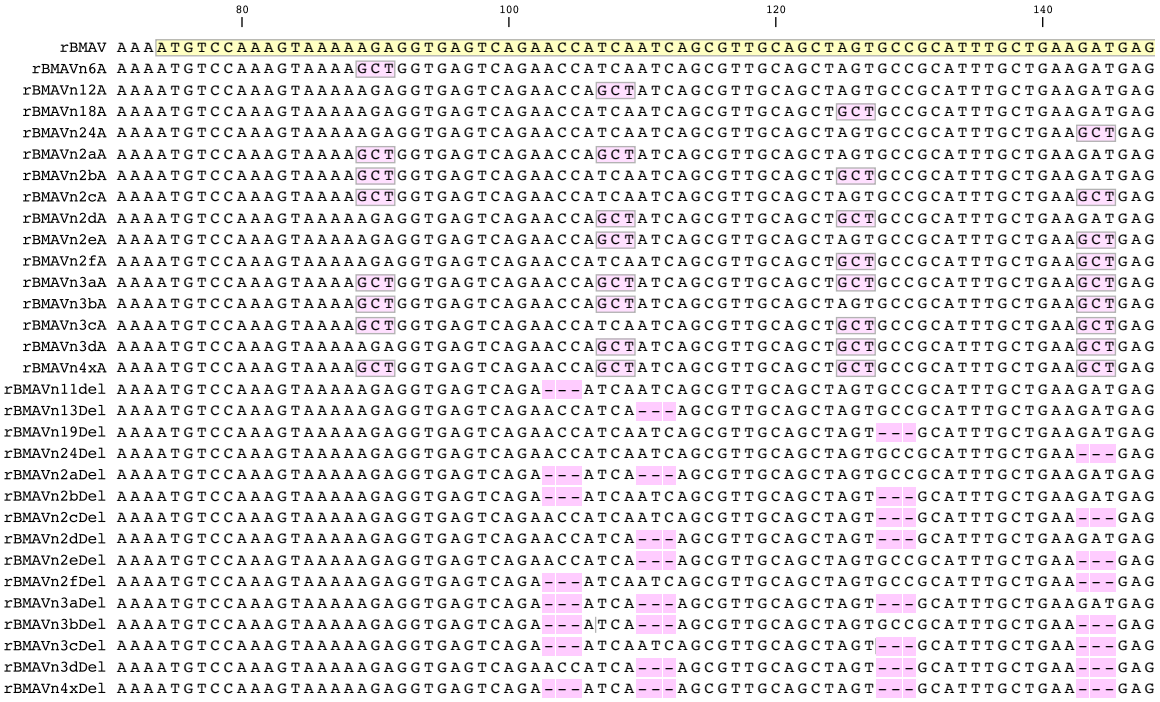
B) rBMAV S segment cRNA – Switch

Alignment of nucleotide sequences is shown, wt N protein ORF is highlighted in yellow, mutated regions in pink.



C) rBMAV S segment cRNA – Point Mutations

Alignment of mutated region of S segment cRNAs is shown, wt N protein ORF is highlighted in yellow, mutated regions in pink.



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