Division of Health Sciences

Molecular Analysis of J-virus and Beilong virus Using Reverse Genetics

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Declaration

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

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Abstract

The emergence of viruses in the family *Paramyxoviridae*, especially those such as *Hendra virus* and *Nipah virus* (NiV) that are zoonotic, highlighted the severity of disease that could be caused by infection with viruses belonging to this family. In addition to causing disease outbreaks, several newly discovered paramyxoviruses were found to have unique genetic features, which provoked renewed interest in the study of previously unclassified or uncharacterised viruses in this family.

J-virus (JPV) was isolated from wild mice, in Queensland, Australia, in 1972, and has been suggested to be a natural respiratory pathogen of mice. *Beilong virus* (BeiPV), another paramyxovirus, was first isolated from human mesangial cells in Beijing, China, in 2003, and was subsequently detected in rat mesangial cells. Following initial characterisation, the genomes of JPV and BeiPV were found to contain two genes, SH and TM, not common to other paramyxoviruses, as well as an extended attachment protein gene. BeiPV has the largest genome in the family *Paramyxoviridae*, which is, in fact, larger than that of any other virus within the order *Mononegavirales*.

The genetic material of paramyxoviruses is not amenable to manipulation via classical genetics; a reverse genetics approach was therefore employed to study the evolution and classification of JPV and BeiPV. Minireplicon systems utilising green fluorescent protein as a reporter were established for JPV, BeiPV and NiV, and were used to better assess the taxonomic status of JPV and BeiPV, and to determine the relationship between these viruses and henipaviruses, which also have exceptionally large genomes. These studies indicate that JPV and BeiPV are closely related and should be classified in the same genus and their replication and transcription machinery is different from that of the henipaviruses.

To gain an understanding of the biology of JPV and BeiPV, viral surface proteins from JPV were expressed and evaluated. Chimeric JPV virions containing recombinant surface proteins were generated and electron microscopy was used to determine the localisation of the proteins encoded by those JPV genes which are uncommon in other paramyxoviruses. Analysis of the attachment protein gene of JPV indicated that the virus was able to assemble an exceptionally large protein (156 kDa) into the virion structure, providing evidence in support of the hypothesis

that JPV and BeiPV may represent an ancient lineage of viruses within the family *Paramyxoviridae*.

In order to determine tissue tropism of JPV during experimental infection and to aid future work with a full-length JPV infectious clone, a real-time PCR assay for JPV was developed and assessed on tissues collected from mice infected with JPV. A multiplex microsphere assay for JPV and BeiPV was developed and used to analyse the seroprevalence of these viruses in Australian and Malaysian rodents. Although there is currently no evidence for disease caused by JPV or BeiPV, this does not preclude the emergence of a zoonotic rodent paramyxovirus related to these viruses. If this were to occur, the tools for virus detection and serological monitoring are now established.

Preface

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General Abbreviations

AAHL	Australian Animal Health Laboratory
AP	alkaline phosphatase
APMV- 2-9	Avian paramyxovirus virus 2-9
BeiPV *	Beilong virus
bp	base pair
BPIV-3	Bovine parainfluenza virus 2
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CDV	Canine distemper virus
CeMV	Cetacean morbillivirus virus
CMV	Cytomegalovirus
CPE	cytopathic effect
DNA	deoxyribonucleic acid
EBOV	Ebola virus
EGFP	enhanced green fluorescent protein
F	fusion protein/ gene
FCS	foetal calf serum
FDLV	Fer-de-Lance virus
FWPV	Fowlpox virus
GFP	green fluorescent protein
H/HN/G	attachment protein/gene
HeV	Hendra virus
HPIV-1-4	Human parainfluenza virus 1-4
hr	hour(s)
HRSV	Human respiratory syncytial virus
IFN	interferon
JPV *	J-virus
kb	kilobase
kDa	kilodalton
L	large protein/gene
LB	Luria-Bertani
M	matrix protein/ gene
m.o.i.	multiplicity of infection
MAb	monoclonal antibody
MenPV *	Menangle virus
MeV	Measles virus
min	minute(s)
MosPV *	Mossman virus
MPRV	Mapuera virus
mRNA	messenger ribonucleic acid
MuV	Mumps virus
MW	molecular weight
N	nucleoprotein/gene
n/µ/m m/L/g	nano/micro/milli metre/litre/gram
NarPV	Nariva virus

NCBI	National Center for Biotechnology Information
NDV	Newcastle disease virus
NiV	Nipah virus
NNS	non-segmented, negative-sense, single-stranded RNA
NNSV	non-segmented, negative-sense, single-stranded RNA virus
OD	optical density
ORF	open reading frame
P	phosphoprotein/ gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	Phocine distemper virus
PoRV	Porcine rubulavirus
PPRV	Pestes-des-petits ruminants virus
RABV	Rabies virus
RNA	ribonucleic acid
rpm	revolutions per minute
RPV	Rinderpest virus
RSV	Respiratory syncytial virus
SARS	severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
SeV	Sendai virus
SH	small hydrophobic protein/gene
ss	single-stranded
SSPE	subacute sclerosing panencephalitis
STAT	signal transducer and activator of transcription
SV-5/10/41	Simian virus 5/10/41
TBS	tris buffered saline
$TCID_{50}$	tissue culture infectious dose 50
TioPV *	Tioman virus
TM	transmembrane protein/gene
TuPV	Tupaia paramyxovirus
UTR	untranslated region
VACV	Vaccinia virus
VACV-ANK	modified Vaccinia Ankara
vRdRp	viral RNA dependent RNA polymerase

* Denotes viruses abbreviated in accordance with the new naming convention used for paramyxoviruses in the 8th ICTV report (Pringle 2005).

CHAPTER 1

Literature Review

1.1 New and Emerging Viral Diseases

Infectious diseases have played a significant role throughout human history. Identification of disease dates back to ancient times and the quest for understanding through science has lead to the discovery of viruses and bacteria as the causative agents of various types of infection and illness. Epidemics and pandemics such as plague in the 1300s and influenza in the 1900s prompted investigations into the treatment and prevention of diseases. Pioneering work by Edward Jenner in 1796 lead to the advent of vaccination and, as technology progresses, basic principles devised to understand and treat disease are being replaced with complex strategies involving molecular biology and genetic manipulation of targeted organisms. Pathogenicity of viruses and our vulnerability to the effects of infectious agents is highlighted through the emergence of new diseases, reappearance of pre-existing disease and, unfortunately, bioterrorism.

Emerging infectious viral diseases are those that have recently appeared in a population as a result of a new virus or the recognition of a previously undetected virus. Emergence of an infectious viral disease may occur due to the extension of the geographic or host range of the virus (Morse 1995). Emerging infectious diseases are often zoonotic, hence having the ability to be transmitted between humans and animals either directly or via a vector. Acts of bioterrorism may result in the release of new or engineered pathogens with increased virulence into the population in an unpredictable manner, resulting in cases of emerging or re-emerging infectious disease (Feldmann *et al.* 2002).

Emerging infectious diseases, and the identification of the pathogens responsible, are frequently coupled with disease outbreaks. The emergence of severe acute respiratory syndrome (SARS) is one such occurrence. Cases of severe atypical pneumonia with unknown etiology were first identified in Guangdong Province, China late in 2002 in an outbreak of disease later designated as SARS. By April 2003 there were 4300 cases of SARS reported to the World Health Organisation, along with 250 SARS-related deaths occurring in 25 different countries (Rota *et al.* 2003). A novel coronavirus was isolated in March 2003 and determined to be the causative agent of the life-threatening respiratory illness. Clinical samples such as blood, serum and tissue were used to inoculate various

continuous cell lines, from which the virus was subsequently isolated (Ksiazek *et al.* 2003). Experimental infection of macaques (*Macaca fascicularis*) proved that the newly discovered virus was the aetiological agent of SARS (Fouchier *et al.* 2003).

The impact re-emerging pathogens can create is adequately demonstrated by the rare, but unpredictable outbreaks of haemorrhagic fevers caused by viruses belonging to the family *Filoviridae*. In 1976, *Ebola virus* (EBOV) emerged in two simultaneous outbreaks of disease in different regions of Africa. One outbreak occurred in the Democratic Republic of Congo (formally Zaire), resulting in 318 cases of haemorrhagic fever with a mortality rate of 79% (Report of an International Commission 1978). The other outbreak occurred in Sudan, where 284 cases were reported with a mortality rate of 53% (WHO/International Study Team 1978). Re-emergence of EBOV subtype Sudan in Uganda (Okware *et al.* 2002) and EBOV subtype Zaire in Gabon (Geisbert *et al.* 2002) and several other incidences of outbreaks occurring sporadically since the 1976 emergence of the virus in the Democratic Republic of Congo, highlight the susceptibility of the human population to a virus that was previously geographically restricted.

In contrast to the punctuated outbreaks of Ebola, the emergence of *Human immunodeficiency virus* (HIV) resulted in an uncontrollable worldwide pandemic. HIV was first discovered in 1983 when a new retrovirus was isolated (Barre-Sinoussi *et al.* 1983). The entire 9,193 nucleotide genome sequence of HIV was published in 1985 (Wain-Hobson *et al.* 1985), and considerable genetic diversity was demonstrated among subtypes of the virus, designated clades A-J. The oldest reported HIV sequence dates back to 1959 and phylogenetically placed this isolate between clades C and D (Zhu *et al.* 1998). Although HIV existed in humans as early as 1959, there was no detectable disease until 1983, emphasising the differences in disease impact resulting from emerging viruses.

The last two decades have yielded the emergence of several new paramyxoviruses, the majority of which were previously unclassified (Wang & Eaton 2001). The discovery of these viruses occurred not only as a result of disease outbreaks but also under circumstances where no disease correlation was evident. New paramyxoviruses have emerged from an extensive range of aquatic and terrestrial animals, demonstrating the vast host range of this family of viruses. Zoonotic viruses were among the emergent species, emphasising the pathogenicity of paramyxoviruses. These emergent viruses provide the opportunity to gain an insight into the evolution, diversity, epidemiology and pathogenesis of paramyxoviruses. Specific examples of new and emerging paramyxoviruses are discussed in Section 1.7.

1.2 Classification of Paramyxoviruses

1.2.1 Order *Mononegavirales*

The order *Mononegavirales* is divided taxonomically into four families- *Filoviridae, Bornaviridae, Rhabdoviridae* and *Paramyxoviridae* (Figure 1.1). All members of these viral families contain a linear, non-segmented, negative-sense single-stranded RNA (NNS) genome varying from 8.9-19.2 kilobases in length (Pringle 2005). The RNA of viruses in the order *Mononegavirales* is contained in a helical nucleocapsid in the enveloped virion and is no longer infectious once deproteinised. The genomes of the four *Mononegavirale* families are organised in a similar order beginning with a 3' non-translated region, followed by core, envelope and polymerase protein genes, and ending in a 5' non-translated region (Pringle 2005). Viral genomes also contain complementarity of the 3' and 5' termini, and transcription occurs via a virion-associated RNA dependent RNA polymerase (Pringle 2005). Morphology of viruses differs between the families.

1.2.2 Family *Paramyxoviridae*

Viruses within the *Paramyxoviridae* family consist of a NNS genome contained exclusively within a nucleocapsid of 18 nm in diameter, 1 μm in length and 5.5 nm in pitch (Lamb & Kolakofsky 2001). The viral RNA has no 5' cap or covalently linked proteins, and the 3' end of the genome is not polyadenylated. Virions are pleomorphic, but usually spherical, range from 150 nm upwards in size and possess an envelope that is derived from the host cell plasma membrane (Lamb *et al.* 2000). Projecting from the viral envelope are two or three types of transmembrane glycoproteins of 8-12 nm in length with a placement of 7-10 nm from each other, depending on the genus. Replication of paramyxoviruses occurs entirely within the cytoplasm of the host cell. Due to the unsegmented single-stranded nature of the viral RNA, and the location of the RNA within a nucleocapsid, paramyxoviruses are genetically stable with mutation the only mechanism whereby variation can occur (Bellini *et al.* 1998). Evidence of recombination has recently been reported in paramyxoviruses but recombination rate is unlikely to be a trait that is set by natural selection to create advantageous or purge deleterious mutations (Chare *et al.* 2003). The family *Paramyxoviridae* is divided into two subfamilies- *Paramyxovirinae* and *Pneumovirinae* (Figure 1.1).

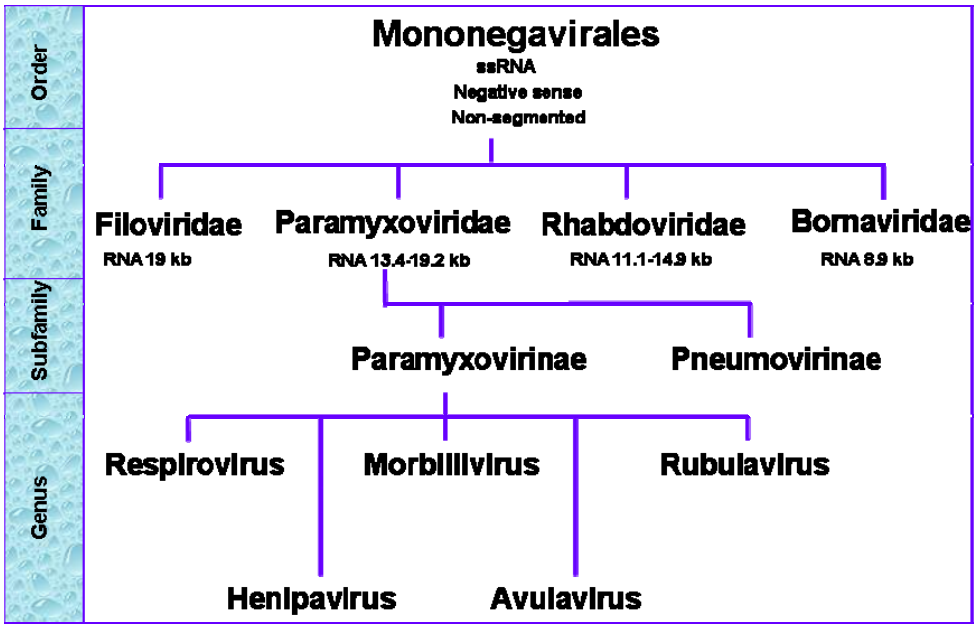


Figure 1.1- Classification of Paramyxoviruses

The order *Mononegavirales* contains four families; *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae* and *Bornaviridae*. The family *Paramyxoviridae* is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The five genera contained within the *Paramyxovirinae* are *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Henipavirus* and *Avulavirus*. This figure was adapted from Wang *et al.* (2003).

Classification of viruses to determine subfamily is based on morphology, genome organisation, biological activity of proteins, and sequence relationship of encoded proteins (Lamb & Kolakofsky 2001). Members of the *Pneumovirinae* are morphologically distinctive from members of the *Paramyxovirinae* in that they possess narrower nucleocapsids and O-linked glycosylation of the attachment protein.

1.2.3 Subfamily *Paramyxovirinae*

The subfamily *Paramyxovirinae* contains viruses with genomes of 15-19 kb in length. The term 'gene', when used in reference to paramyxoviruses, refers to the sequence encoding a single mRNA, even if more than one open reading frame (ORF) is present or more than one protein is encoded (Lamb & Kolakofsky 2001). Members of the subfamily *Paramyxovirinae* contain 6-8 transcriptional units within a highly conserved genome structure. The genome is encapsidated in a nucleocapsid of 18 nm in diameter and a pitch of 5.5 nm. Of the proteins produced by these viruses, the most conserved between *Paramyxovirinae* are the nucleoprotein (N), matrix (M) and polymerase (L) protein. The fusion (F) and attachment (HN/H/G) proteins are less conserved and the phosphoprotein (P) gene is poorly conserved, with the exception of a unique region encoding the V protein-specific region that is not present in the P protein (Lamb *et al.* 2000). Conserved 3' leader and 5' trailer extragenic regions frame the genome termini with 12 nucleotides demonstrating complementarity. Intergenic regions (IGRs) are relatively conserved, with the trinucleotide sequence 3'-GAA-5' predominating, but may also contain sequences of variable length (Bellini *et al.* 1998).

The lipid envelopes of paramyxoviruses contain two surface glycoproteins. The F proteins are present on all viruses and attachment proteins may be designated HN, H or G, based on the presence or absence of haemagglutinin and neuraminidase activity. Surface glycoproteins, in conjunction with the RNA editing mechanism used to translate the P gene, aid in further classifying paramyxoviruses into different genera (Lamb *et al.* 2000). The subfamily *Paramyxoviridae* is currently grouped into five genera. Originally the subfamily was divided into three genera- *Morbillivirus*, *Respirovirus* and *Rubulavirus* (Lamb *et al.* 2000), but recently two other genera were recognised- *Avulavirus* and *Henipavirus* (Mayo 2002). The genus *TuPV-like viruses* has been proposed (Tidona *et al.* 1999), along with *Ferlavirus* (Kurath *et al.* 2004). Several other new or emerging paramyxoviruses cannot be classified as part of any of the existing genera (Wang *et al.* 2003).

Genus *Morbillivirus*

Morbilliviruses are spherical or pleomorphic viruses 150-200 nm in diameter and contain a genome 15.6-15.9 kb in size. The morbillivirus genome contains six transcriptional units, producing eight ORFs (Barrett & Rima 2002). Viruses currently recognised in the *Morbillivirus* genus are *Measles virus* (MeV), *Rinderpest virus* (RPV), *Canine distemper virus* (CDV), Pestes-des-petits ruminants virus (PPRV), *Phocine distemper virus* (PDV) and Cetacean morbillivirus virus (CeMV) (Pringle 2005). During infection, morbilliviruses produce intracytoplasmic and intranuclear inclusion bodies containing viral nucleocapsids. Sequence relatedness between morbilliviruses is low to moderate, depending on protein analysed (Lamb *et al.* 2000). Members of the genus display a greater amino acid homology with members within the genus than with members of other genera within the *Paramyxovirinae*. The P gene of morbilliviruses encodes the P, V and C proteins. Transcripts faithfully transcribed from the P gene produce the P protein, transcripts where one non-templated G residue is inserted at the RNA editing site produce V proteins and the C protein is encoded by an alternate ORF (Lamb *et al.* 2000).

MeV infection results in acute respiratory syndrome followed by systemic spread producing a generalised rash. In rare cases subacute sclerosing panencephalitis (SSPE), a persistent fatal infection of the nervous system, can develop 5-10 years after initial infection (Morrison & Portner 1991) if mutated forms of MeV persist (Reutter *et al.* 2001). MeV is the type species of the genus *Morbillivirus* although a proposal has been made based on antigenic comparison of conserved epitopes in MeV, CDV and RPV that RPV is the archetype morbillivirus, from which CDV and MeV evolved (Norrby *et al.* 1985).

Genus *Respirovirus*

Respiroviruses are spherical or pleomorphic viruses 150-350 nm in diameter and contain a genome of 15.0-15.5 kb in size. The respirovirus genome contains six transcriptional elements separated by a trinucleotide IGR (3'-GAA-5') and all members of this genus produce haemagglutinin and neuraminidase proteins (Banjeree & De 2002). Viruses currently recognised as part of the genus *Respirovirus* are *Sendai virus* (SeV), *Human parainfluenza virus 1* (HPIV-1), *Human parainfluenza virus 3* (HPIV-3), *Bovine parainfluenza virus 3 (*BPIV-3) and *Simian virus 10* (SV-10) (Pringle 2005). SeV, the type species of the genus, was isolated in 1953 (Banjeree & De 2002) and has been fully sequenced (Shioda *et al.* 1986). Relatedness, based on amino acid homology, varies from high to low between

group members. SeV and HPIV-1 have considerable sequence relatedness, but are distinguished by host range, as SeV infects rodents and HPIV-1 infects humans. A similar relationship links HPIV-3 and BPIV-3, where HPIV-3 infects humans and BPIV-3 infects cattle (Lamb *et al.* 2000).

All members of the genus produce C proteins from alternate ORFs within the P gene. Two overlapping ORFs upstream of the RNA editing site within the P gene are designated P-amino 1 and P-amino 2 (Lamb & Kolakofsky 2001). SeV and HPIV-1 utilise the P-amino 2 ORF to produce up to four nested polypeptides, formally identified as C, C', Y1 and Y2 (Curran & Kolakofsky 1989), with the use of four ribosomal initiation sites. Faithful transcription of the P gene results in mRNA encoding the P protein. Addition of one non-templated G residue via the RNA editing site leads to the production of the V protein (Lamb *et al.* 2000). HPIV-1 is a notable exception to the rule as this virus does not edit RNA of the P gene, hence does not produce a V protein. HPIV-3 produces a D protein in lieu of a V protein (Matsuoka *et al.* 1991).

Genus *Rubulavirus*

Rubulaviruses are spherical or pleomorphic viruses 150-250 nm in diameter and contain a genome of 15.2-15.6 kb in size. *Mumps virus* (MuV) is the type species and other members of the genus are *Human parainfluenza virus 2* (HPIV-2), *Human parainfluenza virus 4* (HPIV-4), *Porcine rubulavirus* (PoRV), *Mapuera virus* (MPRV), *Simian virus 5* (SV-5), *Simian virus 41* (SV-41), *Menangle virus* (MenPV) and *Tioman virus* (TioPV) (Pringle 2005). Avian paramyxoviruses were previously classed as rubulaviruses before the creation of the genus *Avulavirus*.

IGRs of variable length separate transcriptional elements of rubulaviruses and all viral members of this genus have an attachment protein with haemagglutinin and neuraminidase activities. The P gene is smaller than that of other paramyxoviruses and is capable of producing a V and W protein in addition to the P protein, but there is currently no evidence supporting the expression of a C protein in any rubulaviruses (Lamb *et al.* 2000). The addition of two non-templated G residues in the RNA editing site of the P gene leads to the production of the P protein, the addition of one non-templated G residue produces the W protein, while a non-edited P transcript produces the V protein. This editing strategy differs from that seen in other viruses within the subfamily *Paramyxovirinae* (Lamb *et al.* 2000). An additional gene encoding a small hydrophobic transmembrane surface protein is located between the F and HN genes of MuV and SV-5 (Ito & Tsurudome 2002).

Genus *Avulavirus*

Viruses belonging to the genus *Avulavirus*, the name derived from avian rubulavirus, are pleomorphic and generally rounded but can sometimes be filamentous. Virions range from 100-500 nm in diameter and contain a genome of 15.1-16.2 kb in size. *Avian paramyxovirus type 1* (APMV-1) was first recognised as a causative agent of disease in 1926 and subsequently identified as a member of the genus *Rubulavirus*. APMV-1 was later termed *Newcastle disease virus* (NDV) (Peeters & Koch 2002). NDV is the type species of *Avulavirus,* accompanied in the genus by eight other avian infecting paramyxoviruses, *Avian paramyxovirus 2-9* (APMV 2-9). All avulaviruses possess haemagglutinin and neuraminidase activity. The P gene encodes the P, V and W proteins (Peeters & Koch 2002).

Complete sequencing of the NDV genome (de Leeuw & Peeters 1999) revealed a genome organisation typical of most paramyxoviruses, but sequencing of the APMV-6 genome uncovered the existence of an extra gene between the F and HN genes (Chang *et al.* 2001). The small hydrophobic (SH) gene of APMV-6 has no homology to any other known proteins in the NCBI database (Chang *et al.* 2001), but the location of the gene between the F and HN genes is the same as the placement of the SH gene in SV-5 and MuV (Ito & Tsurudome 2002). Amino acid sequence homology is greatest between members within the genus *Avulavirus* and these viruses are closely related to the rubulaviruses.

Genus *Henipavirus*

The genus *Henipavirus* currently contains two viral species- *Hendra virus* (HeV) and *Nipah virus* (NiV). Henipaviruses may be spherical, pleomorphic or filamentous, are 40-600 nm in diameter and contain a genome of 18.2 kb in length (Wang & Eaton 2002). Genome length of paramyxoviruses prior to the sequencing of HeV and NiV was described as uniform, varying no more than 5% among all members of the family *Paramyxoviridae* (Lamb *et al.* 2000). Complete sequencing of HeV revealed the viral genome was 15% longer than the genomes of other paramyxoviruses, the size difference due to exceptionally long untranslated regions at the 3' end of mRNA produced by 5 of the 6 genes (Chua *et al.* 2000).

HeV is the type species of the genus. HeV and NiV both lack haemagglutinin and neuraminidase activity, and the genome termini are highly conserved. IGRs separating viral genes contain the sequence 3'-GAA-5' and are absolutely conserved. The F gene of henipaviruses contains a unique sequence indicating endocytosis (Meulendyke *et al.* 2005; Vogt *et al.* 2005) and host cell proteases other than furin are utilised by the viruses to cleave the fusion protein

(Moll *et al.* 2004; Pager *et al.* 2004; Pager & Dutch 2005). The P gene of henipaviruses is capable of encoding a P protein when transcribed exactly, a V protein when a single non-templated G residue is inserted into the RNA editing site or a C protein via an alternate ORF (Wang & Eaton 2002). The conserved QGDNQ motif within the L gene of HeV was replaced with the QGDNE motif (Chua *et al.* 2000), as was the case in NiV (Harcourt *et al.* 2001).

Proposed genus *TuPV-like viruses*

Tupaia paramyxovirus (TuPV) was isolated from a tree shrew (*Tupaia belangeri*), in the late 1970s (Tidona *et al.* 1999) (See Section 1.7). The isolated virus was 300-1000 nm in diameter, pleomorphic and possessed and envelope with 8 nm long surface projections (Tidona & Darai 2002). Sequencing of the 17.9 kb TuPV genome, the N and P/V/C genes in particular, established an evolutionary relationship between this emergent virus and the morbilliviruses (Tidona *et al.* 1999). TuPV contains haemagglutinin but does not possess neuraminidase activity. The IGRs separating genes are conserved, with the trinucleotide sequence 3'-GAA-5', and the 3' non-transcribed leader region is also conserved with a 55 nucleotide sequence. The 5' non-transcribed region is significantly longer than that of other paramyxoviruses, with a length of 590 nucleotides (Tidona & Darai 2002). Sequencing of the L gene revealed the conserved QGDNQ region of the polymerase was replaced with QGDNE (Tidona *et al.* 1999), as reported in HeV and NiV (Chua *et al.* 2000). The genus name *TuPV-like virus* was proposed for placement of viruses phylogenetically related to TuPV, with TuPV as the type species (Tidona & Darai 2002). The International Committee on Taxonomy of Viruses has not approved this genus.

Proposed genus *Ferlavirus*

Fer-de-Lance virus (FDLV) is an emergent reptilian paramyxovirus (see Section 1.7) that was isolated from a Fer-de-Lance viper (*Bothrops atrox*) in 1972 (Clark *et al.* 1979). FDLV was classed as a member of the subfamily *Paramyxovirinae* based on gene length, expression of multiple proteins from P gene, conserved V carboxyl terminal domain and lack of an M2 protein, the later conclusively excluding the virus from the subfamily *Pneumovirinae* (Kurath *et al.* 2004). Comparative analysis of the F gene revealed a highly conserved heptad repeat motif and furin cleavage site typical of other members of the subfamily *Paramyxovirinae* (Franke *et al.* 2001; Franke *et al.* 2006).

The FDLV genome is 15.3 kb in length, obeys the rule of six and contains all genes common to paramyxoviruses, along with an extra gene, designated U. Presence of a unique gene distinguishes FDLV from other paramyxoviruses and based on phylogenetic analysis of the HN and L genes, FDLV is most closely related to the respiroviruses (Kurath *et al.* 2004). A study of 16 paramyxoviruses isolated from reptiles demonstrated strong antigenic relationships between viruses, irrespective of the virus origin. The novel U gene was also located in viruses isolated from two different snakes. Based on these findings the suggestion was made that snake paramyxoviruses should comprise a new genus within the subfamily *Paramyxovirinae* (Kurath *et al.* 2004). The name proposed was *Ferlavirus*, with FDLV the type species. The International Committee on Taxonomy of Viruses has not approved this genus.

1.3 Virus Structure

Paramyxovirus virions pleomorphic and are composed of two structural components; the helical ribonucleoprotein core and the lipoprotein bilayer membrane, acquired from the host cell that constitutes the envelope. Virions are sensitive to heat, lipid solvents, ionic and non-ionic detergents, formaldehyde and oxidising agents and exhibit a buoyant density of 1.18-1.20 g/cm^2 in a sucrose gradient (Lamb *et al.* 2000)

The NNS genomic RNA is always tightly encased by nucleocapsid (N) proteins in a left-handed coil with distinct 'herringbone' morphology. The nucleocapsid, in conjunction with highly phosphorylated P proteins and multifunctional L protein that possesses catalytic polymerase activity, forms a complex that has RNA-dependent RNA polymerase activity. Nucleocapsids are 18 nm in diameter, 1 μm in length and 5.5 nm in pitch (Lamb & Kolakofsky 2001), never disassemble naturally and are able to withstand high salt and gravity forces of caesium chloride density gradient centrifugation.

Projecting 8-12 nm from the lipid bilayer of the viral envelope are two or three glycoproteins, glycosylated by N-linked carbohydrate side chains (Lamb *et al.* 2000). All paramyxoviruses possess attachment (HN, H or G) proteins and transmembrane F proteins that are activated by proteolytic cleavage (Conzelmann 1998). In addition, an SH protein is present in some rubulaviruses (Ito & Tsurudome 2002). The most abundant protein, the matrix (M) protein is basic, slightly hydrophobic and is associated with the inner surface of the lipid envelope. A paramyxovirus virion is schematically represented in Figure 1.2.

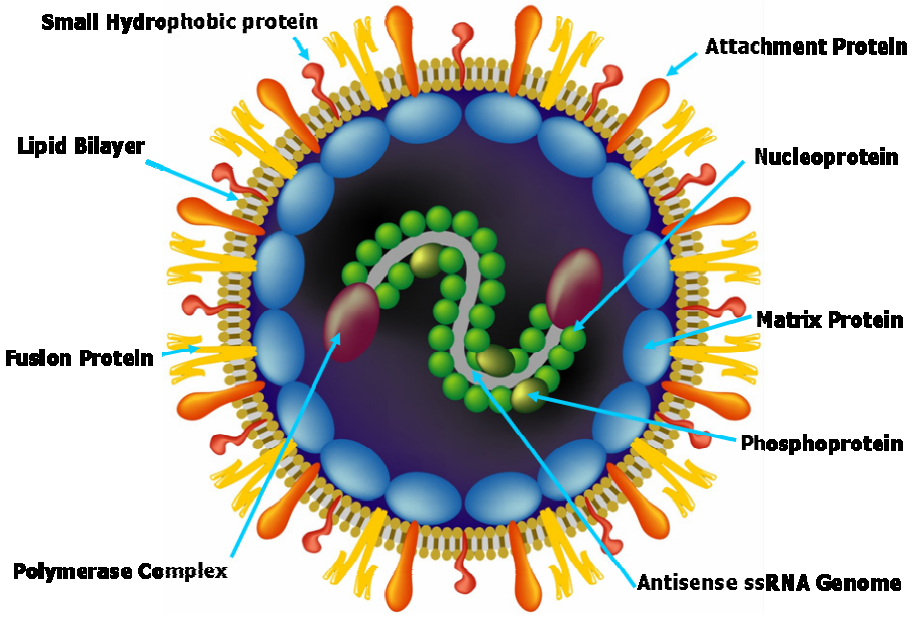


Figure 1.2- Schematic Representation of a Paramyxovirus

The schematic paramyxovirus diagram illustrates the location of the small hydrophobic (SH) protein, lipid bilayer, fusion (F) protein, polymerase (L) complex, attachment (HN/H/G) protein, nucleoprotein (N), matrix (M) protein, phosphoprotein (P) and the single-stranded (ss) RNA genome. This figure was supplied by Dr. Ian M. Mackay, Sir Albert Sakzewski Virus Research Centre (July, 2004).

1.4 Paramyxovirus Genome Characteristics

1.4.1 Genome organisation

The genome organisation of the *Paramyxovirinae* is conserved, although some species within the subfamily possess additional genes, and is as follows- 3'-N-P/V/C-M-F-HN-L-5, where HN is the attachment protein and may be either HN, H or G. Paramyxovirus genes are defined as sequences encoding a single mRNA, even if the sequence contains more than one ORF or encodes more that one protein (Lamb & Kolakofsky 2001). Genes are separated by non-transcribed IGRs that are generally conserved trinucleotide sequences, but are also present in variable in length (1-85 nucleotides in rubulaviruses) and nucleotide composition. A schematic representation of a paramyxovirus genome is given in Figure 1.3 and the specific genome organisations for the type species of each genus are displayed in Figure 1.4.

1.4.2 Genome replication and the 'rule of six'

Viral RNA of paramyxoviruses is required to be bound by a helical nucleocapsid in order to be infectious. Electron microscopy analysis of SeV nucleocapsids revealed helices of left-handed symmetry, which suggested a stoichiometric relationship between six nucleotides of RNA and one N protein subunit (Egelman *et al.* 1989). For efficient replication, SeV RNA must contain a total number of nucleotides that is a multiple of six, which was dubbed the 'rule of six' (Calain & Roux 1993).

The rule of six does not exclude other requirements for efficient replication, such as total minimum RNA length and *cis*-acting sequences. The RNA polymerase initiates more efficiently when *cis*-acting promotor sequences are found in the correct context with relation to N subunits, determined by the length of the entire genome (Hausmann *et al.* 1996). Association of N monomers with hexameric genomic nucleotide sequences begins with the first nucleotide at the 5' end of the genome and continues until reaching the 3' end, until the genome is precisely covered by N subunits. Most members of the subfamily *Paramyxovirinae* obey the rule of six, but the requirement for N subunit association correlates with whether the virus edits the P gene (Kolakofsky *et al.* 1998).

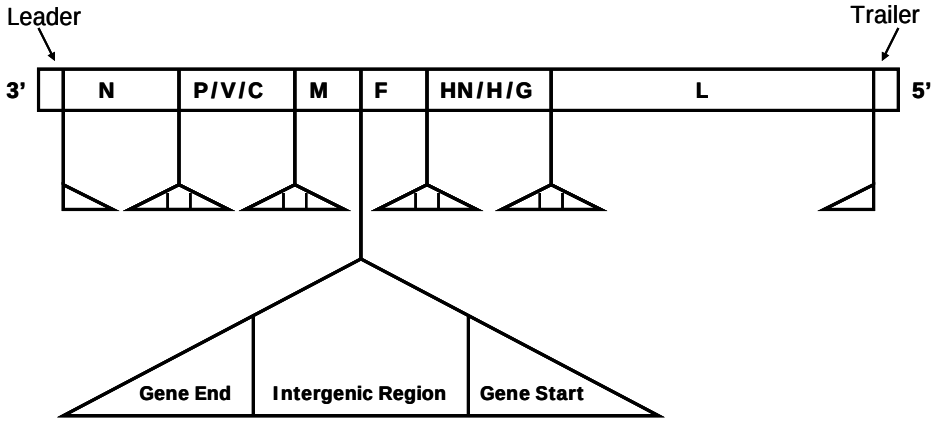


Figure 1.3- Paramyxovirus Genome Organisation

Paramyxoviruses contain non-segmented, negative-sense, single-stranded RNA genomes. The 3' terminal region contains an untranslated leader sequence and the 5' end contains an untranslated trailer region. Genes common to all paramyxoviruses are the nucleoprotein (N) gene, phosphoprotein (P) gene, matrix (M) gene, fusion (F) gene, attachment (HN/H/G) gene and the large (L) gene. Gene junctions consist of the gene end signal of the upstream gene, an intergenic region and the gene end signal of a downstream gene. This figure has been adapted from Lamb & Kolakofsky (2001).

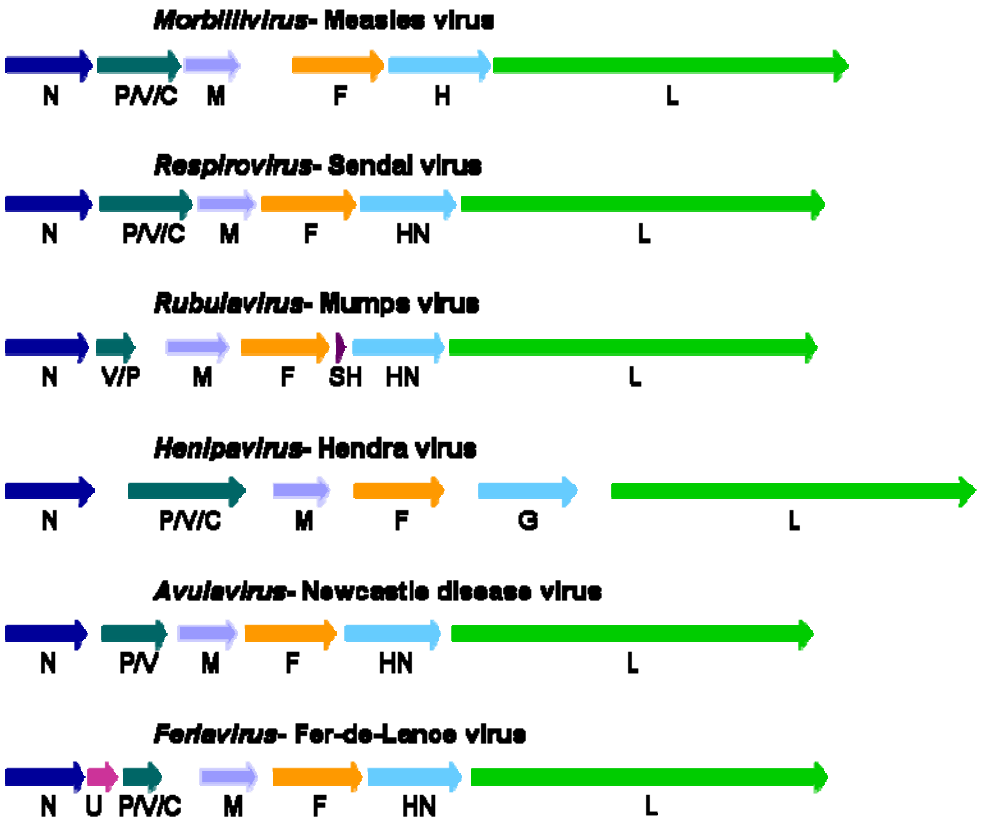


Figure 1.4- *Paramyxovirinae* Genome Organisation

The genome organisation of paramyxoviruses within the *Paramyxovirinae* is represented via illustration of the type species for each genus. The genus *Ferlavirus* has been suggested (Kurath *et al.* 2004), but has not been approved by The International Committee on Taxonomy of Viruses. The schematic representation of the *Fer-de-Lance virus* genome displays the location of the novel U (unknown) gene not common to other paramyxoviruses. Representative genomes are drawn to scale.

The requirement for the rule of six may have evolved to maintain genome stability, protecting against insertion of nucleotides into RNA editing sites during replication (Hausmann *et al.* 1996). Paramyxoviruses editing P mRNA either obey the rule of six or confront selective disadvantage (Conzelmann 1998).

1.4.3 Coding Capacity

A distinct feature of RNA replication is high mutation rate leading to deleterious mutations. A compact genome organisation and high coding capacity of genes offers a selective advantage for an RNA virus as the genome is replicated rapidly and frequently (Domingo & Holland 1997). The average coding capacity of genomes within the *Paramyxoviridae* prior to the isolation of the henipaviruses was 92%. Exceptionally long untranslated regions in the genomes of henipaviruses significantly reduced the coding capacity within the subfamily to 82%. As a high genome coding capacity appears to be beneficial, the possibility exists that the henipaviruses represent an ancient lineage of paramyxoviruses (Wang *et al.* 2003). The coding capacity of paramyxoviruses is extended by the ability of the P gene to encode several proteins (see Section 1.5.2 and 1.6.4).

1.5 Paramyxovirus Genes and Protein Products

Members of the *Paramyxovirinae* subfamily contain a conserved genome with the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F) attachment (HN/H/G) and large (L) genes present in all viruses (see figure 1.3).

1.5.1 Nucleocapsid (N) protein gene

The N protein functions in the encapsidation of viral RNA, and associates with the P and L proteins during replication and transcription. The N protein also interacts with the M protein during assembly. The P protein functions as a chaperone to prevent uncontrolled encapsidation of non-viral RNA, allowing the N protein to bind selectively to the genome and antigenome (Errington & Emmerson 1997). Encapsidated RNA is resistant to degradation by RNase. The N protein is not a classical RNA binding protein, but levels of the N protein in the cytoplasm function in switching between transcription and replication of the viral genome. Binding of antibodies to the N protein releases P proteins (Morgan *et al.* 1984), proving the interaction between the two proteins. Regions of the N protein capable of P protein interaction are variable between paramyxoviruses. The N protein region of NDV that binds the P protein differs from the binding regions in SeV and MeV (Kho *et al.*

2004). Strong hydrophobic forces maintain N-RNA and N-N interactions and the domain F-X4-Y-X4-S-Y-A-M-G (where X represents any amino acid residue) is found in a strong hydrophobic region near the middle of the N protein (Lamb & Kolakofsky 2001).

The monomeric N protein, N^O, consists of two regions. The N-terminal domain, N_{CORE}, constitutes 80% of the N protein and is relatively conserved. In contrast, the C-terminal domain, N_{TAIL}, which constitutes the remaining 20% of the N protein, is hypervariable. Although N_{TAIL} is poorly conserved, this region is always highly charged and mostly negative (Lamb & Kolakofsky 2001). The RNA binding domains are located in the conserved N_{CORE} domain (Mountcastle *et al.* 1974), and this N-terminal domain is also required for correct folding of the N protein for the formation of a 'herringbone-like' structure (Kho *et al.* 2003). The N_{TAIL} domain is dispensable for assembly, may be utilised by the N protein in the transition between helical states and contains phosphorylation and antigenic sites (Morgan *et al.* 1984). Conservation of long, unstructured regions of N proteins within the *Paramyxovirinae* may be of functional significance (Karlin *et al.* 2003).

1.5.2 Phosphoprotein (P) gene

The P gene of the *Paramyxovirinae* represents an example of viruses compacting as much genetic information into genes as possible. The P gene encodes a vast array of gene products ranging from truncated, modified and full length proteins, through a variety of mechanisms. Translation via alternate ORF and RNA editing (to be discussed in Section 1.5.4) enable the production of P, V, C, C', D, I, W, X, Y1, Y2 and Z protein products, depending on the genus. The P and V proteins are produced as a result of either faithful transcription of RNA or RNA editing. All viruses in the subfamily *Paramyxovirinae*, except HPIV-1, contain an RNA editing site (Lamb & Kolakofsky 2001). HPIV-3 produces a D protein in lieu of a V protein (Matsuoka *et al.* 1991). C proteins are produced from alternate ORFs in most genera.

There are two major types of P gene coding strategy observed in *Paramyxovirinae* members- Type A and Type B (Wang *et al.* 2003). The Type A coding strategy, used by all paramyxoviruses except rubulaviruses and avulaviruses, involves an unedited version of the P gene encoding a P protein. The V protein is translated when a single non-templated G reside is inserted into the RNA editing site, which has a consensus sequence of 5'-AAAAAGGG-3'.The C protein is produced by internal translation initiation (Wang *et al.* 2003). The Type B coding strategy is used by the rubulaviruses, which do not encode a C protein. An

unedited version of the P gene encodes the V protein, and the addition of two non-templated G residues in the RNA editing site, which has a consensus sequence of 5'-TTTAAGAGGGG-3', results in the production of a P protein (Wang *et al.* 2003). Type A and B coding strategies of paramyxoviruses are represented in Figure 1.5.

P protein

The P proteins of viruses within the genus *Rubulavirus* are the smallest within the subfamily *Paramyxovirinae*, and viruses within the genus *Henipavirus* contain the largest P proteins (Wang *et al.* 1998). The P protein consists of two distinct domains, based on sequence conservation. The P N-terminal (PNT) domain contains sites for phosphorylation, is poorly conserved and is involved in chaperoning of newly synthesised N proteins during replication. The P C-terminal (PCT) domain is highly conserved and has a critical role in positioning of the L protein on the transcription template (Blanchard *et al.* 2004).

The P protein is a component of the polymerase complex and is natively unstructured in MeV and morbilliviruses, but folded in rubulaviruses (Karlin *et al.* 2002). Unstructured P proteins undergo 'induced folding' upon binding to N and L proteins. N protein binding to the P protein of MeV creates a 4-helix bundle, but MuV P protein persistently lacks tertiary structure in the N binding domain, indicating the rubulaviruses may have evolved a slightly different binding mechanism from other paramyxoviruses (Kingston *et al.* 2004). The PCT domain of the P protein exists as a tetramer (Tarbouriech *et al.* 2000a). Coiled coils can form dimers, trimers and tetramers depending on packing of hydrophobic side chains at helix interfaces and the coiled coil region of the P protein conserved in the subfamily *Paramyxovirinae* is sufficient for oligomerisation (Curran *et al.* 1995). Consistent with this observation, SeV was found to contain an oligomerisation domain with a homotetrameric coiled coil structure (Tarbouriech *et al.* 2000b). Phosphorylation of the P protein is directed by cellular kinases and SeV and HPIV-1 are highly phosphorylated. HPIV-1 also requires two separate regions on the P protein to enable efficient interaction with the L protein (Bousse *et al.* 2001), as precise protein interactions are essential for polymerase activity.

V protein

The V- and P-coding regions upstream of the RNA editing site are identical, and the C-terminal domains of the V protein, located downstream of the editing site, are highly conserved amongst members of the *Paramyxovirinae* (Lamb & Kolakofsky 2001).

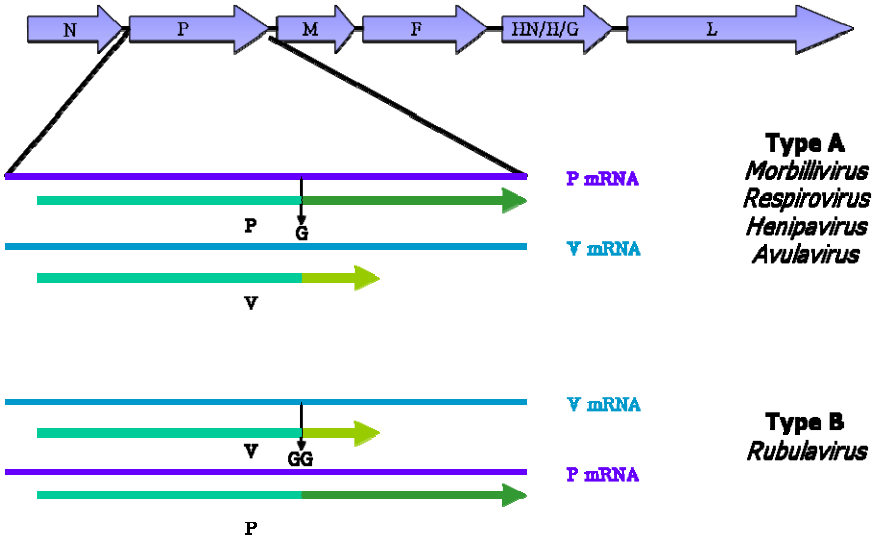


Figure 1.5- RNA Editing Mechanism Used by Paramyxoviruses

Pseudotemplated addition of nucleotides co-transcriptionally into mRNA occurs in all members of the *Paramyxovirinae*, except HPIV-1 and is confined exclusively confined to the P gene. Insertions into mRNA occur in the middle of the P gene, resulting in a frameshift that allows ribosomal access to a second ORF. The morbilliviruses, respiroviruses, henipaviruses and avulaviruses all encode a P protein when the P gene is faithfully transcribed. The V protein is encoded when a single, non-templated G residue is inserted into the mRNA transcript. The rubulaviruses encode a V protein from the faithfully transcribed P gene and a P protein is encoded when two non-templated G resides are inserted into the mRNA transcript. This illustration was adapted from Wang *et al.* (2003).

The C-terminal domain contains a region where 15 out of 48 amino acids, seven of which are cysteine residues, are perfectly conserved between paramyxovirus V proteins (Figure 1.6). This highly conserved C-terminal region of the V protein resembles a zinc finger domain and is capable of binding two zinc atoms. The zinc-binding capability of SeV is involved in viral pathogenesis. This was demonstrated experimentally when SeV lacking the V protein was less virulent in mice, with virus clearance from the lungs of the mice occurring efficiently (Huang *et al.* 2000).

The V protein is capable of functioning in interferon (IFN) signalling inhibition (Nishio *et al.* 2005), cell cycle alterations, inhibition of double-stranded DNA signalling and prevention of apoptosis (Nanda & Baron 2006). Host organisms inhibit and eliminate viral infection by sacrificing virus-infected cells by apoptosis. SV-5 is capable of preventing viral induced apoptosis (Wansley & Parks 2002), but deletions in the conserved C-terminal region of the SV-5 V protein increased CPE and apoptosis in infected HeLa cells, indicating that the full-length V protein is required in the prevention of programmed cell death (Sun *et al.* 2004). The tumour suppressor homologue p73 is thought to be an inducer of apoptosis through a mitochondrial pathway. The MeV V protein inhibits the transcriptional activity of p73, hence, preventing apoptosis (Cruz *et al.* 2006). Controlled killing of cells has been demonstrated with recombinant paramyxoviruses, such as SV-5 with a mutant V protein (Parks *et al.* 2002), eluding to the possibility of therapeutic vaccine vectors to target the destruction of cancerous cells.

Interferons are a family of soluble cytokines mediating cellular resistance to viruses. IFN signalling interference is a common method utilised by paramyxoviruses to circumvent host anti-viral responses, but the mechanism whereby evasion of host cell defences is achieved is virus-specific (Young *et al.* 2000). The signal transducer and activator of transcription (STAT) family of proteins are critical components of the IFN signalling pathway, involved in constitutive expression of certain genes, such as caspases, and are involved in processing of T cell antigens.

The paramyxovirus V protein is able to function as an IFN antagonist (Kubota *et al.* 2005). The V protein of SV-5 targets STAT1 for proteasome-mediated degradation in human cells (Didcock *et al.* 1999), but not mouse cells. The V protein of SV-41 and MuV decrease levels of STAT1 and the V protein of HPIV-2 decreases levels of STAT2 (Gotoh *et al.* 2002).

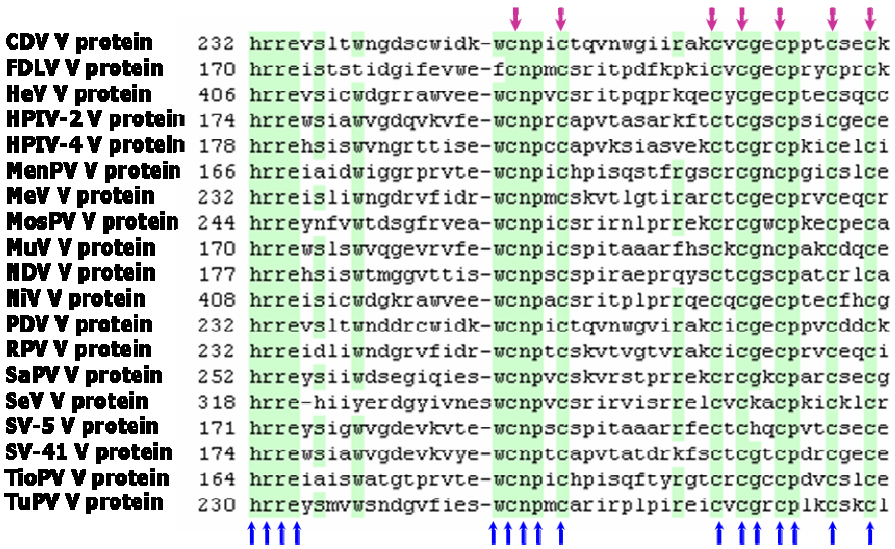


Figure 1.6- Conserved C-terminal Region of the V Protein

The highly conserved C-terminal region of the paramyxovirus V protein resembles a zinc finger domain and is capable of binding two zinc atoms. Within this region, fifteen out of forty-eight amino acids, seven of which are cysteine residues, are perfectly conserved. Conserved amino acids are indicated by blue arrows and cysteine residues are indicated by green arrows. GenBank database accession numbers of paramyxoviruses used in this alignment are as follows- CDV (AAQ96302), FDLV (NP899657), HeV (NP047108), HPIV2 (P23057), HPIV4 (P21740), MenPV (AAK62279), MeV (Q9EMA9), MosPV (NP958050), MuV (NP054709), NDV (Q06428), NiV (NP112023), PDV (JQ1565), RPV (Q03340), SaPV (AAF63742), SeV (BAC79135), SV-5 (P11207), SV-41 (P36315), TioPV (NP665866) and TuPV (Q9QM81).

In addition to proteasome degradation, inactivation of STAT is achieved by ubiquitin ligase destruction by SV-5, HPIV-2 and MuV V proteins, prevention of nuclear import of IFN by STAT with MeV V protein and sequestration of STAT by HeV and NiV V proteins (Horvath 2004).

A co-dependent relationship exists between STAT1 and STAT2. SV-5 and MuV require STAT2 to target STAT1 and HPIV-1 requires STAT1 to target STAT2. The V protein of henipaviruses is able to form a tripartite complex with STAT1 and STAT2, inhibiting IFN signalling through sequestration of STAT (Rodriguez *et al.* 2004). Overactivity of STAT3 is associated with diseases such as cancer, arthritis, lupus, autoimmunity, cardiac hypertrophy and obesity. MuV V protein targets the destruction of STAT3 via ubiquitin ligase resulting in the inhibition of a diverse range of cellular responses. MuV is able to invade the immune system and replicate in activated T lymphocytes, perhaps indicating that the MuV V protein may be utilised as a treatment in disease where overactive STAT3 is implicated (Horvath 2004).

C protein

Several paramyxoviruses are capable of expressing a group of non-structural proteins, the C proteins, from an alternate ORF within the P gene, upstream of the RNA editing site (see Section 1.5.4) and are not affected by addition of non-templated G residues into mRNA transcripts (Bellini *et al.* 1998). Rubulaviruses do not encode a C protein, morbilliviruses and henipaviruses encode one C protein and respiroviruses are capable of encoding a group of several C-like proteins. SeV, a respirovirus, encodes four C-like proteins, designated C, C', Y1 and Y2. Transcription of the Y proteins of SeV is initiated by a ribosomal shunt mechanism (Latorre *et al.* 1998b) that enables the ribosome to utilise codons other than AUG to begin transcription. Homology of C proteins within a genus is high, but insignificant between genera (Lamb & Kolakofsky 2001). C proteins are abundant in virus infected cells, distributed evenly in the cytoplasm of SeV infected cells (Cadd *et al.* 1996), and localised with nucleocapsids in MeV infected cells (Reutter *et al.* 2001).

The C protein has been implicated as playing several roles during paramyxovirus infection. Research on MeV suggests that the MeV C protein is an infectivity factor, supporting the production of infectious virus particles (Devaux & Cattaneo 2004) and sustaining long term infection by inhibiting apoptosis (Takeuchi *et al.* 2005). The MeV C protein prevents early cell death and has been demonstrated to be necessary for efficient replication of MeV *in vitro*, and in

macaques (Takeuchi *et al.* 2005). Alternatively, studies have demonstrated that truncating the MeV C protein results in optimal virus growth (Miyajima *et al.* 2004), and abrogating the expression of the expression of the C protein increases RNA synthesis (Reutter *et al.* 2001). One of the functions of the C protein is to limit genome amplification and mRNA synthesis, and deletion of the C protein restricted the replication of MeV in human peripheral blood mononuclear cells (Escoffier *et al.* 1999). Binding of the SeV C protein to the L protein has also been suggested as a possible means whereby RNA synthesis is decreased (Kato *et al.* 2004). Synthesis of mRNA is inversely proportional to the level of C protein expression (Curran *et al.* 1992) and identification of a coiled coil motif presents structural evidence that oligomerisation of C proteins may be involved in this inhibition of transcription (Malur *et al.* 2004). The inhibitory effect of replication may be attributed to the C protein increasing the selectivity of the polymerase replication complex for promoter *cis*-acting elements, hence governing the activity of the polymerase complex (Tapparel *et al.* 1997). The C proteins play a critical role in regulation of virus assembly, where they facilitate incorporation of envelope and M proteins into virions, perhaps by acting as chaperones to convert newly synthesised M proteins into assembly-initiating forms (Hasan *et al.* 2000). Inhibition of RNA synthesis in SeV occurs through the C protein affecting promotor strength and is dependent on co-expression of the N and L proteins (Cadd *et al.* 1996). The C and C' proteins of SeV are versatile and not functionally equivalent, and are capable of functioning in a positive manner early during infection, but later in infection they exert a negative influence to inhibit RNA accumulation (Latorre *et al.* 1998a).

The C proteins are relatively absent in primary infection and more abundant as infection progresses in order to restrain genome synthesis, hence preventing CPE and death of host cells. The smallest C protein, Y1, elicits an anti-apoptotic effect in SeV (Koyama *et al.* 2003). The SeV C protein is an IFN antagonist (Kato *et al.* 2004) and antiviral activity through the action of IFN stimulated tyrosine phosphorylation of STAT is circumvented as C protein associates with STAT1 (Garcin *et al.* 2004; Gotoh *et al.* 2004). The C protein of HPIV3 inhibits the activation of STAT1 by targeting phosphorylation (Malur *et al.* 2005). STAT1 targeting by C proteins can occur after nuclear translocation. The C protein is also capable of inhibiting the activity of IFN-α, IFN-β and IFN-γ, independent of STAT association (Gotoh *et al.* 2004). The counteraction of IFN by C proteins may play a role in viral pathogenesis during acute phase of infection and also the establishment of persistent infection.

1.5.3 Matrix (M) protein gene

The M protein is the most abundant protein of the paramyxovirus virion and has the capability to maintain the virion structure even after dissolution of the overlying viral envelope. The M protein is basic, not glycosylated, and is somewhat hydrophobic, although hydrophobic domains of sufficient length required for transversing of the lipid membrane are not present (Lamb & Kolakofsky 2001). As a central organiser of morphogenesis, the M protein interacts with integral membrane glycoproteins, the lipid bilayer and nucleocapsids. Modification by acetylation and phosphorylation produces M proteins capable of differing interactions with glycoproteins and nucleocapsids. Dephosphorylation is associated with M protein maturity and is correlated with viral budding from the host cell. As M proteins are produced at half the level represented in the virion, the proteins may be rate-limiting (Morrison & Portner 1991). Communication between viral components on the host cell surface and nucleocapsids is mediated by M protein interaction with actin filaments (Giuffre *et al.* 1982).

Shutdown of M production in preparation for export is a key component of viral assembly and budding from a cell, but the M protein alone is not sufficient for initiation of this process. Interaction of M proteins with the cytoplasmic tails of the fusion and attachment proteins is required during the budding process (Ghildyal *et al.* 2005), as is nucleocapsid interaction. The cytoplasmic tails of the glycoproteins have critical, yet redundant roles of association with the M protein as HN and F appear to be interchangeable (Schmitt *et al.* 2002). Viral export in SV-5 is facilitated by F and HN, but in SeV, where F also enhances budding, HN plays a suppressive role (Sugahara *et al.* 2004).

The absence of M protein results in uncontrolled membrane fusion activity, and M inactivation or mutation has been implicated in persistent infection where viral budding fails to occur (Lamb & Kolakofsky 2001). The M protein is not absolutely required for virus spread and is often defective in cases where MeV infection has progressed to the fatal disease SSPE (Cattaneo *et al*. 1988). During MeV infection, wild type virus is cleared by the host immune system but assembly-deficient virions are tolerated, resulting in persistent infection. Highly fusogenic virions propagate deep within the brain parenchyma, initiating SSPE, which ultimately leads to death (Cathomen *et al*. 1998).

1.5.4 Fusion (F) protein gene

Amalgamation of paramyxoviruses with host cells is directly mediated by the F protein and occurs at the host plasma membrane. Viral fusion involves

docking of the membrane to the target membrane, F protein activation, merger of membranes, pore formation, pore expansion and is mediated by the F protein along with attachment proteins (Morrison 2003). The transmembrane domain of the F protein of *Respiratory syncytial virus* (RSV) has been identified as the region important for transport of the protein to the plasma membrane, control of directional trafficking and virus budding (Brock *et al.* 2005). Interaction of the F protein with attachment proteins is required to draw cells into the appropriate juxtaposition for fusion to occur (Horvath *et al.* 1992). The requirement for co-expression of attachment proteins with the F protein for fusion is not stringent in SV-5 (Bagai & Lamb 1995), but is absolutely necessary in virtually all other paramyxoviruses. The F protein of CDV is an important determinant of persistent infection (Plattet *et al.* 2005). The interaction between the F and HN proteins takes place at the cell surface and is triggered by HN recognition of receptors, the F and HN proteins are not transported to the membrane as a complex (Li *et al.* 2004). Cell surface receptor recognition by the G protein of HeV plays a pivotal role in the fusion process, as destruction of host cell receptors with proteases abolishes fusion capability of the henipavirus (Bossart *et al.* 2001).

F proteins are type 1 glycoproteins possessing a hydrophobic transmembrane domain at the C-terminus that anchors the protein to the lipid envelope. Glycosylated side chains are important in F protein folding, as is a structural cylindrical cavity, the F pocket, that contains a hydrophobic region, creating a flexible domain (Plemper *et al.* 2003). The F pocket represents a conserved region of the paramyxovirus F protein. The F protein is synthesised as an inactive precursor, F_0, and proteolytic cleavage of this protein results in two polypeptides, F_1 and F_2, linked by a disulfide bond (Lamb & Kolakofsky 2001). The F_1 protein of CDV and MeV may also be cleaved again, generating F_{1a} and F_{1b} fragments (von Messling *et al.* 2004b). Furin cleavage occurs via recognition of the protease of a conserved multibasic site defined as R-X-K/R-R (Hosaka *et al.* 1991). Inactive F_0 precursor proteins delivered to the plasma membrane require cleavage of a single basic residue and this cleavage is performed by extracellular host proteases, often located in the respiratory tract. The crystal structure of SV-5 revealed that the structural differences between the inactive F_0 precursor and post-fusion conformation of the F protein are profound (Yin *et al.* 2005; Yin *et al.* 2006). The method of F protein cleavage utilised by paramyxoviruses is correlated with tissue tropism. Cleavage of F proteins with extracellular host proteases in NDV strains containing multibasic residues confines viral infection to the respiratory tract, whereas virulent strains of NDV are capable of producing systemic infection due to

the presence of intracellular cleavage by furin (Glickman *et al.* 1988). The addition of N-linked sugars to F proteins occurs only in the F_2 subunit of morbilliviruses, but other genera contain 3-6 sites of N-linked glycosylation, distributed between both F_1 and F_2 subunits. N-linked oligosaccharides are required for biological stability of MeV F proteins (Alkhatib *et al.* 1994).

The F protein contains two heptad repeats, consistent with an α-helical coiled coil conformation (Chambers *et al.* 1990). HR1 and HR2 interact with one another to form a 6-helix bundle (Baker *et al.* 1999), and the formation of this bundle pulls viral and cellular membranes towards each other to facilitate fusion. Heptad repeats are conserved, and HR2 of NiV and HeV are interchangeable (Xu *et al.* 2004). Glycine residues are important in maintenance of fusion level (Horvath & Lamb 1992), but also act as α-helix destabilising resides. Substitution of glycine residues for alanine resides leads to an increase in the α-helix nature of a peptide, resulting in increased fusogenic properties (Bagai & Lamb 1997). The balance between fusion and replication must be maintained as high fusion activity is deleterious to cell viability.

1.5.5 Small hydrophobic (SH) protein gene

The presence of an additional gene flanked by the F and HN genes was discovered in the genome of the avulavirus APMV-6 and the rubulaviruses SV-5 and MuV. The SH protein, first identified in SV-5, contains an extensive hydrophobic region, allowing the protein to span the lipid membrane of the virus once (Hiebert *et al.* 1985). An analogous SH gene was later discovered in the genome of MuV (Elango *et al.* 1989) and although the SH genes of both MuV and SV-5 are located in the same position between the F and HN genes, no sequence homology exist between the two viral genes.

The SH protein of MuV is an integral membrane protein, anchored through the lipid bilayer of the virus and into the cytoplasm via a region located in the C-terminus. The MuV SH protein may have a role in viral pathogenesis, mediated through inhibition of TNF-α-induced NF-κB activation (Wilson *et al.* 2006). Only a small amount of SH protein is present in MuV infected calls, and in cells infected with the Edmondson strain of MuV there is no SH protein detected at all (Takeuchi *et al.* 1996). The Edmondson strain of MuV does not contain the SH protein, indicating that the SH gene is not essential for viral replication. Amino acid disparity of the putative SH protein in MuV is high, with up to 23% diversity observed during the comparison of six strains of the virus (Takeuchi *et al.* 1991). The SH protein of

MuV may function in modifying the permeability of the host cell plasma membrane, hence performing the function of a viroporin.

In contrast to MuV, the SH protein of SV-5 is anchored to the viral membrane via an N-terminus hydrophilic region, and the C-terminal portion of the protein extends extracellularly. To examine the function of the SH protein, a recombinant SV-5 with the SH gene deleted (rSV5△SH) was constructed (He *et al.* 1998). The recombinant virus did not exhibit altered morphology and did not differ phenotypically from SV-5. Infection of cells with either wild-type SV-5 or rSV5△SH revealed no difference in growth rate, infectivity or plaque size produced in cells, leading to the conclusion that the SH protein is not required for viral growth in tissue culture cells (He *et al.* 2001). Although not required for viral replication in cells, the SH protein is implicated in the prevention of apoptosis. SV-5 is capable of replicating in MDBK cells for up to 40 days, producing little CPE. Infection of MDBK cells with rSV5△SH increased the level of CPE, leading to apoptosis of the cells and the hypothesis that the SH protein prevents programmed cell death, possibly through the inactivation of apoptosis inducing caspases 2 and 3, was proposed (He *et al.* 2001). Further studies revealed rSV5△SH induced cell death by activating a pathway involving TNF-α, leading to apoptosis (Lin *et al.* 2003). Prevention of apoptosis by the SH gene results in survival of the infected host cell, and ultimately survival of the virus.

The avulavirus APMV-6 contains a SH gene and the product of this gene is an integral membrane protein containing two potential N-linked glycosylation sites (Chang *et al.* 2001). The C-terminal portion of the APMV-6 SH protein extends to the outside of the cell membrane, similar to that of SV-5, but the protein does not have any homology to other SH proteins. The SH protein of HPMV-6 contains a second hydrophobic region 20 amino acids downstream of the hydrophobic region located at the protein terminus (Chang *et al.* 2001).

1.5.6 Attachment (HN/H/G) protein gene

The attachment proteins of the *Paramyxovirinae* are multifunctional type II transmembrane glycoproteins that facilitate the binding of virions to host cells and promote membrane fusion. Attachment proteins consist of, from N-terminus to C-terminus, a cytoplasmic domain, a hydrophobic membrane anchoring domain, a stalk region, and a globular head that extends to the extracellular region. Attachment proteins are classified into three groups based on the presence or absence of haemagglutinin and neuraminidase activity (Bellini *et al.* 1998). The attachment proteins of respiroviruses, rubulaviruses and avulaviruses are designated HN, as

they exhibit both haemagglutination and neuraminidase activity. The morbillivirus and henipavirus attachment proteins are designated H and G, respectively.

Co-translationally, the attachment proteins are inserted into the membrane of the rough endoplasmic reticulum, transported through the Golgi membranes via cellular secretory and membrane proteins, and translocated to the cellular membrane (Morrison & Portner 1991). During translocation, oligosaccharides may be added to, or modified, on the attachment protein. H proteins are only glycosylated with N-linked oligosaccharides, whereas G proteins contain 3-6 available sites for N-linked, or O-linked addition of oligosaccharides. Attachment proteins appear to have evolved in two distinct lineages from one common progenitor protein, as the HN protein of NDV displays characteristics of the primitive progenitor (Morrison & Portner 1991).

The HN protein mediates attachment of viruses to host cells by binding to cellular sialic acid-containing proteins or glycolipids. Enzymatic cleavage of sialic acid is mediated by neuraminidase and this catalytic reaction is thought to prevent virus particles from undergoing self-aggregation during budding from the host cell (Lamb & Kolakofsky 2001). The HN protein contains a single hydrophobic domain close to the N-terminus, and amino acid residues encoded by the conserved hexapeptide sequence NRKSCS are thought to interact, forming the sialic acid binding site (Jorgensen *et al.* 1987). Receptor binding to one site on the NDV HN protein modulates activity of a second binding site to promote continued receptor binding (Porotto *et al.* 2006). The functional HN protein consists of disulfide-linked homodimers that interact to form a non-covalently associated tetramer.

The NDV HN protein can be synthesised as a biologically inactive precursor, HN_o, where removal of 90 amino acid residues from the C-terminus is required to activate the protein. The HN protein is a major antigenic determinant and is responsible for influencing the virulence and tropism of NDV. Reduced receptor recognition and neuraminidase activity inherent to avirulent HN proteins translate into differences in viral virulence and tropism of NDV, and this is independent of F cleavability (Panda *et al.* 2004). N-linked glycoproteins are important in folding, transport and biological activities of HN proteins and loss of glycans from NDV attachment proteins leads to a reduction in virulence. Loss of glycans from HN proteins may expose T cell epitopes that allow virus infected cells to be targeted by cytotoxic T lymphocytes, hence facilitating viral recognition and clearance by the host.

Sialic acid is not the primary receptor for H proteins, as is the case for HN proteins, although predictions of glycosidic activity for the H protein were proven in

RPV and PPRV (Langedijk *et al.* 1997). MeV, CDV and RPV all share a common cellular receptor, SLAM (CD150) (Ono *et al.* 2001a; Ono *et al.* 2001b; Baron 2005), which is thought to play a role in the generalised immunosuppression of the host upon infection. The structure of the H protein was predicted to consist of a globular head domain with a six-bladed β-propeller fold set connected to an anchored membrane stalk (Langedijk *et al.* 1997). The HeV G protein contains the amino acid sequence TIHHCS as a replacement for the conserved NRKSCS sequence of the putative sialic acid binding site (Yu *et al.* 1998b). The lack of sequence homology in this functionally important region is consistent with the fact that no neuraminidase activity has been detected for HeV.

1.5.7 Large (L) protein gene

The L gene accounts for 40-50% of the coding capacity of a paramyxovirus genome, yet produces the least amount of protein (about 50 copies of L protein per virion) as the location of the gene is the most promotor distant on the transcriptional map (Bellini *et al.* 1998). The L protein is a multifunctional polypeptide, catalysing many steps in RNA synthesis and processing, and is also involved in methylation, capping and polyadenylation. Phosphorylation of SeV P proteins has implicated the L protein as a kinase associated with the viral core (Einberger *et al.* 1990), but as phosphorylation occurs in the absence of the L protein, evidence of the involvement of the L protein is not conclusive. The N-terminal region of the L protein is involved in binding of the P protein and contains an active site for RNA polymerisation, whereas the C-terminal region of the protein is not required for binding to RNA, but is important in interaction with the N protein (Parks 1994).

The L gene is highly conserved among other non-segmented, negative-sense, single-stranded RNA viruses (NNSV). Sequence comparisons of SeV, NDV and MeV L genes revealed six conserved domains (I-VI) separated by variable regions, suggesting the structure of a concatenated functional domain (Poch *et al.* 1990). The MeV L protein contains distinct sequence elements that are postulated to be from an ancestral polymerase (Blumberg *et al.* 1988), and as MeV and SeV share high sequence homology, there is speculation that the two viruses evolved from a putative common ancestor. Domain II of the L protein is essential for RNA recognition. Domain III of the L protein contains a pentapeptide motif, QGDNQ, thought to contain an active site for phosphodiester bond formation. Morbilliviruses contain three conserved regions, A, B and C separated by two hinge regions of low identity. The QGDNQ domain is present in the B region and is flanked by hydrophobic resides (Poch *et al.* 1990). Mutational analysis of the conserved

QGDNQ motif of RPV revealed that conservation of D and N residues is absolutely required to retain polymerase function (Chattopadhyay *et al.* 2004). The conserved QGDNQ motif of paramyxovirus was found to be altered in HeV, NiV, TuPV (Wang *et al.* 2001) and *Mossman virus* (MosPV) (Miller *et al.* 2003). These four viruses contain the sequence QGDNE. This amino acid change introduces a second negative charge into a functionally important area. The possibility exists that the henipavirus sequence motif represents an ancient form of catalytic domain. As the Q amino acid is highly conserved in all NNSV, but different in HeV, NiV, MosPV and TuPV, it may be concluded that this residue is not directly involved in catalytic function (Chattopadhyay *et al.* 2004).

1.6 Gene Expression of Non-Segmented, Negative-Sense, Single-Stranded RNA Viruses

1.6.1 Transcriptional stop/start mechanism

All paramyxovirus RNA synthesis begins with a single entry of the viral RNA-dependent RNA polymerase (vRdRp) at the 3' end of the genome, with transcription of the 3' leader beginning before mRNA synthesis. Initiation of RNA synthesis is dependent on the distance between the genome 3' end and gene start signal (Cordey & Roux 2006). Gene junctions of paramyxovirus genomes consist of a gene termination signal in the upstream gene, an IGR and a gene initiation signal in the downstream gene (Lamb & Kolakofsky 2001). The strength of the replication promotor is important in determining initiation of mRNA synthesis (Vulliemoz *et al.* 2005). A scanning model predicts that ribosomes initiate translation at the first AUG codon that is in favourable context according to the "Kozak" sequence 5'-GCC^{A}or$_{G}$CCAUGG-3' (Kozak 1986). Monocistronic mRNA produced by the vRdRp contains a 5' cap (Abraham *et al.* 1975) and is polyadenylated by a stuttering mechanism that reiteratively copies a short run of 4-7 uridines (U tract) leading to the termination and release of the mRNA. After termination of release of mRNA, the vRdRp remains attached to the template before reinitiating transcription at the downstream gene (Lamb & Kolakofsky 2001). This stop/start mechanism of transcription is not perfect and failure of vRdRp to reinitiate transcription at a downstream site results in a gradient of mRNA production inversely proportional to the distance from the 3' end of the genome (Figure 1.7) (Lamb & Kolakofsky 2001), and this transcription gradient is maintained throughout infection (Plumet *et al.* 2005). The vRdRp may also read through IGRs, generating polycistronic mRNA.

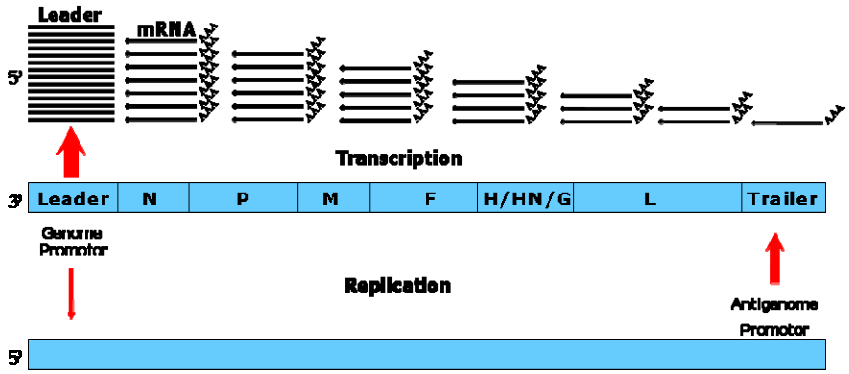


Figure 1.7- Paramyxovirus Transcription and Replication

The stop/start model for transcription is not perfect and failure of viral RNA-dependent RNA polymerase to reinitiate transcription at a downstream site results in a gradient of mRNA production inversely proportional to the distance from the 3' end of the genome. Transcription and replication occurs via promotor sequences for polymerase entry, existing on the 3' end of both genome and antigenome. The relative promotor strength is represented by the arrow size and relative amounts of polyadenylated mRNA transcripts are shown. This figure has been adapted from Conzelmann (1998).

The length of IGR sequence plays a role in termination of transcription and polyadenylation but is not a determining factor alone (He & Lamb 1999). Mutagenesis studies on the L gene on *Human respiratory syncytial virus* (HRSV) indicate that *cis*-acting gene end signals and the *trans*-acting polymerase function together, determining the efficiency of transcript termination (Cartee *et al.* 2003). The U tract might serve as a genomic spacer to ensure minimum distance between gene stop and start signals are maintained at junctions with short IGRs (Rassa *et al.* 2000). Regulation of viral gene expression is possible through the distance of the gene from 3' end of genome, as transcription of downstream genes cannot be more abundant that that of upstream genes.

1.6.2 Intergenic regions

Morbilliviruses, respiroviruses and henipaviruses contain IGRs exactly three nucleotides in length with a highly conserved sequence (usually 3'-GAA-5'). Avulaviruses and rubulaviruses, in contrast, contain IGRs variable in length and sequence. Sequencing of MenPV revealed an IGR of 85 nucleotides separating the F and HN gene (Bowden *et al.* 2001), expanding the extent of variability in length of IGRs, previously believed to range from 1-47 nucleotides (Lamb & Kolakofsky 2001).

TioPV, in addition to MenPV, contains IGRs significantly exceeding the previous upper limit of 47 nucleotides for the genus *Rubulavirus*. All IGRs of TioPV begin with a C residue, an identical residue as observed in the beginning of the trinucleotide IGRs of non-rubulaviruses. TioPV and MenPV are the only rubulaviruses lacking haemagglutination and neuraminidase activities, which, when taken into consideration with IGR sequence information, may indicate TioPV represents an evolutionary intermediate between the rubulaviruses and non-rubulaviruses (Wang *et al.* 2003). The sequence variability of IGRs may provide an additional level of transcriptional control beyond that resulting from the distance of genes from the 3' end promotor (Rassa & Parks 1999).

1.6.3 Genome termini

The coding region of the paramyxovirus genome is flanked by a 3' leader region and a 5' trailer region. The 3' leader region of the genome contains *cis*-acting signals for encapsidation, replication and transcription, and is bipartite in nature, with the terminal twelve nucleotides almost identical in the genome and antigenome (Lamb & Kolakofsky 2001). These two hexameric elements function as replication

promotors, and spacing between promotors in genome termini is crucial due to hexamer phasing patterns (Egelman *et al.* 1989). Synthesis of leader RNA differs from that of downstream mRNA transcripts in that the leader is not capped and does not contain a poly (A) tail. The leader and N gene junction plays a vital role in the switch between transcription and replication (Conzelmann 1998), and expression of the 3' leader has been reported to control the induction of apoptosis in SeV (Garcin *et al.* 1998). The trailer region of the SeV genome has been implicated in the inhibition of apoptosis, resulting in optimal virus replication (Wiegand *et al.* 2005).

1.6.4 RNA editing

RNA editing, the pseudotemplated addition of nucleotides co-transcriptionally into mRNA, occurs in all members of the *Paramyxovirinae* subfamily, except HPIV-1 and is confined exclusively to the P gene. The expression of two proteins from the P gene of SV-5, that differed by only two G resides, verified for the first time the insertion of two non-templated G resides into the transcribed mRNA (Thomas *et al.* 1988). Insertions into mRNA occur in the middle of the P gene, resulting in a frameshift that allows ribosomal access to a second ORF. The mechanism responsible for RNA editing, known as "stuttering", was elucidated in SeV when RNA editing was predicted to occur as the viral polymerase reiteratively copied one of three C residues on the genome template, then slipped backward before the next nucleotide was added (Vidal *et al.* 1990). The stuttering mechanism of RNA editing is reliant on the viral polymerase temporarily pausing during transcription at a stretch of pyrimidine residues (5'-A_nG_n-3'). *Cis*-acting sequences located upstream from the editing site, in particular two nucleotides immediately upstream, modulate the pattern of G insertion into the mRNA (Hausmann *et al.* 1999b), which differs between paramyxovirus P genes. During RNA editing, realignment of the stuttering polymerase downstream, rather than upstream, leads to nucleotide deletions, as opposed to insertions (Jacques *et al.* 1994). The stuttering mechanism of RNA editing utilised by the polymerase in the P gene is similar to the mechanism used for mRNA polyadenylation, suggesting an evolutionary relationship between the two (Hausmann *et al.* 1999a).

1.6.5 RNA replication

Propagation of paramyxoviruses involves virus adsorption and entry into a host cell, protein production, genome replication, assembly and release of progeny virions, with aspects of viral replication taking place in the cytoplasm. Virus adsorption into a host cell resulting from attachment (HN/H/G) proteins binding to

cellular receptors, is mediated by the F protein, and occurs at the neutral pH of the cell membrane. Fusion of the virus with a host cell releases helical nucleocapsids into the cytoplasm (Lamb & Kolakofsky 2001).

The Kingsbury-Kolakofsky model of transcription and replication (Blumberg *et al.* 1991) dictates that a promotor sequence for polymerase entry exists on the 3' end of both genome and antigenome, mRNA is produced via a polarity gradient of transcription, and relative amount of mRNA versus genomic RNA is modulated by a virus-encoded protein. All viral RNA synthesis begins at the 3' end of the genome, but cannot be initiated until primary transcription and translation provides critical levels of N and P proteins. The switch from transcription to replication may be delayed until the N protein has accumulated to threshold levels (Plumet *et al.* 2005). The vRdRp replicates the genome via a complementary RNA antigenome, which is always encapsidated by N proteins. Synthesis of antigenome from genome and, conversely, genome from antigenome, results when the vRdRp ignores junction signals and editing sites within RNA. N proteins bind to the nascent strand of RNA, preventing recognition of termination signals by vRdRp, a process that is self-regulatory and is governed by the availability of N subunits (Lamb & Kolakofsky 2001).

The 3' terminal regions of the genome and antigenome contain *cis*-acting promotor sequences recognised by the vRdRp via a scanning mechanism (Kolakofsky *et al.* 2004) as the polymerase does not dissociate from the RNA template. Two discontinuous regions essential in promotor function, conserved region 1 and 2 (CRI and CRII), identified in SV-5 (Murphy *et al.* 1998), were not confined to the leader region of the genome as CRII is in the coding region of the L gene. Genome length is significant (Calain & Roux 1993) and promotor sequences in hexamer positions 14, 15 and 16 have been demonstrated to be conserved, with the sequence 5'-CGNNNN-3' in SV-5 (Murphy & Parks 1999), 5'-$(GNNNN)_3$-3' in SeV (Tapparel *et al.* 1998) and 5'-$(GNNNAN)_2$GNNNCN-3' in MeV (Walpita 2004), indicating the importance of promotor alignment along the same face of the helical nucleocapsid. The 3' end of the antigenome contains a stronger promotor than that of the genome, the genome also contains negatively-acting signals (Keller & Parks 2003) accounting for the higher ratio of genomic molecules to antigenomic molecules during replication. The MeV M protein has been shown to be a negative regulator of viral polymerase activity, having an equally repressive effect on mRNA transcription and genome replication (Reuter *et al.* 2006).

Genome synthesis and viral assembly occur concurrently (Gubbay *et al.* 2001). Free N subunits associate with template RNA, forming nucleocapsids, and

integral membrane proteins are synthesised in the endoplasmic reticulum of the host cell. Host cell enzymes and molecular chaperones assist in folding and conformational maturation of glycoproteins, and carbohydrate chains are modified in the Golgi apparatus. Viral glycoproteins are transported to the plasma membrane, which is devoid of host cell proteins, and progeny virus are released by budding (Lamb & Kolakofsky 2001). A schematic diagram of viral replication is given in Figure 1.8.

1.7 New and Emerging Paramyxoviruses

Fer-de-Lance virus

The extensive host range of paramyxoviruses, previously considered being restricted to mammals and birds, became apparent following the emergence of a virus capable of infecting reptiles. A disease outbreak occurred in 1972 in a Swiss serpentarium where a new virus was isolated from a Fer-de-Lance viper (*Bothrops atrox*) and subsequently termed FDLV, named after the infected reptilian host (Clark *et al.* 1979). FDLV was isolated from embryonic snake eggs seven days after inoculation with lung tissue obtained from diseased vipers. CPE was observed in the form of syncytia after reptilian cells lines were inoculated with FDLV (Clark *et al.* 1979). The optimum temperature for viral replication was determined to be 28-30°C (Mayr *et al.* 2000). Preliminary analysis of FDLV revealed a single-stranded, non-segmented RNA genome, while electron microscopy displayed pleomorphic virions. Structural properties of FDLV, in combination with evidence demonstrating the virus was antigenically distinct from other mammalian and bird paramyxoviruses, placed the first reptilian virus isolated in the family *Paramyxoviridae*, intermediately between the subfamilies *Paramyxovirinae* and *Pneumovirinae* (Clark *et al.* 1979).

Molecular analysis of 16 paramyxoviruses isolated between 1972 and 1998 from 14 different reptilian host species was undertaken in an attempt to clarify genetic relatedness and taxonomic position of the new viruses (Ahne *et al.* 1999). Classification of the viruses was dependant on electron microscopy and physiochemical studies, such as sensitivity to chloroform, haemagglutination properties and syncytia formation. Phylogenetic relationships were established based on the comparison of a 518 nucleotide segment of the L gene along with a 352 nucleotide segment of the HN gene (Ahne *et al.* 1999).

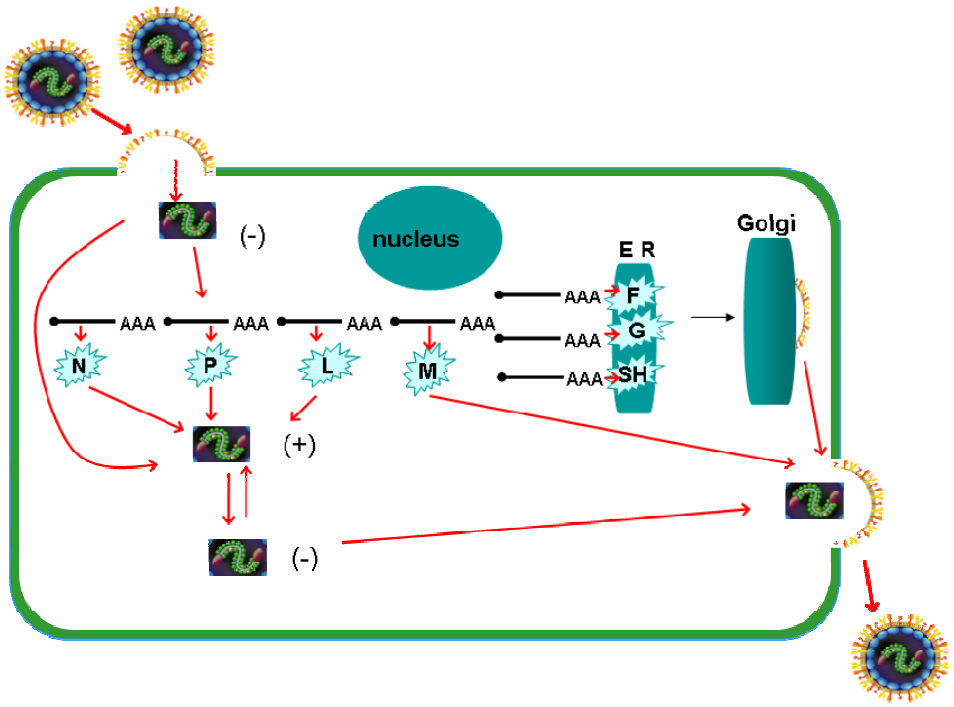


Figure 1.8- Paramyxovirus Infectious Cycle

Paramyxoviruses attach to host cells through the binding of attachment proteins to cellular receptors, a process mediated by the fusion protein. Upon entry, transcription and translation of viral RNA occurs in the cytoplasm. The polymerase proteins associate with the viral RNA and integral membrane proteins are synthesised in the endoplasmic reticulum (ER). Carbohydrate chains of glycoproteins are modified in the Golgi apparatus, and then the glycoproteins are transported to the plasma membrane. Genome synthesis and viral assembly occur concurrently, and progeny virions are released by budding. This figure has been adapted from Lamb & Kolakofsky (2001).

The study revealed the existence of two distinct subgroups of viruses likely to represent divergent viral species. Reptilian paramyxoviruses share strong antigenic relationships, regardless of origin, although a correlation between subgroup and geographical location was observed (Ahne *et al.* 1999). Viruses isolated from 18 different snake species displaying respiratory symptoms and neuronal disease were determined to be paramyxoviruses based on electron microscopy, CPE and haemagglutination inhibition (Franke *et al.* 2001).

The FDLV genome is 15,378 nucleotides in length and is consistent with other paramyxoviruses in that the common N, P, M, F, HN and L genes were present, along with conserved CCT or CTT IGRs observed in members of the henipavirus, morbillivirus and respirovirus genera. A unique feature observed in FDLV was the presence of an extra gene, designated 'U' for 'Unknown', between the N and P genes (Kurath *et al.* 2004). This new U gene codes for a protein with a molecular mass of either 19.4 or 16.6 kDa, depending on the translation initiation codon utilised. The protein has no similarity to any protein sequences in the National Center for Biotechnology Information (NCBI) database and could possibly function as a small transmembrane protein (Kurath *et al.* 2004). Two other FDLV isolates, Biti-CA98 and Gono-GER85, isolated from snakes (*Bitis atropos* and *Gonosoma oxycephala*, respectively), were found to also contain the U gene (Kurath *et al.* 2004).

Tupaia paramyxovirus

TuPV was isolated from primary kidney cells of an apparently healthy tree shrew (*Tupaia belangeri*) captured near Bangkok, Thailand in the late 1970s (Tidona *et al.* 1999). Primary kidney cells from the tree shrew were cultured and spontaneously degenerated after seven days, producing a cytopathic effect (CPE) that manifested as syncytia formation and cell lysis. CPE was also observed when clarified supernatant from primary kidney cells was transferred to continuous tupaia fibroblast or kidney cell lines, indicative of the presence of an infectious agent contaminating the culture (Tidona *et al.* 1999). Tupaia fibroblast and kidney cells were the only cell types capable of supporting growth of TuPV. Pleomorphic enveloped particles and helical nucleocapsids distinctive of paramyxoviruses were observed by electron microscopy. No cross-reactivity was observed between TuPV and other paramyxoviruses such as HPIV-1, SeV, MeV and MuV (Tidona *et al.* 1999). Sequencing of the exceptionally large 17,904 nucleotide TuPV genome

revealed that the organisation of the P/V/C gene was most similar to that of HeV, morbilliviruses and respiroviruses. The TuPV 5' trailer sequence, at 590 nucleotides, is significantly longer in comparison to other members of the subfamily *Paramyxovirinae*, whose trailer sequences range from 25-114 nucleotides (Tidona *et al.* 1999).

Hendra virus

In September 1994, HeV caused an outbreak of severe respiratory disease resulting in the death of 14 horses and a horse trainer at a stable near Brisbane, Australia. The index case was a pregnant mare that died of acute respiratory disease (Murray *et al.* 1995). After the death of the mare, 14 out of 21 sick horses died or were euthanased. Clinical signs of illness were fever up to 41°C, depression, respiratory distress and frothy nasal discharge. At the stable, a 49-year-old male trainer and a 40-year-old male stablehand developed an influenza-like illness with symptoms of lethargy, headache and vertigo. The stablehand gradually recovered over a period of six weeks but the condition of the trainer rapidly deteriorated and proved fatal. Both the trainer and stablehand had been in close contact with the index case of the outbreak (Murray *et al.* 1995). Isolation HeV, which was initially named *Equine morbillivirus*, was achieved when Vero cells were inoculated with spleen and lung tissue from two affected horses. These Vero cells experienced CPE, with the formation of syncytia. Analysis of the virus by electron microscopy revealed a pleomorphic, enveloped structure with virions ranging in size from 38-600 nm (Murray *et al.* 1995). Serological surveying of 1600 horses and 90 humans (Murray *et al.* 1995) did not identify neutralising antibodies to HeV. Later findings demonstrated that the natural reservoir for the virus are pteropid bats in the genus *Pteropus* (Halpin *et al.* 2000).

HeV was determined to be the cause of a second human fatality, when a 35-year-old male farmer from MacKay, Australia, died as a result of severe encephalitis preceded by epileptic activity (O'sullivan *et al.* 1997). Closer inspection into circumstances surrounding the case uncovered prior illness in the man corresponding with the time two horses had died on his property. The farmer had cared for the horses and had assisted in necropsies before developing a brief aseptic meningitic disorder with sore throat, vomiting, headache, drowsiness and stiff neck. The initial illness and death of the two horses occurred in August 1994, 13 months before the reoccurrence of illness and death in the farmer, and also before the Hendra stable outbreak of HeV (Murray *et al.* 1995). Retrospective findings from serology, electron microscopy, PCR and immunohistochemistry implicated HeV as

the cause of disease in the horses. Another fatal equine case of HeV infection occurred in 1999, in Cairns, Australia, in a 9-year-old thoroughbred mare (Field *et al.* 2000). Clinical signs of disease prior to euthanasia of the horse were depression and oedema of the face, lips and neck (Field *et al.* 2000). Histological analysis of lung tissue obtained from the horse displayed haemorrhage with erythrophagocytosis, oedema, emphysema and syncytia formation in endothelial cells (Murray *et al.* 1995). Two more incidences of HeV were reported in Queensland, Australia, in 2004 and each resulted in the death of one horse. A veterinarian involved in the autopsy of one of the horses was infected with HeV and developed a flu-like illness but later recovered (Field *et al.*).

HeV was originally classified as a member of the genus *Morbillivirus* based on partial sequencing of the M gene (Murray *et al.* 1995). The P gene of HeV was deduced to have the potential to encode the proteins P, V and C. This data in combination with serological evidence demonstrating the lack of cross-reactivity between HeV and morbilliviruses indicated that the virus was not a morbillivirus (Wang *et al.* 1998). Sequencing of the N gene (Yu *et al.* 1998a) consolidated the exclusion of HeV from the genus *Morbillivirus*, as did sequencing of the G gene (Yu *et al.* 1998b) determining that the HeV attachment protein did not possess haemagglutination or neuraminidase activity. Sequencing of the entire 18,234 nucleotide HeV genome revealed a sequence variation in a highly conserved region of the L protein (Chua *et al.* 2000). Paramyxovirus L proteins contain the sequence motif QGDNQ, which is absolutely conserved in all NNSV. The sequence of this motif in HeV was determined to be QGDNE (Chua *et al.* 2000). The QGDNQ motif is important for polymerase function and sequencing results have subsequently determined that TuPV, NiV and MosPV also contain the QGDNE sequence change. HeV has been designated as the type species of the genus *Henipavirus*.

Menangle virus

In 1997, *Menangle virus* (MenPV) was isolated during an outbreak of reproductive disease in pigs at a piggery in New South Wales, Australia. The piggery experienced a decrease in farrowing rate from 82% to 62% and there were 27% less piglets born alive during the outbreak. Mummified piglets were born and stillborn piglets were frequently deformed, having severe degeneration of the brain and spinal cord (Philbey *et al.* 1998). No symptoms of disease were observed in postnatal pigs. The outbreak began in April of 1997 and lasted for 21 weeks (Love *et al.* 2001).

Virus isolation was achieved by inoculation of BHK21 cells with heart, lung and brain tissue from affected piglets, where CPE was observed in the form of vacuolation of cells and syncytia formation. MenPV was classed as a paramyxovirus based on electron microscopic visualisation of pleomorphic particles and 'herringbone' nucleocapsids. Serum samples collected from cattle, sheep, birds, rodents, cats and a dog were all negative for MenPV antibodies (Kirkland *et al.* 2001). Two piggery workers became ill during the outbreak with an influenza-like illness in conjunction with a red, spotty rash. Other symptoms included malaise, chills, fever, sweating, headache, with weight loss observed after disease progression. Both of these workers had intense occupational exposure to infected pigs and serological analysis revealed the presence of MenPV antibodies in both cases (Chant *et al.* 1998). Serum samples were obtained from 250 humans and the only MenPV positive samples were those corresponding to the two piggery workers (Kirkland *et al.* 2001). The likelihood of MenPV infection of humans appears to be low without the prior amplification of the virus in pigs (Hooper 2001).

A colony of fruit bats (*Pteropus poliocephalus*) were found to be roosting within 150 m of the piggery and these animals were speculated to be the potential source of MenPV infection in the pigs (Kirkland *et al.* 2001). Serum was collected from bats and determined to be positive for MenPV antibodies. Control of the MenPV outbreak and eradication of the virus from the piggery resulted following the administration of a serological testing and segregation program. Development of protective immunity against MenPV by sows also aided in reducing development of disease (Kirkland *et al.* 2001).

MenPV was reported to lack haemagglutinin and neuraminidase activity (Philbey *et al.* 1998). The neuraminidase encoding region of the attachment protein of paramyxoviruses contains seven conserved residues, but only two or three of these residues are present in MenPV, suggesting that MenPV does not possess the same degree of neuraminidase activity as detected in other rubulaviruses and respiroviruses (Bowden *et al.* 2001). MenPV is closely related to *Tioman virus* (TioPV), a novel virus isolated from the urine of fruit bats (*Pteropus hypomelanus*) (Chua *et al.* 2001), and both MenPV and TioPV have been tentatively classified in the genus *Rubulavirus* (Pringle 2005).

Nipah virus

In 1998, a severe disease outbreak affecting humans and pigs occurred just outside the town of Ipoh in peninsular Malaysia. The causative agent of the outbreak was initially thought to be *Japanese encephalitis virus* (JEV), a mosquito-

borne viral disease enzootic to the region, but aspects of the outbreak did not fit with the characteristics of JEV. JEV does not cause death in pigs, and furthermore, humans that had been previously immunised against JEV were shown to succumb to the new disease. The NiV outbreak claimed the lives of 105 people of the 265 that presented with infection, attributing a 40% mortality rate to the virus. The majority of cases were males who had direct contact with pigs or pig products indicating NiV was a zoonotic agent capable of crossing the species barrier between pig and human (Wong *et al.* 2002). Symptoms of disease in infected humans ranged from mild headache, fever, dizziness and reduced level of consciousness. Symptoms in pigs were those of porcine 'respiratory and encephalitis syndrome' consisting of a distinct loud barking cough, paralysis, abnormal movement and fever. Meningitis may also occur in pigs with oedema and infiltration of white blood cells (Wong *et al.* 2002).

NiV was isolated five days after inoculation of Vero cells with cerebrospinal fluid taken from two of the first three patients that died at the University of Malaya Medical Centre (Chua *et al.* 1999). The emergent virus was ultimately termed "Nipah" after Kampung Sungai Nipah (Nipah river village) where specimens were obtained that yielded the first viral isolates (Wong *et al.* 2002). The first of the three patients was a 51-year-old male that presented with acute confusion and pain associated with myoclonus of the left arm. The condition of the patient deteriorated after development of hypotension and bradycardia and he died six days after admission to hospital. The second patient was a 34-year-old male that presented with fever, drowsiness and lethargy and was comatose two days after hospital admission. He died five days after onset of coma after development of hypotension. The third patient was a 52-year-old male who was admitted to hospital with headache, nausea, fever and chills and died within five days (Chua *et al.* 1999). The brain was the most severely affected tissue and vasculitic vessels displayed vessel wall necrosis and thrombosis. Endothelial ulceration with varying degrees of inflammation and fibrinoid necrosis was detected in infected blood vessels along with extravascular parenchyma (Wong *et al.* 2002). More than one million pigs were culled over the course of the outbreak (Ahmad 2000) as authorities saw this as the only means possible to control the spread of disease.

In March 1999, an outbreak of pneumonia occurred in eleven abattoir workers in Singapore. These eleven patients, who all worked at the same abattoir, presented with fever, headache and drowsiness, with one case resulting in fatality after disease progression lead to cardiac arrest (Paton *et al.* 1999). Serum was obtained from all patients diagnosis was made after detection of IgM antibodies to

NiV (Paton *et al.* 1999). Pigs from NiV affected areas of Malaysia had been imported to the abattoir in Singapore and the outbreak was controlled when the abattoir was closed and importation of pigs ceased. NiV was also found to be the causative agent responsible for outbreaks of fatal febrile encephalitis in Bangladesh in 2001, 2003, 2004 and 2005 (Hsu *et al.* 2004; Epstein *et al.* 2006; Harcourt *et al.* 2005) and in India in 2001 (Chadha *et al.* 2006). Fruit bats in the genus *Pteropus* have been identified as the natural reservoir host for both NiV and HeV (Hsu *et al.* 2004; Sendow *et al.* 2006). Serological, virological and molecular studies have revealed the presence of NiV or NiV-like viruses in fruit bat populations in Malaysia, Cambodia and Thailand (Reynes *et al.* 2005; Olson *et al.* 2002; Wacharapluesadee *et al.* 2005; Chua *et al.* 2002).

Sequencing of the 18,246 nucleotide genome (Chua *et al.* 2000) revealed the presence of N, P, M, F and G genes (Harcourt *et al.* 2000), and cross-neutralisation experiments of NiV revealed that the novel virus was closely related to HeV (Chua *et al.* 2000). Characterisation of the genome revealed a P gene structure similar to that of HeV, and the expression of a V protein was determined to occur by the RNA editing mechanism utilised by the respiroviruses and morbilliviruses (Chua *et al.* 2000). Cleavage of the F protein precursor into active subunits is usually mediated by furin in members of the subfamily *Paramyxovirinae*, but the conserved sequence recognised by this protease is not present in NiV, or HeV, indicating that these viruses may possibly utilise different host cells proteases for the activation of the F protein (Harcourt *et al.* 2000). The functionally important conserved amino acid sequence, QGDNQ, in the polymerase gene of NiV is replaced with QGDNE, as is also the case in HeV (Harcourt *et al.* 2001).

Both NiV and HeV have been classified as a Category C critical biological agent for public health preparedness, along with other emerging biological threat agents that present a possible bioterrorism risk (Rotz *et al.* 2002). The NiV outbreaks in Malaysia caused widespread panic before the etiological agent was determined and controlled, leading to suggestions that NiV may be a potential agent of bioterrorism (Lam 2003).

1.8 Rodent Paramyxoviruses

SeV, an extremely contagious respiratory virus, is one of the most important pathogens of rodents, and this member of the genus *Respirovirus* has been indispensable in paramyxovirus research since isolation from laboratory rodents in the 1950s (Baker 1998). At least four novel paramyxoviruses, *Nariva virus* (NarPV) (Tikasingh *et al.* 1966), MosPV (Campbell *et al.* 1977), *J-virus* (JPV) (Jun *et*

al. 1977) and *Beilong virus* (BeiPV) (Li *et al.* 2006), have been isolated from rodents since the 1960s.

1.8.1 *Nariva virus*

NarPV was isolated from forest rodents (*Zygodontomys b. brevicauda*), trapped on Bush Bush Island in the Nariva swamp, Eastern Trinidad, in 1962 and 1963 (Tikasingh *et al.* 1966). NarPV was isolated from mouse brains and when the virus infected Vero and BHK-21 cells, formation of syncytia was observed. Haemagglutination and cell binding studies indicate NarPV may not use sialic acid as a receptor for attachment (Karabatsos *et al.* 1969). NarPV virus particles were visualised as 130-250 nm pleomorphic structures capable of haemagglutination of red blood cells (Walder 1971). Intracytoplasmic inclusion bodies containing helical nucleocapsids engulfed within the matrix of lysosomes of brain cells were observed, and brain lesions were comparable to those of MeV infection (Walder *et al.* 1971). Electron microscopic analysis of NarPV enabled categorisation of this virus as a member of the family *Paramyxoviridae*.

The pathogenesis of NarPV was studied in hamsters, an animal closely related to the natural rodent host of the virus. Infection of suckling hamsters with NarPV resulted in acute necrotising encephalitis with multinucleated cells observed in ependyma and neutrophils (Roos & Wollmann 1979). Weanling hamsters infected with NarPV exhibited less cerebral parenchymal necrosis than suckling hamsters. NarPV produced non-productive infection in the weanlings, but despite the lack of infectious virus particles, the animals developed fatal encephalitis. The maturity of the host immune system appears to be important in NarPV pathogenesis, and age-related changes in susceptible neural cells, such as changes in cell surface receptors and cellular enzymes, may be critical in disease progression (Roos & Wollmann 1979).

1.8.2 *Mossman virus*

MosPV was isolated in northern Queensland, Australia, on three occasions from 1970-1971. The virus was isolated from pooled organs of a female rat (*Rattus leucopus*), trapped near Mossman, Queensland, a pool of mites (*Laelaps echidninus*) and pooled organs from a male rat (*Rattus fuscipes*) (Campbell *et al.* 1977). Infection of BHK-21 cells with MosPV produced CPE with syncytia formation, a hallmark of paramyxovirus infection. Electron microscopic analysis of MosPV displayed viral particles and free nucleocapsids, which when taken into account with

cellular based experiments, lead to the conclusion that MosPV was a paramyxovirus (Campbell *et al.* 1977).

Sequencing of MosPV revealed a genome of 16,650 nucleotides in length, with arrangement of genes consistent with that of the henipaviruses (Miller *et al.* 2003). Characterisation of the genome uncovered several features that may assist in classification MosPV. The MosPV 3' leader region of the genome was found to contain 55 nucleotides, consistent with all other members of the *Paramyxovirinae* and the IGRs that separate the viral genes were conserved, although not invariant, trinucleotide sequences.

The coding strategy determined for the P gene was similar to that of the henipaviruses, morbilliviruses and respiroviruses (Miller *et al.* 2003). The F gene of many paramyxoviruses contain the conserved sequence R-X-K/R-R (Hosaka *et al.* 1991) that directs cleavage of the F protein by furin, but the MosPV sequence deviates from the conserved sequence and instead contains AGNKK as the cleavage sequence. The conserved sequence of respiroviruses and rubulaviruses, NRKSCS, in the attachment protein is thought to form part of the sialic acid binding site (Jorgensen *et al.* 1987). Similar to henipaviruses and morbilliviruses, MosPV lacks conservation of this site. The lack of conservation of the hexapeptide motif in the attachment protein, along with the failure to detect any neuraminidase activity, resulted in the attachment gene of MosPV to be designed G (Miller *et al.* 2003). The conserved QGDNQ motif in the L gene was replaced with QGDNE, an observation also made in the henipaviruses and TuPV (Wang *et al.* 2001). MosPV is yet to be classified into a genus within the subfamily *Paramyxovirinae*, but phylogenetically appears to be positioned between the morbillivirus and henipavirus genera, along with *Salem virus* and TuPV (Miller *et al.* 2003).

1.8.3 *J-virus*

The trapping of wild mice (*Mus musculus*) during a study investigating the pathology of feral rodents in northern Queensland, Australia, in 1972 (Mesina *et al.* 1974) lead to the isolation of JPV on four separate occasions, via kidney autoculture (Jun *et al.* 1977). The four moribund mice from which kidneys were obtained, all displayed extensive haemorrhagic lung lesions. Passaging of JPV in mouse kidney and lung primary cell lines, and continuous cell lines from mouse kidney (CSL217), human (MRC5 and Hep2), pig kidney (PS), baby hamster kidney (BHK-21) and monkey kidney (Vero) resulted in CPE characterised by destruction of the cell monolayer and vacuolated syncytia formation (Jun *et al.* 1977). Cell lines where CPE was not observed were bovine kidney (MDBK), human (HeLa and Chang liver)

and rat fibroblast (RT). CPE was associated with eosinophilic cytoplasmic inclusions, but no involvement of the nucleus was detected.

Electron microscopy exposed a viral nucleocapsid of 15 nm diameter, with 'herringbone' morphology, typical of paramyxoviruses. Evidence of soluble or virus-associated haemagglutinin was not detected and haemadsorption using standard techniques and erythrocytes from a variety of species was not observed (Jun *et al.* 1977). Complement fixation assays were used in an attempt to detect a serological relationship between JPV and a range of paramyxoviruses, such as HPIV-1-4, NDV, MeV, MuV, NarPV and MosPV, but all produced negative results.

Intranasal or subcutaneous inoculation of mice, pigs and rats with JPV did not produce clinical signs of disease in these animals, although neutralising antibodies against the virus were detected. Viraemia, in conjunction with mild lethargy was observed in rats and mice inoculated with JPV, and varying degrees of haemorrhagic interstitial pneumonia was discovered in the animals upon autopsy, three weeks post-infection (Jun *et al.* 1977). Serological surveillance by serum neutralisation tests among Australian mammals detected JPV antibodies in 27/96 wild mice, 17/123 wild rats, 13/107 pigs, 1/157 cattle and 2/91 humans, but a similar survey using complement fixation experiments on American mammals failed to detect JPV reactive antibodies in any of 55 laboratory mice, 45 humans and 5 hamsters (Jun *et al.* 1977). Serum neutralisation experiments, when taken into account with viral characterisation and animal experiments, suggest JPV is a natural respiratory pathogen of feral *Mus musculus* in Queensland, Australia.

Molecular characterisation of JPV

The entire genome of JPV has been sequenced and is 18,954 nucleotides in length (Jack *et al.* 2005). The increase in genome size of JPV over that of other paramyxoviruses is due to the presence of two additional genes between the fusion and attachment genes, and an extensive second ORF within the attachment protein gene. The genome organisation of JPV is 3'-N-P/V/C-M-F-SH-TM-G-L-5' and is shown schematically in Figure 1.9. JPV has been classed as member of the family *Paramyxoviridae*.

At 18,954 nucleotides, the genome of the JPV is compatible with the 'rule of six' (Calain & Roux 1993). The terminal end sequences of paramyxoviruses customarily display complementarity of 11-13 nucleotides (Lamb & Kolakofsky 2001). Complementarity between the genome termini of JPV is imperfect, differing by two nucleotides, but this feature is not extremely unusual, as MeV and MosPV also display imperfect complementarity, also differing by two nucleotides.

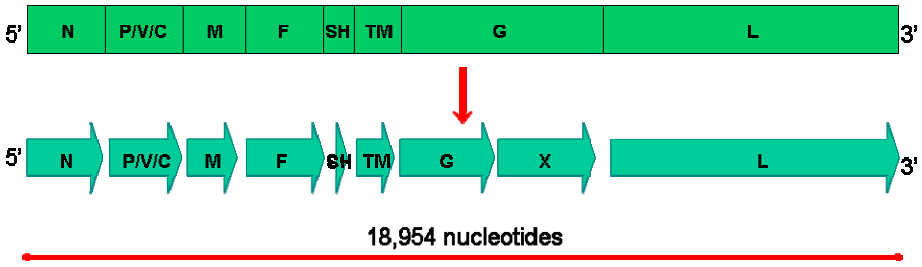


Figure 1.9- Schematic Diagram of JPV Genome and Encoded Proteins

The genome of JPV contains eight genes and is 18,954 nucleotides in length. The increase in genome size of JPV over that of other paramyxoviruses is due to the presence of two additional genes between the fusion and attachment genes, and a second ORF within the attachment protein gene. The genome organisation is shown and arrows represent encoded proteins. The putative 'X' protein produced from ORF-X is also shown.

The 3' leader sequence of JPV is 55 nucleotides, matching the length of that of other paramyxovirus leader sequences, and the 5' trailer sequence is 36 nucleotides, similar to the 33 nucleotide trailer region of the henipaviruses. JPV genes are separated by trinucleotide IGRs, with the bases CTT making up the nucleotide sequence in all gene junctions but that between the 3' leader and N gene where the sequence is TTT (Jack *et al.* 2005). The genome of JPV consists of eight genes and may produce up to twelve proteins.

The JPV N protein contains 522 amino acids and has a predicted molecular mass of 58.37 kDa (Jack *et al.* 2005). The P gene of JPV has the capacity to encode four putative proteins. The highly acidic P protein contains 499 amino acids, has a predicted molecular mass of 53.68 kDa, and is translated after faithful transcription of the P gene. There are a large number of potential sites for phosphorylation on the P protein. The C protein contains 152 amino acids, with a predicted molecular mass of 17.72 kDa, and is translated via an alternate ORF within the P gene. The V protein is produced as a result of the addition of one non-templated G residue into the RNA editing site of the P gene and contains 292 amino acids, with predicted molecular mass of 31.27 kDa (Jack *et al.* 2005). The W protein contains 310 amino acids, has a predicted molecular mass of 33.74 kDa, and is produced as a result of the addition of two non-templated G residues into the RNA editing site of the P gene (Jack *et al.* 2005).

The M protein of JPV contains 340 amino acids, has a predicted molecular mass of 37.69 kDa, and is structurally similar to M proteins of other known paramyxoviruses. The F protein is a type I glycoprotein containing 544 amino acids, with a predicted molecular mass of 59.74 kDa (Jack *et al.* 2005). The cleavage site of the F protein of JPV is monobasic, with the sequence GVPGVR. Alteration of this sequence is consistent with experimental evidence that the JPV F protein is unable to be cleaved by furin (Miller 2004).

The SH protein, consisting of 69 amino acids, with a predicted molecular mass of 7.66 kDa, is translated from the novel SH gene of JPV (Miller 2004). SH proteins have been identified in SV-5 (Hiebert *et al.* 1985), MuV (Elango *et al.* 1989), and APMV-6 (Chang *et al.* 2001), with hydrophobic regions present in these proteins functioning as transmembrane anchors. Although the positioning of the JPV SH gene is analogous to that of the SH gene of SV-5 and MuV, no sequence homology is observed between the JPV SH protein and any other paramyxovirus protein. Structurally, the JPVP SH protein is similar to other SH proteins, having a predicted transmembrane domain at the N-terminus. Identification of the JPV SH protein in infected cells has been achieved by radio-immunoprecipitation, but the location of

the protein is yet to be determined. Dicistronic forms of SH mRNA, F-SH and SH-TM were detected (Miller 2004).

The other novel gene within the JPV genome, TM, produces a type II transmembrane glycoprotein of 258 amino acids in length, with a predicted molecular mass of 29.09 kDa, and this protein lacks homology with any other proteins listed in the NCBI database. The TM protein, which is basic in nature, contains two potential sites for O-linked glycosylation and has a putative transmembrane domain. The TM protein was identified by western blot in JPV infected cells, and indirect immunofluorescence experiments supported the prediction that the TM protein is an integral membrane protein with the C-terminal region exposed at the cell surface (Miller 2004).

The attachment gene produces a protein containing 709 amino acids, with a predicted molecular mass of 78.13 kDa, and also contains an extraordinarily long untranslated region of 2,218 nucleotides after the stop codon (Jack *et al.* 2005). The untranslated region of the attachment gene consists of a 2,115 nucleotide second ORF, which has been designated ORF-X, beginning directly after the stop codon. Paramyxoviruses with HN attachment proteins contain six of the seven conserved residues believed to be neuraminidase actives sites (Langedijk *et al.* 1997). This conservation pattern is also present in the JPV G protein. The conserved hexapeptide sequence NRKSCS, thought to be the sialic acid binding site (Jorgensen *et al.* 1987), is changed to NRRSCS in the JPV G protein. The conservation of the sialic acid binding site, along with the conservation of six out of seven resides of the neuraminidase active site suggests the attachment protein of JPV would have haemagglutination and neuraminidase activities. In contrast to predictions, experimental evidence suggested the absence of either haemagglutination or neuraminidase activities, and the JPV attachment protein was hence designated as a G protein (Miller 2004). The JPV G protein contains a putative hydrophobic transmembrane domain and five potential sites for the addition of N-linked carbohydrates. Analysis of mRNA transcripts by northern blot revealed monocistronic G mRNA transcripts. JPV may utilise an unconventional mechanism to translate a protein from ORF-X, either individually, or as part of a G-X fusion product of 1,414 amino acids in length. No evidence of X synthesis or translation was detected by western blot, indirect immunofluorescence and radioimmunoprecipitation in JPV infected cells (Miller 2004).

The L gene of JPV produces a protein containing 2,204 amino acids, with a predicted molecular mass of 254.46 kDa. The six conserved domains essential for polymerase activity are all present in JPV. JPV contains the conserved QGDNQ

sequence (Jack *et al.* 2005), not the altered QGDNE sequence observed in the other four paramyxoviruses with large genomes.

Experiments to determine the pathological effect of JPV were repeated in BALB/c mice. Intranasal and aerosol routes of inoculation were used to infect mice with JPV in two separate studies. Mice infected with JPV did not display any clinical signs of disease, consistent with observations in the original JPV pathogenicity experiments (Jun *et al.* 1977). JPV was isolated from the lungs of infected mice and moderate serum neutralising antibody titres were evoked, but in contrast to previous experiments, virus was not able to be isolated from any tissue apart from lung, viraemia did not occur, and there was no evidence of extensive haemorrhagic lung lesions (Miller 2004). Conflicting results between the two animal experiments may have resulted from attenuation of JPV due to adaptation of the virus to growth in cell culture or that BALB/c mice may be less susceptible to JPV infection than *Mus musculus*, the mouse species that is believed to be a natural host of the virus.

1.8.4 *Beilong virus*

A routine search for sequences related to JPV using BLASTx on the NCBI server revealed two putative novel cDNAs, termed Angrem 104 (AF367870) and Angrem 52 (AY040225) (Liang *et al.* 2003), from human mesangial cells with significant homology to JPV P, M and F genes (Jack *et al.* 2005). The relationship between the Angrem cDNA sequences to other paramyxoviruses was reported (Schomacker *et al.* 2004; Basler *et al.* 2005), but the close relationship to JPV was not ascertained by these groups as the JPV genome sequence had not yet been deposited in the NCBI database. Although non-retroviral RNA integration into a eukaryotic genome has been reported (Crochu *et al.* 2004), contaminating viral RNA in the mesangial cells was believed to be the more likely source of the homologous paramyxovirus sequence.

A virus was later isolated from the human mesangial cell line from which the cDNA sequences Angrem 104 and Angrem 52 were originally obtained. The isolated virus produced syncytia in Vero cells, and upon examination of cell cultures and supernatant by electron microscopy, pleomorphic virions were observed (Li *et al.* 2006). The virions observed by negative contrast electron microscopy were enveloped, possessed surface projections, and contained 'herringbone-like' RNP, features characteristic of paramyxoviruses. The new paramyxovirus was named BeiPV to represent the joint collaboration between the **Bei**jing and Gee**long** researchers that lead to the isolation and characterization of the virus. Concurrently, isolation of this paramyxovirus from human mesangial cells, obtained from the

American Type Culture Collection, was unsuccessfully attempted (Basler *et al.* 2005). Later, it was discovered that a rat mesangial cell line, from the same laboratory in which the human mesangial cells were cultured, carried BeiPV, which is believed to be the original source of the virus (Li *et al.* 2006).

Molecular characterisation of BeiPV

The genome of BeiPV has been completely sequenced and at 19,212 nucleotides in length, the genome is the largest in the family *Paramyxoviridae* sequenced to date, which also is the largest in the order *Mononegavirales*. The genome organisation of BeiPV is similar to that of JPV and is schematically presented in Figure 1.10. BeiPV, like JPV, contains the two novel genes, SH and TM, and an extended G gene. The BeiPV genome length is compatible with the 'rule of six' (Calain & Roux 1993). Complementarity between the genome termini of BeiPV is imperfect, with differences at three nucleotides. The 3' leader sequence of BeiPV is 55 nucleotides, matching the length of that of other paramyxovirus leader sequences, and the 5' trailer sequence is 25 nucleotides, similar to the trailer region of the rubulaviruses. BeiPV genes are separated by conserved trinucleotide IGRs, with the bases CTT making up the nucleotide sequence in all gene junctions (Li *et al.* 2006).

The BeiPV N protein contains 522 amino acids, and has a predicted molecular mass of 58.09 kDa (Li *et al.* 2006). The P gene of BeiPV has the capacity to encode four putative proteins, P, C, V and W. The P protein contains 496 amino acids, has a predicted molecular mass of 53.60 kDa, and is translated after faithful transcription of the P gene. The V protein is produced as a result of the addition of one non-templated G residue into the RNA editing site of the P gene and contains 292 amino acids, with predicted molecular mass of 31.90 kDa. The W protein contains 313 amino acids, has a predicted molecular mass of 34.46 kDa, and is produced as a result of the addition of two non-templated G residues into the RNA editing site of the P gene. The C protein contains 160 amino acids, with a predicted molecular mass of 18.44 kDa (Li *et al.* 2006). The M protein of BeiPV contains 340 amino acids, and has a predicted molecular mass of 37.43 kDa, and the F protein is a type I glycoprotein containing 544 amino acids, with a predicted molecular mass of 58.98 kDa. The cleavage site of the F protein of BeiPV is monobasic, with the sequence KLGNVK (Li *et al.* 2006). The BeiPV F gene cleavage site is most similar to that of HeV and is not expected to be cleaved by furin.

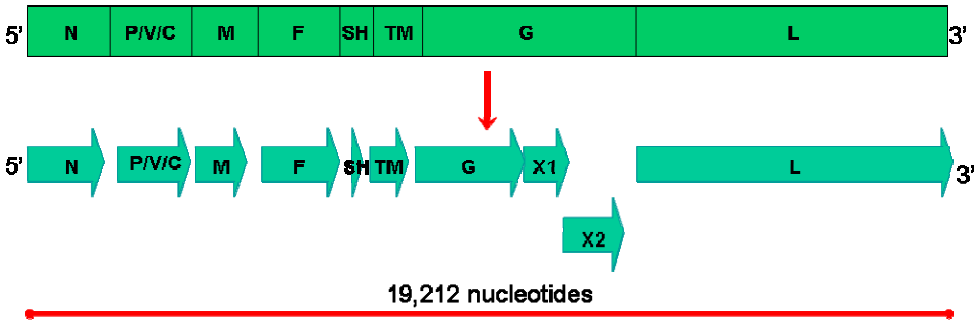


Figure 1.10- Schematic Diagram of BeiPV Genome and Encoded Proteins

The genome of BeiPV contains eight genes and is 19,212 nucleotides in length. The increase in genome size of BeiPV over that of other paramyxoviruses is due to the presence of two additional genes between the fusion and attachment genes, and an untranslated region within the attachment protein gene. The genome organisation is shown and arrows represent encoded proteins. The putative 'X' proteins produced from ORF-X1 and ORF-X2 are also shown

The L gene of BeiPV produces a protein containing 2,172 amino acids with a predicted molar mass of 249.97 kDa. BeiPV, like JPV, contains the conserved QGDNQ sequence, not the altered QGDNE sequence observed in the other four paramyxoviruses with large genomes (Li *et al.* 2006).

The gene encoding the attachment protein of BeiPV is the largest of all paramyxovirus attachment protein genes identified to date. The attachment gene produces a protein containing 734 amino acids, with a predicted molecular mass of 81.43 kDa, and also contains an extraordinarily long untranslated region of 2,284 nucleotides after the stop codon (Li *et al.* 2006). The untranslated region of the attachment gene consists of two additional ORFs, which have been designated ORF-X1 and ORF-X2. ORF-X1 is present in the same reading frame as the G ORF and may encode a protein of 299 amino acids. ORF-X2, overlapping ORF-X1 by 31 nucleotides, is present in an alternate reading frame and may encode a protein of 394 amino acids. Currently, there is no evidence to suggest that proteins are encoded by ORF-X1 and ORF-X2 (Li *et al.* 2006).

The BeiPV SH and TM genes are similar in size and location to those of JPV. The SH protein, consisting of 76 amino acids, encodes a protein with a predicted molecular mass of 9.05 kDa, and the TM protein, consisting of 254 amino acids, encodes a protein with a predicted molecular mass of 28.73 kDa (Li *et al.* 2006). The function of these proteins is unknown.

1.9 Genetic Engineering of Non-Segmented, Negative-Sense, Single-Stranded RNA Viruses

1.9.1 Reverse genetics

Viral research by classical genetics associates a particular phenotype with a corresponding gene, and can be accomplished relatively easily, whereas the use of reverse genetics to genetically manipulate the viral genome and rescue infectious virus particles from entirely engineered clones, while not as straightforward, is extremely propitious. Viruses can be generated that have never before existed in nature, and desired mutations can be introduced to enable phenotypic studies, provided the mutations are not lethal. Reverse genetics permits the study of roles of viral components in replication and their contribution to pathogenicity, attributes difficult to determine via conventional virology. The unique gene expression strategy utilised by NNSV involves replication of the ribonucleoprotein complex and sequential synthesis of mRNA and neither the genomic, nor antigenomic RNA

genome is capable of initiating an infectious cycle. Development of reverse genetics systems for RNA viruses within this replication category has provided a powerful tool for genetic manipulation and study of those viruses.

The establishment of a technique to introduce recombinant RNA into the genome of a segmented negative-sense, single-stranded RNA virus was achieved through the manipulation of influenza virus (Luytjes *et al.* 1989). Production of the genetically manipulated influenza virus was dependent on co-transfection with influenza virus polymerase proteins and support virus. The chimeric influenza virus could be passaged in cell culture (Luytjes *et al.* 1989). The first reverse genetics system to be established for a NNSV resulted from the manipulation of *Rabies virus* (RABV), a member of the family *Rhabdoviridae*, in 1994 (Schnell *et al.* 1994). A reverse genetics system was developed with the supposition that engineering the RABV genome would provide an insight into virus-host interactions, viral neurotropism, latency and pathogenesis. The deduction was made that RABV genome and antigenome transcripts would both be capable of initiating productive infection, when supplied in conjunction with N, P and L polymerase proteins. Construction of the RABV plasmid using the antigenome was favoured in order to prevent interference with encapsidation of RNA and translation of proteins, resulting from hybridisation of negative-sense genomic RNA and positive-sense RNA from protein-encoding plasmids, produced simultaneously during transcription (Schnell *et al.* 1994). Hence, the antigenome of RABV was cloned in the opposite direction to the T7 terminator and hepatitis delta virus (HDV) ribozyme sequence. The HDV ribozyme sequence provides a template for autocatalytic cleavage, generating a precise and predictable cleavage site in the plasmid (Perrotta & Been 1990). Transfection of the RABV plasmid, in addition to N, P and L support plasmids, resulted in the formation of transcriptionally active nucleocapsids, and subsequent assembly and budding of infectious RABV. Only extremely low levels of infectious RABV particles were produced during reverse genetics experiments, although the presence of one virally infected cell in a 3.2 cm tissue culture dish was deemed sufficient for re-isolation of the engineered virus (Schnell *et al.* 1994). Recovery of infectious virus was enhanced by implementing a freeze/thaw cycle to release virions from infected cells.

The first member of the family *Paramyxoviridae* for which a reverse genetics system was developed was MeV (Radecke *et al.* 1995). The construction of the MeV reverse genetics system was similar to that of the RABV system, and recovery of infectious MeV was successful. Viral production in transfected cells was monitored via formation of syncytia, a hallmark of paramyxovirus infection. MeV

rescue from plasmids encoding the genomic RNA opposed to antigenomic RNA was low, this result in accordance with the theory that hybridisation of positive and negative RNA transcripts interferes with viral production. The expression of genes inserted into the MeV genome was determined not to be detrimental, as the expression of the firefly luciferase reporter gene did not reduce MeV infectivity (Radecke *et al.* 1995). Although the efficiency of viral rescue using the MeV reverse system displayed a strong inverse correlation to the size of the replicon engineered, the recovery of a full-length infectious virus with a long genome is possible. The EBOV genome was engineered to express the enhanced green fluorescent protein (EGFP) gene between the viral NP and VP35 genes, generating a replicon of 19,773 nucleotides, and was successfully recovered (Towner *et al.* 2005). The GFP protein expressed from the recombinant EBOV genome was used to visualise virus infection, a technique also used for the visualisation of CDV infection in ferrets (von Messling *et al.* 2004a).

In general, reverse genetics of paramyxoviruses implies the ability to recover infectious virus from a cDNA copy of the NNS genome. Establishment of a reverse genetics system involves the construction of a plasmid containing the viral antigenome, and support plasmids containing the N, P and L genes. Transfection of cells with protein expression plasmids occurs in conjunction with transfection of the plasmid containing the full-length viral genome, all plasmids being under the control of a T7 promotor (Neumann *et al.* 2002). Supply of T7 polymerase enables synthesis of viral RNA and the virus replication cycle is initiated. Generation of infectious viral particles from cloned cDNA is schematically presented in Figure 1.11. Recently, the recovery of infectious MeV from a reverse genetics system was achieved through transfection of the MeV genome in two or three segments (Takeda *et al.* 2006). Up to six foreign genes were successfully incorporated into the MeV infectious clone containing three segments, and five of the foreign proteins were expressed by the recombinant MeV (Takeda *et al.* 2006).

The T7 bacteriophage polymerase initiates transcription at a specific base, possessing stringent specificity for the T7 promotor sequence, hence is highly selective. The natural T7 transcription terminator sequence functions efficiently in artificial plasmids. The T7 promotor and terminator sequences are easily engineered into expression vectors, and all these vectors require to enable expression of foreign genes is the T7 RNA polymerase. *Vaccinia virus* (VACV) has been used to supply the T7 RNA polymerase to cells transfected with plasmids under the control of T7 promotors.

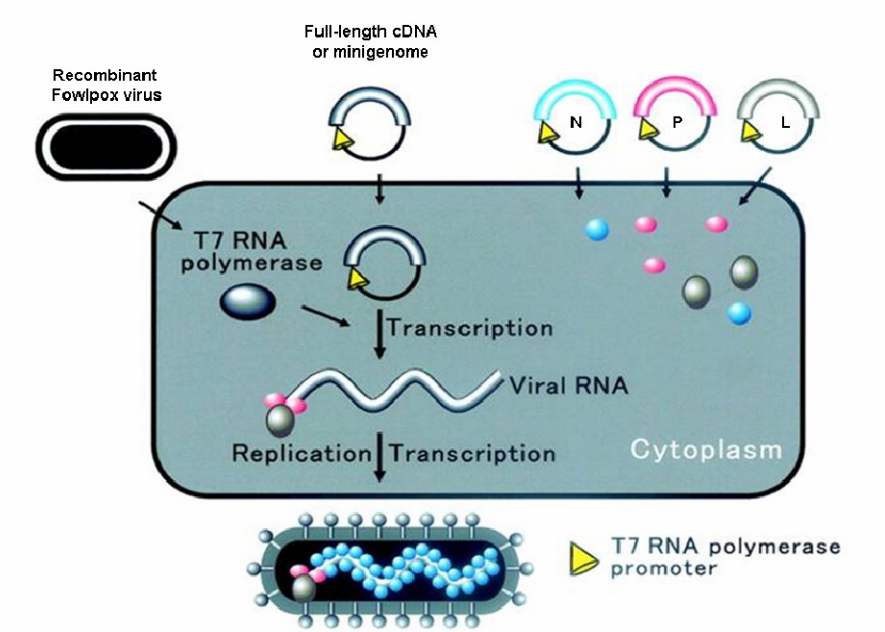


Figure 1.11- Generation of Infectious Paramyxoviruses by Reverse Genetics

Cells are transfected with a plasmid containing the full-length viral genome and support plasmids encoding the N, P and L polymerase genes. Plasmids contain a T7 promotor and the T7 polymerase is supplied through infection with a recombinant virus, such as *Vaccinia virus* or *Fowlpox virus*. The T7 polymerase enables synthesis of viral RNA. The viral genome is transcribed and polymerase proteins are translated. Replication and transcription of the newly synthesised viral RNA is then initiated and infectious virions are produced. This figure was adapted from Neumann *et al.* (2002).

DNA encoding the bacteriophage T7 RNA polymerase gene was ligated to the VACV transcriptional promotor and integrated into the VACV genome (Fuerst *et al.* 1986). The recombinant VACV retained infectivity and stably expressed T7 RNA polymerase in mammalian cells. As T7 localises in the cytoplasm, transfected plasmids do not have to enter the nucleus for transcription, and mRNA produced does not have to be transported back to the cytoplasm for translation (Fuerst *et al.* 1986).

VACV, although highly effective in delivering T7 RNA polymerase to susceptible cells, is highly cytotoxic. Modifications to VACV were implemented to reduce the cytotoxic effect on cells, such as creating modified Vaccinia Ankara (VACV-ANK), a highly attenuated VACV with a restricted host range (Sutter *et al.* 1995). The T7 RNA polymerase gene was also integrated into the *Fowlpox virus* (FWPV) genome, under the control of the VACV early/late promotor (Britton *et al.* 1996). This virus, fpEFLT7pol, is able to stably express T7 RNA polymerase in avian and mammalian cells, but as FWPV undergoes abortive replication in mammalian cells, no infectious virus is produced in these cells. FWPV has a longer replication cycle than VACV, allowing increased time for synthesis of foreign gene products and CPE is only observed at high multiplicity of infection (m.o.i.) of virus (Britton *et al.* 1996). The effects of FWPV-T7 and VACV-ANK-T7 on cells were compared, and FWPV-T7 was found to be non-cytopathic, in contrast to VACV-ANK-T7, which was highly cytopathic, multiplied rapidly and produced CPE in 3-5 days, even when used at a low m.o.i. (Das *et al.* 2000).

An alternate method to viral infection for providing T7 RNA polymerase for expression of genes under the control of T7 promotors is the utilisation a stably transfected cell line. A BHK cell line stably expressing T7 RNA polymerase, BHK/T7-9, was created to eliminate detrimental effects of viral infection on cells used in reverse genetics experiments (Ito *et al.* 2003). Homologous recombination between the transfected full-length genome and support plasmids mediated by DNA polymerase of VACV frequently occurs, but this adverse feature of VACV infection is eliminated in the BHK system.

1.9.2 Minigenome versus full-length genome

A minireplicon system may be created as the first step towards the engineering of a full-length infectious clone by replacing the entire coding region of a viral genome with a reporter gene. Optimisation of conditions for rescue of infectious virus from cloned cDNA is achieved by first rescuing a reporter gene. Rescue of a synthetic genome was first achieved with a SeV construct (Park *et al.* 1991).

Minireplicon systems allow the function of *cis* and *trans*-acting sequences at the terminal ends of the viral genome to be determined, and also the functionality of the support plasmids can be tested. Through analysis of components of viral polymerase in a minigenome, the N protein, previously hypothesised to alter the dynamic balance between replication and transcription, was found to have limited effect on viral RNA synthesis (Fearns *et al.* 1997). The function of accessory proteins, such as V or C, may be analysed. The V protein of SeV was determined completely dispensable for viral replication in a model where the protein was knocked out (Nagai 1999), and, in a minireplicon system, the MeV (Parks *et al.* 2006) and SV-5 (Lin *et al.* 2005) V proteins were found to repress genome replication. Both V and C proteins have been implicated in CDV pathogenesis (von Messling *et al.* 2006). A definite advantage of a minireplicon system over a reverse genetics system is that no infectious viral particles are generated, allowing functional studies of viruses classified as biosafety level (BSL)-4 to be undertaken without biocontainment restrictions (Halpin *et al.* 2000).

The development of a reverse genetics system containing a full-length infectious clone has obvious advantages over that of a system containing a minigenome. Attributes uncovered through the study of individual viral genes and proteins may not necessarily correspond to those observed in the natural viral life cycle. Functions of viral proteins required early in the natural life cycle are bypassed in a minireplicon system, as transfected plasmids are not under the control of viral specific promotors and polymerases (Nagai 1999). Viral models where genes are knocked out, or substituted with alternate genes, are able to be created with the use of reverse genetics. The generation of attenuated viruses for use as vaccines or vectors is also possible, as is ascertaining the role genes play in viral virulence (Ito *et al.* 2003).

1.10 Research Significance and Objectives

1.10.1 Aims of this study

1- To characterise JPV and BeiPV using reverse genetics

Pursuing characterisation of JPV and BeiPV in relation to viral pathogenesis or functions of accessory proteins may only be possible by observing the authentic viral life cycle. In contrast, determination of evolutionary relatedness of JPV, BeiPV and NiV was possible through the establishment of a minireplicon system. Expression of the GFP reporter gene in the minigenome was reliant upon

co-expression of N, P and L genes, which were supplied in the form of support plasmids. Creation of minireplicon systems for JPV and BeiPV also functioned as the initial stage in establishment of complete reverse genetics systems for the viruses.

2- To explore the localisation and function of the novel SH and TM proteins of JPV

The two novel genes identified, SH and TM, in addition to the untranslated region located within the G gene, contributed to the exceptionally long genome of JPV. The function of these encoded gene products has yet to be determined. Cloning and transfection of the JPV SH and TM genes aimed to facilitate analysis of protein expression and localisation. Fusion of the two sequential ORFs of the G gene, via mutation and cloning, created an extended G gene encoding a significantly larger attachment protein. The functionality of the extended JPV G attachment protein was analysed using an electron microscopy technique.

3- To establish assays for the detection of JPV and BeiPV

A JPV specific real-time PCR assay was developed to assist in monitoring the tissue tropism of JPV in experimentally infected animals. The real-time PCR assay was also developed with the goal of monitoring infectious virus production from engineered clones in a reverse genetics system, and to be used as a potential diagnostic tool for future applications in disease investigation. The multiplex microsphere assay assessed the seroprevalence of JPV and BeiPV in sera samples taken from the Australian and southern Asian rodent population.

1.10.2 Significance

JPV was isolated from wild mice in Queensland, Australia, in 1972 and has been suggested to be a natural respiratory pathogen of mice. BeiPV was isolated from human mesangial cells, and subsequently detected in rat mesangial cells. The emergence of several other paramyxoviruses, especially those such as HeV and NiV, which are zoonotic and have caused fatalities in humans, provoked renewed interest in the characterisation of previously unclassified viruses. The potential of paramyxoviruses to cause lethal infection in a variety of host organisms should not be underestimated, nor should the possibility that uncharacterised viruses may possess zoonotic capability.

JPV and BeiPV have been classified within the family *Paramyxoviridae*. These viruses are unique in that both contain two additional genes, TM and SH,

along with an extensive untranslated region within the G gene. With a genome consisting of 19.2 kb, BeiPV is the largest known virus within the family *Paramyxoviridae*. Characterisation of JPV and BeiPV and their novel genes may facilitate an understanding of disease pathogenesis of the viruses and assist in ascertaining the evolutionary relationship between these viruses and other paramyxoviruses.

CHAPTER 2
Development of Reverse Genetics Systems for JPV and BeiPV

2.1 Introduction

The emergence of several paramyxoviruses, especially those such as HeV and NiV which are zoonotic and have caused fatalities in humans, have provoked renewed interest in the characterisation of paramyxoviruses previously unclassified or not associated with known diseases (Wang & Eaton 2001). JPV and BeiPV are two recently characterised paramyxoviruses of rodent origin. Complete sequencing of the JPV (Jack *et al.* 2005) and BeiPV (Li *et al.* 2006) genomes revealed a unique genome organisation never before observed in the *Paramyxovirinae*. Further characterisation of JPV and BeiPV may provide a better understanding of the viral evolution of the *Paramyxovirinae* and also aid in the classification of these viruses.

In this study reverse genetics systems were established for NiV, JPV and BeiPV. For each virus a minigenome was generated where all protein coding regions were replaced with a negative-sense copy of the EGFP gene. The viral leader, trailer, N gene 3' UTR and L gene 5' UTR all remained intact in the minigenome. The vRdRp complex is sufficient for transcription of a minigenome, and each of the N, P and L proteins that constitute the polymerase complex are absolutely required. The N, P and L genes of JPV and BeiPV were cloned and in conjunction with the minigenomes form the GFP minireplicon system. Minigenomes of JPV and BeiPV of differing lengths were constructed to determine if these viruses obey the 'rule of six' in an *in vitro* replication assay. To analyse the effect of the C protein on the JPV and BeiPV minireplicon systems, both JPV and BeiPV P genes were constructed with the start site of the C ORF silenced. The effect of the C protein on replication of the minigenome was determined when JPV and BeiPV minireplicon systems were supplemented, on separate plasmids, with the C protein in a dose dependant manner.

2.2 Materials and Methodology

2.2.1 Production of viral cDNA

Virus and cell culture

Vero cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Invitrogen, USA), supplemented with 10% (v/v) foetal calf serum (FCS) (Thermo Trace, Australia), 2 mM glutamine (Invitrogen), 10 mM HEPES (MP Biomedicals, USA) and penicillin (JRH Biosciences, USA) and streptomycin (Sigma, Australia) antibiotics. Media supplemented with 10% FCS, glutamine and HEPES will be referred to as EMEM-10 from herein.

JPV was supplied to the AAHL in RK13 cells by the Queensland Department of Primary Industries. BeiPV was isolated at AAHL (Li *et al.* 2006).

Antibodies and conjugates

The JPV rabbit antiserum, supplied by Gary Crameri, was obtained from rabbits immunised at AAHL. The monoclonal antibodies (MAbs) anti-RGS-His_6 (Qiagen, Germany) and anti-GST (Amersham Biosciences, Australia) were used for the detection of the RGS-His_6 and GST tags, respectively. The goat anti-mouse (Chemicon, USA) and goat anti-rabbit (Silenus, Australia) alkaline phosphatase (AP) conjugates were used for the detection of MAbs and rabbit sera, respectively.

Virus infection

Vero cell monolayers were propagated in 150 cm^2 flasks. Media was removed from flasks and 100 μL of JPV or BeiPV (1 x 10^6 $TCID_{50}$/mL) in 1 mL of PBS-A (Ca++/Mg++ free PBS) was used to inoculate cells. Cells were incubated at 37°C, with rocking, for 1 hr. After incubation, inoculum was replaced with 30 mL of pre-warmed EMEM-10. Cells were incubated at 37°C, until the formation of syncytia was evident. Upon observation of syncytia, virus culture supernatant was purified and the Vero cell monolayer was harvested by scraping cells into the PBS-A. Either purified virus or virus infected Vero cells were used for RNA extraction.

Virus purification

Virus culture supernatant was collected in a 50 mL falcon tube and centrifuged at 2500 rpm in an IEC Centra-7R centrifuge (210 rotor) for 10 min at 4°C to remove cell debris. A 20 mL syringe and 19G x 1 ½ " needle was used to fill a bell top tube (Beckman, USA) with virus culture supernatant. Tubes and space filler caps

were placed into a 55.2 Ti rotor (Beckman) and centrifuged at 50,000 rpm for 30 min at 10°C under vacuum, in a Beckman L8-80M ultracentrifuge. After centrifugation, supernatant was removed from the tube and the crude virus pellet was resuspended in 350 μL RLT buffer (QIAGEN, Germany).

RNA extraction

RNA was extracted from crude virus pellets (from above) using the QIAshredder column and RNeasy kit (both from QIAGEN), according to manufacturer's instructions. Viral RNA was extracted from virus infected Vero cells (from above) using the QIAamp Viral RNA Mini kit (QIAGEN), according to manufacturer's instructions.

Synthesis of cDNA

Viral cDNA was synthesised from viral RNA using the Omniscript Transcriptase kit (QIAGEN). Each 40 μL reaction contained 1 μL of JPV or BeiPV RNA, 4 μL of 10X buffer, 4 μL of 5 mM dNTPs, 4 μL of a 1:50 dilution of random hexamers (Invitrogen), 2 μL of 10 U/μL RNAsin RNase inhibitor (Promega, USA), 2 μL of Omniscript reverse transcriptase and H_20. The reverse transcription reactions were incubated at 37°C for 1 hr, then cDNA was stored at -20°C.

2.2.2 Construction of protein expression vectors

Construction of bacterial expression vectors

Three bacterial expression vectors were created for routine cloning and protein expression. The pHMN vector contained a His_6 tag, the pBAN vector a biotin tag and the pGAN vector a GST tag.

The JPV TM gene was amplified from JPV cDNA by PCR, using DJ01 (*Xho*I, *Mlu*I) and DJ02 (*Not*I, *Hind*III) primers. Restriction sites included in primers are displayed in parentheses and primer sequences are listed in Appendix 1. The pRSET-C vector (Invitrogen) and JPV-TM PCR product were digested with *Xho*I and *Hind*III, and ligated together. After cloning, the JPV-TM gene was removed from the pRSET-C vector by digestion with *Mlu*I and *Not*I, creating the pHMN vector.

A fragment of the JPV L gene was amplified from JPV cDNA by PCR using DJ39 (*Sac*I, *Asc*I) and DJ40 (*Not*I, *Eco*RI) primers. The pDW363 vector (Tsao *et al.* 1996) and JPV L fragment PCR were digested with *Sac*I and *Eco*RI, and ligated together. After cloning, the JPV L gene fragment was removed from the pDW363 vector by digestion with *Asc*I and *Not*I, creating the pBAN vector. Inserts containing

either *Asc*I/*Not*I or *Mlu*I/*Not*I restriction sites may be cloned into this vector, but once an insert containing *Mlu*I is cloned into *Asc*I restriction site, the restriction sequence is disrupted and the insert cannot be removed with either enzyme.

The JPV L gene fragment was also used for the modification of the pGD3 vector. The pGD3 vector (Wang *et al.* 1996) and JPV L fragment PCR were digested with *Sac*I and *Eco*RI and ligated together. After cloning, the JPV L gene fragment was removed from the pGD3 vector by digestion with *Asc*I and *Not*I, creating the pGAN vector. As with the pBAN vector, inserts containing either *Asc*I/*Not*I or *Mlu*I/*Not*I restriction sites may be cloned into pGAN.

Construction of mammalian expression vectors

The mammalian expression vector pCI-neo (Promega) was routinely used for transfection experiments. The pCI-neo vector contains both CMV and T7 promotors, and JPV inserts were ligated into *Mlu*I and *Not*I sites of this vector.

Minigenome studies utilised the pUC19 vector (Invitrogen). The *Nar*I restriction site was deleted from the pUC19 vector by digesting the vector with *Nar*I, blunting ends with Klenow polymerase (Promega) and re-ligating the modified vector. The pUC19dNar vector created was used for expression of GFP minigenomes.

The JPV TM gene was amplified from JPV cDNA by PCR, using DJ49 (*Spe*I, *Mlu*I) and DJ50 (*Not*I, *Xho*I) primers. The pTM1 vector (Halpin *et al.* 2004) and JPV-TM PCR were digested with *Spe*I and *Xho*I, and ligated together. After cloning, the JPV-TM gene was removed from the pTM1 vector by digestion with *Mlu*I and *Not*I, creating the pTM1 vector.

2.2.3 Expression of recombinant polymerase proteins in *E. coli*

Plasmids and bacterial strains

The three vectors, pHMN, pBAN and pGAN, were used for the expression of recombinant proteins. The *E. coli* strains MC1061 (F^- *araD139* Δ(*ara-leu*)*7696* Δ*lac174 galU* $^-$ *galK* $^-$ *hsr* $^-$ *hsm* $^+$ *strA*) and BL21(DE3) (Invitrogen) were routinely used for recombinant protein expression.

Preparation of electrocompetent *E. coli* cells

A single colony from freshly plated electrocompetent *E. coli* cells was used to inoculate 2 mL LB broth (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract (Difco Laboratories, USA), 1% (w/v) NaCl, pH 7.0). The bacterial culture was incubated for 5 hr, at 37°C, with shaking. After incubation, 1 mL of culture was used

to inoculate 20 mL of LB broth. The 20 mL culture was incubated overnight, at 37°C, with shaking.

After incubation, 12 mL of overnight culture was used to inoculate 1.2 L of pre-warmed LB broth (1:100 dilution). The culture was incubated at 37°C, with shaking, until the OD_{600} (UV-1201 Spectrophotometer, Shimadzu, Japan) reached 0.6-0.9. Once bacterial cells were in log phase of growth, cultures were placed on ice. The cultures were transferred to 250 mL pre-cooled centrifuge tubes and centrifuged at 4000 *g* for 15 min, at 4°C. Bacterial pellets were resuspended in 10 mL of sterile, ice-cold 10% glycerol and centrifuged at 4000 *g* for 15 min, at 4°C. The glycerol wash was repeated three times. After the final wash, bacterial pellets were resuspended in 2.5 mL of 10% glycerol and divided into Eppendorf tubes in 50 μL aliquots. Competent cells were snap frozen in liquid nitrogen and stored at -80°C.

Recombinant protein expression

Recombinant proteins were produced by transforming competent cells with expression vectors. For expression of proteins in the pHMN vector, BL21(DE3) competent cells were transformed. For expression of proteins in either the pBAN or pGAN vectors, MC1061 cells were transformed. Purified plasmid DNA was transformed into 50 μL of competent cells by electroporation at 1.8 kV using Gene Pulser (Bio-Rad, USA). After electroporation, cells were allowed to recover in 250 μL SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) for 1 hr, at 37°C, with shaking. Once recovery time was complete, transformed cells were spread onto LB agar plates containing 100 μg/mL ampicillin, and incubated at 37°C, overnight.

A single colony from the transformation plate was used to inoculate 2 mL of LB broth containing 100 μg/mL ampicillin. LB broth was additionally supplemented with 50 μM D-Biotin for cultures containing pBAN plasmids. Cultures were incubated at 37°C, overnight, with shaking.

Following overnight incubation, 1 mL of culture was used to inoculate 49 mL LB broth (1:50 dilution), supplemented with ampicillin and D-Biotin as above. Culture was incubated at 37°C, with shaking, until the OD_{600} reached 0.6-0.9. To induce expression, 500 μL of 100 mM isopropyl β-D-thiogalactoside (IPTG) was added to the culture. The cultures were incubated at 37°C, with shaking, for a further 4 hr and then harvested.

2.2.4 Expression of recombinant polymerase proteins in mammalian cells

Transfection of JPV polymerase proteins in Vero cells

Genes were cloned into the pCIneo vector for protein expression in mammalian cells. Vero cells were seeded into 6 well plates, at 1 X 10^6 cells per well in 3 mL EMEM-10 and incubated overnight at 37°C with 5% CO_2. For each transfection, Lipofectamine 2000 (Invitrogen) was added to the plasmid DNA in a 3:1 ratio (3 μL Lipofectamine: 1 μg DNA), then serum free EMEM was added to bring the final sample volume to 500 μL. Routinely, 5 μg of DNA was used for each transfection. After incubation for 30 min at room temperature, a further 500 μL of serum free EMEM was added to the sample. This transfection mix was added to the Vero cells and incubated at 37°C with 5% CO_2 for 6 hr, after which the transfection mix was removed and replaced with EMEM-10.

2.2.5 Western blot analysis of recombinant polymerase proteins

Preparation of bacterial cell lysates for SDS-PAGE

Upon completion of induction (see section 2.2.3), bacterial cultures were centrifuged at 5000 rpm in JA 25.5 rotor (Beckman) for 10 min at 4°C. The bacterial pellet was resuspended in 2 mL of Native Binding Buffer (NBB) (20mM NaPO4, 500 mM NaCl, pH7.8). The sample was kept on ice and sonicated three times, using the Microson Ultrasonic Cell Disruptor (Misonix, USA). After sonication, the cell lysate was centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and pellet was resuspended in 1 mL of NBB. Cell lysates were stored at -20°C.

Preparation of mammalian cell lysates for SDS-PAGE

To prepare mammalian cell lysates, media was removed from cells and replaced with 1 mL of PBS-A. Cells were scraped from plates with a cell scraper and collected with PBS-A in eppendorf tubes. Cells were centrifuged at 2000 rpm for 2 min and PBS-A was removed. The cell pellet was resuspended in 100 μL of RIPA buffer (1 mM PMSF, 1% sodium deoxycholate, 50 mM Tris-HCl, pH 7.4, 1% Triton-X, 0.1% SDS, 150 mM NaCl and H_2O). Cells resuspended in RIPA buffer were lysed on ice for 20 min and clarified by centrifugation at 13,000 rpm for 10 min at 4°C. After centrifugation, supernatant was collected into a new eppendorf tube and either analysed be western blot immediately or stored at -20°C for later analysis.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS gels were cast in Mini-Protean™ III dual slab cell apparatus (Bio-Rad). Gels for standard protein analysis were 75 mm in thickness and preparative gels for protein purification were 1.5 cm in thickness. Polyacrylamide separating gels contained 4.7 mL, 4 mL, 3.3 mL, 2.4 mL, or 1.4 mL of 30% (w/v) acrylamide/bis-acrylamide solution (Bio-Rad) in 2.7 mL, 3.3 mL, 4 mL, 5 mL or 6 mL MilliQ® H_20 (Millipore, USA) for 8%, 10%, 12%, 15% or 18% gels, respectively. Added to the polyacrylamide solution was 2.5 mL of 1.5 M Tris-HCl pH 8.8, 100 μL of 10% (w/v) SDS, 50 μL of 10% (w/v) ammonium persulfate (Bio-Rad) and 5 μL of N, N, N', N' tetramethylethylenediamine (Bio-Rad). Polyacrylamide stacking gel containing 5% acrylamide/bis-acrylamide was prepared similarly using 1.2 mL of 0.5 M Tris-HCl pH 6.8.

Protein samples were analysed under reducing conditions. A 6X solution of reducing SDS-PAGE loading buffer (0.04% (w/v) bromophenol blue, 6% (w/v) SDS, 190 mM Tris-HCl pH 6.5, 45% (v/v) glycerol, 15% (v/v) 2-β mercaptoethanol) was added to protein lysates, and lysates were boiled at 100°C, for 5 min prior to loading on the gel. Once lysates were loaded on the gel, proteins were separated by electrophoresis, at 200 V, in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3) for 30-60 min using a Power Pac 3000 (Bio-Rad).

Following electrophoresis, SDS gels were rinsed in H_20 for 5 min then either stained or further analysed by Western blot. Gels were stained in BioSafe™ Coomassie Blue G250 Stain (Bio-Rad). Stained gels were preserved by soaking in gel drying solution (30% (V/V) methanol, 5% (v/v) glycerol) for 30 min, then sealing between sheets of gel drying film (Promega).

Western blot

Proteins separated by SDS-PAGE were transferred from gels onto Immobilon-P transfer membrane (0.45 μM pore size) (Millipore, USA). Proteins were transferred from gels to membranes in transfer buffer (1.44% (w/v) glycine, 0.3% (w/v) Tris, 20% (v/v) methanol) using a Mini-Protean II Trans-blot® cell (Bio-Rad) and a Power Pac 3000 (Bio-Rad) set on 250 mA, for 1 hr. Upon completion of the transfer, the Western blots were incubated in a blocking solution of 5% (w/v) skim milk in Tris-buffered saline (10 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 0.05% (v/v) Tween 20 (TBS-T) at room temperature for 1 hr or at 4°C, overnight.

After the blocking incubation, the membranes were incubated for 1 hr at room temperature with the primary antibody, diluted in TBS-T. Prior to addition of the secondary antibody, the membranes were washed three times with TBS-T for 5

min. The secondary antibody, containing an AP conjugate, was also diluted in TBS-T and incubated on the blots for 1 hr at room temperature. After antibody incubations, the blots were washed three times with TBS-T for 5 min and once with H_2O for 5 min. When monitoring the production of antibodies, lysates containing insoluble bacterial proteins were loaded onto preparative 1.5 cm thick SDS gels and separated by electrophoresis. Proteins were transferred to membranes (as above) and the blots were cut into strips of 5 mm width. Membrane strips were individually incubated with different antibodies.

An alkaline AP solution containing 33 μL 5-bromo-4-chloro-3-indolyl-phosphate (Promega) and 66 μL nitro blue tetrazolium (Promega) in 10 mL AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was prepared. The blots were incubated in development solution until the desired colour intensity was reached, and then were rinsed in H_2O to stop the colour development reaction from proceeding.

Purification of insoluble proteins

Lysates containing insoluble bacterial proteins were loaded onto preparative 1.5 cm thick SDS gels and separated by electrophoresis, as described above. Upon separation, gels were rinsed in H_20 and stained with ice-cold 0.3 M KCl. Protein bands were excised from the gels with a scalpel, diced into small pieces and placed into an eppendorf tube. To solubilise the protein, 600 μL of elution buffer (50 mM NH_4HCO_3, 0.1% SDS) was added to the gel pieces. The gel pieces and buffer solution were incubated overnight at room temperature on a rotating wheel. Protein elutions were removed and stored at -20°C and a further 600 μL of elution buffer was added to the remaining gel pieces. The gel pieces were incubated once again at room temperature overnight on rotating wheel and protein elutions were removed and stored at -20°C. Quantitation of the level of protein in elutions was determined by SDS-PAGE where samples were loaded in conjunction with a molecular mass marker.

2.2.6 Preparation of monospecific antiserum against the JPV L protein

PCR amplification of a predicted antigenic region of the JPV L gene

Primers were designed to amplify predicted antigenic regions of the JPV L gene. Protein antigenicity was predicted by analysing the Kyte and Doolittle hydrophilicity profile (Sci Ed Central Clone Manager 7, version 7.11, USA) (Kyte & Doolittle 1982) of the L protein and selecting a hydrophilic region for amplification

(Figure 2.1A). Two antigenic fragments, JPV L1 (Figure 2.1B) and JPV L2 (Figure 2.1C) were predicted. The L1 gene fragment was amplified by PCR using DJ30 (*Mlu*I) and DJ31 (*Not*I) primers and the L2 gene fragment was amplified by PCR using DJ31 (*Mlu*I) and DJ32 (*Not*I) primers. Both PCR reactions utilised the pHMN-JPV-L clone as the template.

Recombinant protein expression and purification

The predicted antigenic JPV L gene fragments were cloned into the *Mlu*I and *Not*I sites of the pHMN, pBAN and pGAN expression vectors. The L protein was expressed from the pHMN vector and purified. The protein concentration was determined by SDS-PAGE analysis.

Immunisation

Two female New Zealand White rabbits (ID numbers 517 and 518), age 4 months (Institute of Medical and Veterinary Science, Australia) were used for antibody production. Animals were housed in the small animal facility of the secure area of AAHL and procedures were assessed and approved by the AAHL Animal Ethics committee.

Prior to immunisation, blood samples were taken from each rabbit via an ear bleed. Xylocaine ointment (AstraZeneca, Australia) was applied to the shaven, 70% ethanol cleaned ear of the rabbit. Vaseline (Lever Rexona, Australia) was applied to the ear to prevent dispersal of blood, and Oil of Wintergreen (Sunspirit Oils, Australia) was applied to promote vasodilation of blood vessels. The lateral ear vein was nicked with a scalpel and blood droplets were collected in 9.5 mL Vacutainer SST tubes (Becton Dickinson, USA). Upon completion of the ear bleed, pressure was applied to the ear to allow clotting. Vacutainer tubes were gently inverted and blood was allowed to clot for 1 hr at room temperature, followed by a further 3 hr at 4°C. Tubes were then centrifuged at 1200 rpm in IEC Centra-7R centrifuge (210 rotor) for 10 min at 4°C, to separate the serum from the blood clot. Serum was collected and stored in 1 mL aliquots, at -80°C.

For immunisations, 100 μg of JPV L protein in 500 μL of PBS and 500 μL of Montanide ISA 50V (Seppic, France) adjuvant was prepared. Rabbits were immunised with 500 μL of protein/adjuvant solution by intramuscular injection with a 23 G X 1" needle, in each cranial thigh. Rabbits were boosted with an additional 100 μg of the JPV L protein six weeks after the initial immunisation. This boost was performed by Eric Hansson and Gary Crameri.

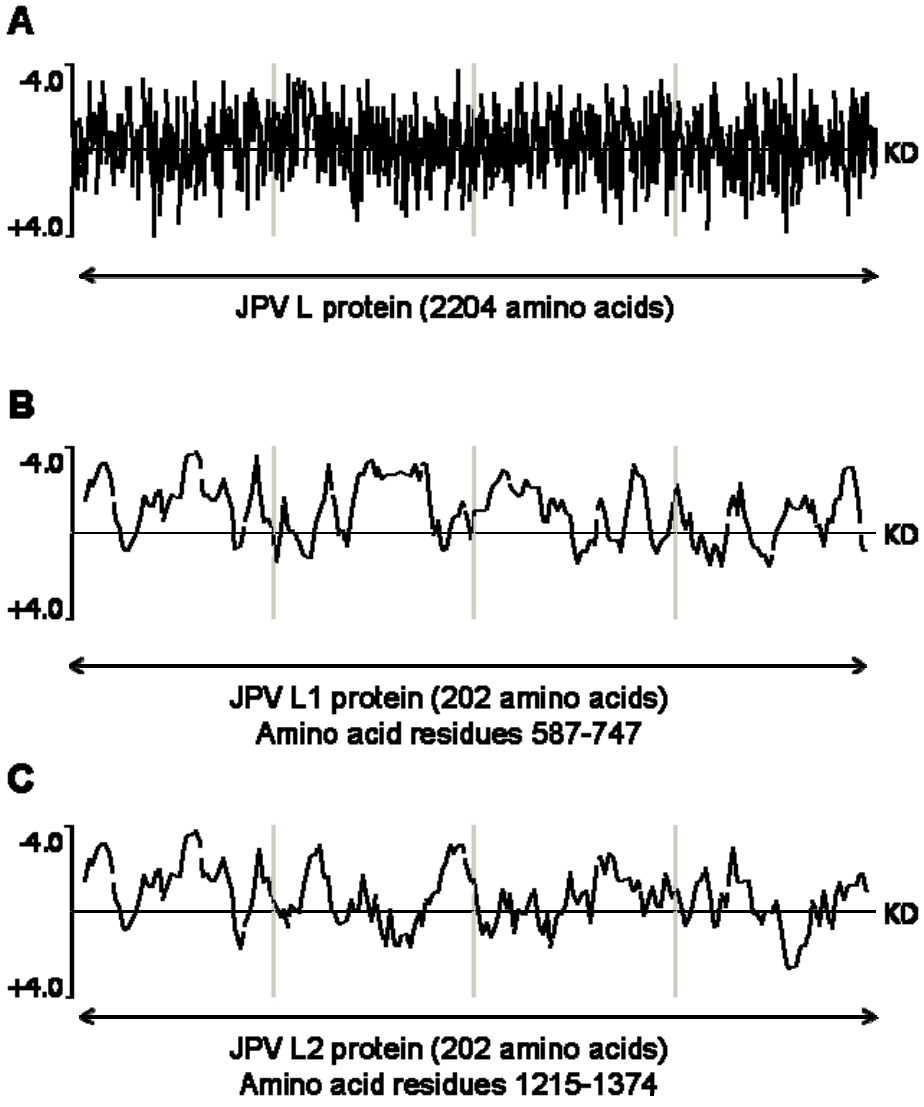


Figure 2.1- Kyte & Doolittle Analysis of the JPV L Protein

Kyte & Doolittle values fall within a range of +4 to -4, with hydrophilic residues having a negative score. Kyte and Doolittle hydrophilicity plots are shown for the full-length JPV L protein (A), the JPV L1 fragment encompassing amino acid residues 587-747 of the JPV L protein (B) and the JPV L2 fragment encompassing amino acid residues 1215-1374 of the JPV L protein (C).

Blood samples were collected from rabbits on days 21, 29 and 48, via ear bleeding as described above. Final blood samples were collected on day 51 via cardiac puncture. Rabbits were anesthetised with 500 μL Ketamine (Ilium Veterinary Ethical, Australia) and 500 μL Xylazil-20 (Ilium Veterinary Ethical, Australia) via intramuscular injection in the cranial thigh with a 23 G X 1" needle. Once anesthetised, rabbits were terminally bled by cardiac puncture with 16 G X 1 ½ " needle. The terminal bleeds were performed by Gary Crameri. Blood was transferred to Vacutainer tubes, and serum samples were collected, as described above.

2.2.7 Construction of a NiV minigenome with a GFP reporter gene

Design of NiV minigenome

The NiV minigenome pNiV-CAT, a gift from P. Rota (Halpin *et al.* 2004), was used as a template for the construction of the pNiV-GFP minigenome. The NiV GFP minigenome contained the same features as the JPV GFP minigenome (See Chapter 2, Section 2.2.8). The NiV N, P and L support plasmids were a generous gift from P. Rota (Halpin *et al.* 2004).

PCR amplification of the NiV genome termini

A DNA fragment containing the NiV L 5' UTR, NiV trailer region and T7 promotor sequence was amplified from the pNiV-CAT minigenome with the DM04 (*Not*I, *Bam*HI) and RSP (*Hind*III) primers. A second DNA fragment containing the NiV leader region, NiV N 3' UTR, hepatitis delta virus ribozyme sequence and two T7 terminator sequences was amplified from the pNiV-CAT minigenome with the USP (*Sac*I) and DM05 (*Nco*I, *Bam*HI) primers.

Construction of NiV GFP minigenome expression plasmid

The DNA fragment containing the NiV L 5' UTR, NiV trailer region and T7 promotor sequence, representing the 5' termini of the NiV minigenome, was cloned into the *Bam*HI and *Hind*III sites of a pUC19d*Nar* expression vector, creating the pNiV5' plasmid. The DNA fragment containing the NiV leader region, NiV N 3' UTR, hepatitis delta virus (HDV) ribozyme sequence and two T7 terminator sequences, representing the 3' termini of the NiV minigenome, was digested with *Eco*RI and cloned into the *Eco*RI and *Sma*I sites of the pNiV5' plasmid, creating the pNiV3'5' plasmid. The pCI-EGFP plasmid was a gift from Luke Lambeth (Lambeth *et al.* 2006) and contained the GFP reporter gene. The GFP reporter gene was removed

from the pCI-EGFP plasmid by digestion with *Nco*I and *Not*I, and was then ligated into the *Nco*I and *Not*I sites of the pNiV3'5' plasmid, generating the pNIV-GFP minigenome. The pNIV-GFP minigenome differed from the pNiV-CAT minigenome in that the A residue at nucleotide 92 of the NiV genome was substituted with a G residue, due to a PCR induced mutation.

2.2.8 Establishment of a JPV minireplicon system

Design of the GFP minigenome

The 5' end of the GFP gene was flanked by the N gene 3' UTR, gene start signal, viral leader, *Hepatitis delta virus* (HDV) cleavage sequence and two T7 terminator sequences. The 3' end of the GFP gene was flanked by the L gene 5' UTR, gene stop sequence, viral trailer and T7 promotor (Figure 2.2).

Construction of the JPV GFP minigenome expression plasmid

The pNiV-GFP minigenome served as a template for the construction of the JPV minigenome. The JPV leader region and N 3' UTR were amplified from JPV cDNA using the DM08 and DM09 (*Nco*I) primers. This PCR product was used as a template in a subsequent PCR reaction, with the DM03 (*Nar*I) and DM09 (*Nco*I) primers. The DM03 primer extended the genome specific PCR into the HDV region and added a *Nar*I site to the product. The PCR product containing the 3' terminus of the JPV minigenome was cloned into the *Nar*I and *Nco*I sites of the pNiV-GFP minigenome, hence replacing the 3' NiV terminus with the 3' JPV terminus. A DNA fragment containing the JPV L 5' UTR and JPV trailer region was amplified from JPV cDNA with the DM13 (*Not*I) and DM12 (*Hind*III) primers. This PCR product, representing the 5' terminus of the JPV minigenome, was cloned into the *Not*I and *Hind*III sites of the pNiV-GFP plasmid, completing the replacement of the NiV termini with the JPV termini, and generating the pJPV-GFP minigenome. One T residue at position 18,843 of the JPV genome was omitted from the pJPV-GFP plasmid during cloning to ensure the minigenome length was exactly divisible by six.

A JPV minigenome that was not divisible by six was also generated. The JPV minigenome contained an *NcoI* restriction site within the N gene 3' UTR. The pJPV-GFP plasmid was linearised by digestion with *NcoI*, overhangs created during digestion were filled in with Klenow polymerase, and then the plasmid was ligated with T4 ligase. The resulting plasmid, pJPV-GFP(+4), was no longer divisible by six.

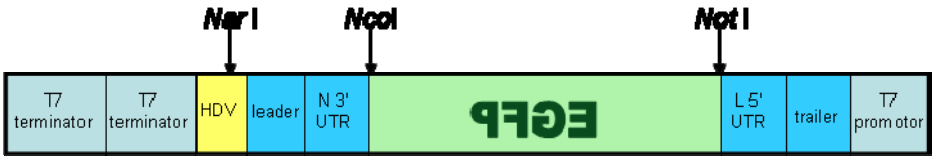


Figure 2.2- GFP Minigenome Construct

The schematic diagram of the GFP minigenome indicates the positions of the T7 terminator, hepatitis delta virus ribozyme (HDV) sequence, leader, N 3' untranslated region (UTR), EGFP, in reverse orientation, L 5' UTR, trailer and T7 promotor.

PCR amplification and cloning of JPV N, P, PdC, L and C ORFs

The JPV N ORF was amplified from JPV cDNA by PCR using DJ18 (*Mlu*I) and DJ08 (*Not*I) primers. The JPV P ORF was amplified from JPV cDNA using DJ19 (*Mlu*I) and DJ10 (*Not*I) primers. The JPV N and P ORFs were cloned into the pTM1 expression vector, creating pTM1-JPV-N and pTM1-JPV-P, respectively. The JPV C ORF was amplified by PCR from the JPV-P clone using DJ52 (*Mlu*I) and DJ53 (*Not*I) primers. The JPV C ORF was cloned into the pTM1 vector, creating pTM1-JPV-C.

The JPV C ORF was silenced in the JPV P gene, creating the JPV-PdC clone. A fragment of the P gene was amplified by PCR using DJ43 and PJ03 primers. The DJ43 primer contained a t1874c mutation to silence C protein ATG start codon. This PCR product was used as a template for another PCR reaction, using DJ52 (*Mlu*I) and PJ03 primers. The JPV-PdC PCR product and pTM1-JPV-P clone were both digested with *Mlu*I and *Nco*I, and the JPV-PdC fragment replaced the 5' region of the JPV-P gene, silencing the C gene, and creating the pTM1-JPV-PdC clone.

The JPV L ORF was amplified in five fragments; fragment 1 using DJ23 (*Mlu*I) and DJ41 (*Nsi*I, *Not*I) primers, fragment 2 using PJ60 (*Nsi*I) and DJ24 (*Sph*I, *Not*I) primers, fragment 3 using DJ25 (*Sph*I) and DJ26 (*Ppu*MI, *Not*I) primers, fragment 4 using DJ27 (*Ppu*MI) and DJ42 (*Age*I, *Not*I) primers, and fragment 5 using PJ38 (*Age*I) and DJ28 (*Not*I) primers. The construct pHMN-JPV-L was created by inserting fragments 1-5 in a sequential manner, using the unique 3' restriction site in combination with *Not*I as the 5' restriction site. The JPV L ORF was removed from the pHMN-JPV-L construct by digestion with *Mlu*I and *Not*I, and then cloned into the pTM1 vector, creating pTM1-JPV-L.

2.2.9 Establishment of a BeiPV minireplicon system

Construction of the BeiPV minigenome expression plasmid

The pJPV-GFP minigenome served as a template for the assembly of the BeiPV minigenome. The BeiPV leader region and N 3' UTR were amplified from BeiPV cDNA using the DM10 and DM11 (*Nco*I) primers. This fragment was used as a template in a subsequent PCR reaction, with the DM03 (*Nar*I) and DM11 (*Nco*I) primers. The PCR product containing the 3' terminus of the BeiPV minigenome was cloned into the *Nar*I and *Nco*I sites of the pJPV-GFP minigenome, thereby replacing the 3' JPV terminus with the 3' BeiPV terminus. A DNA fragment containing the BeiPV L 5' UTR and BeiPV trailer region was amplified from BeiPV cDNA using the

DM13 (*Not*I) and DM12 (*Hind*III) primers. This fragment, representing the 5' terminus of the BeiPV minigenome, was cloned into the *Not*I and *Hind*III sites of the pJPV-GFP plasmid, completing the replacement of the JPV termini with the BeiPV termini, and generating the pBeiPV-GFP minigenome. Two G residues at positions 19,101 and 19,102 of the BeiPV genome were omitted from the pBeiPV-GFP plasmid during cloning so that the minigenome was divisible by six. The plasmid pBeiPV-GFP(+4), which was no longer divisible by six, was constructed using the same method as for pJPV-GFP(+4).

PCR amplification and cloning of BeiPV N, P, PdC, L and C ORFs

The BeiPV N ORF was amplified with DM23 (*Mlu*I) and DM24 (*Not*I) primers, and the BeiPV P ORF was amplified with DM25 (*Mlu*I) and DM26 (*Not*I) primers. Both BeiPV N and P products were cloned into the pTM1 vector, using the *Mlu*I and *Not*I restriction sites. The BeiPV PdC gene, where a silent mutation in the P gene knocked out the ATG start codon, was amplified with DM16 (*Mlu*I) and DM17 (*Age*I) primers. The region between the *Mlu*I and *Age*I sites of the pTM1-BeiPV-P plasmid was replaced with the BeiPV-PdC fragment, creating the pTM1-BeiPV-PdC plasmid. The BeiPV C ORF was amplified by PCR from the BeiPV-P clone, using DM43 (*Mlu*I) and DM44 (*Not*I) primers. The BeiPV C ORF was cloned into the pTM1 vector, creating pTM1-BeiPV-C.

The BeiPV L ORF was cloned in multiple fragments. The 5' section of the BeiPV L gene was amplified from BeiPV cDNA using DM18 (*Mlu*I) and DM22 (*Pvu*II, *Not*I) primers, then cloned into the *Mlu*I and *Not*I sites of the pTM1 vector, creating the pTM1-BeiPV-L1 plasmid. The central region of the BeiPV L gene was amplified with B96 and B145 primers, and cloned into the *Sac*I and *Not*I sites of the pGAN vector, generating pGAN-BeiPV-L2. The 3' section of the BeiPV L gene was amplified by PCR using B35 and DM19 (*Not*I) primers and cloned into the *Sty*I and *Sac*I sites of the pGAN-BeiPV-L2 plasmid, generating pGAN-BeiPV-L2-L3. The BeiPV-L2-L3 fragment was cloned into the *Sal*I and *Not*I sites of the pTM1-BeiPV-L1 plasmid, generating pTM1-BeiPV-L1-L2-L3. To complete the cloning of the BeiPV L gene, the 900 bp region between the *Sal*I sites of the BeiPV L ORF, which the pTM1-BeiPV-L1-L2-L3 plasmid lacked, was amplified by PCR using B152 and B153 primers. The PCR product spanned the 1.4 kb region between the *Sac*II and *Nhe*I sites of the L gene (encompassing the *Sal*I to *Sal*I region) and was cloned into the *Sac*II and *Nhe*I sites of the pTM1-BeiPV-L1-L2-L3 plasmid, generating the pTM1-BeiPV-L clone.

Sequencing

All clones were confirmed by sequencing using the BigDye Terminator v1.0 kit and ABI PRISM 377 DNA sequencer (both from Applied Biosystems, USA) in accordance with manufacturer's recommendations.

2.2.10 Minigenome functional studies

FWPV-T7 infection of Vero cells

The fpEFLT7pol virus (FWPV-T7) that stably expressed T7 RNA polymerase was kindly provided by M. Skinner (Britton *et al.* 1996).

Vero cells were seeded into 96 well plates at 20,000 cells per well, in 200 μL EMEM-10 and incubated overnight at 37°C with 5% CO_2. After overnight incubation, media was removed and the cells were inoculated with 100 μL FWPV-T7 in 2% FCS, at an m.o.i. of 5. The cells were incubated at 37°C for 2 hr.

Transfection of Vero cells with minigenome and support plasmids

After infection with FWPV-T7, virus inoculum was removed from the cells and replaced with transfection mix. The optimum ratio of plasmid concentrations in the minireplicon assay were pre-determined to be a mixture of 0.5 μg minigenome plasmid, 0.3 μg N plasmid, 0.2 μg P plasmid and 0.1 μg L plasmid. For each minireplicon assay, Lipofectamine 2000 (Invitrogen) was added to the plasmid DNA in a 3:1 ratio (3 μL Lipofectamine: 1 μg DNA) and then serum free EMEM was added to bring the final sample volume to 50 μL. After incubation for 30 min at room temperature, a further 100 μL of serum free EMEM was then added to the sample. This transfection mix was added to the FWPV-T7 infected cells and allowed to incubate at 37°C with 5% CO_2 for 6 hr, after which time the transfection mix was removed and replaced with EMEM-10. Each minireplicon assay was performed in replicates of eight and each experiment was carried out at least three times. Transfected cells were incubated at 37°C with 5% CO_2 for 72 hr and then observed using the IX71 microscope (Olympus, USA).

Quantitation of GFP expression

Cells were observed with the IX71 microscope (Olympus) and images were captured by the high resolution DP70 camera (Olympus) using the analysis® (Soft Imaging System, Germany) program. Cells expressing GFP were quantified using the Universal TEM Imaging Platform iTEM (Soft Imaging System) program. Briefly, individual fluorescent cells were detected by threshold analysis and subsequently

counted to determine the number of positive cells in each well. To ensure repeatability between images, all procedures were performed as a macro function with fixed parameters. The same magnification was used for each experiment and eight replicate wells were analysed for each condition.

2.3 Results

2.3.1 Production of the JPV L monospecific antibody

Expression of recombinant JPV L protein

The JPV L1 and L2 gene fragments were cloned into the pHMN vector and the recombinant L protein was expressed in *E. coli*. Proteins from cell lysates were separated by SDS-PAGE and a protein with a molecular mass of approximately 23 kDa was detected. The recombinant JPV L2 protein was expressed at a high level in the insoluble fraction of the cell lysate (Figure 2.3A). The recombinant JPV L1 protein failed to be expressed in *E. coli*, and was subsequently omitted from further experiments (data not shown). The 23 kDa JPV L2 protein (which will be referred to as JPV L from herein) was excised, and eluted from the SDS-PAGE gel. After several elutions (Figure 2.3B), sufficient protein, as determined by comparison with a known standard, was purified for immunisation of rabbits.

Generation of antiserum

The development of specific antibodies against the JPV L protein was evaluated by Western blot. Sera collected from rabbits on day 21 was analysed against the $(His)_6$-tagged JPV L protein (the same protein used to immunise the rabbits) and a non-related viral protein, the $(His)_6$-tagged SARS Nc protein. The Western blot demonstrated that antibodies in the sera from both rabbits readily detected the JPV L protein, but not the SARS Nc protein (Figure 2.4). These results indicated that the rabbits had mounted an immune response against the JPV L protein. Blood was collected from rabbits on days 0, 21 29, 48 and 51.

To further demonstrate the specificity of the rabbit anti-sera, the samples were monitored against a GST-tagged JPV L protein. The Western blot provided evidence that antibodies in sera from both rabbits detected the JPV L protein (Figure 2.5). Sera from both rabbits, at days 21 through 51, contained monospecific antibodies against the recombinant JPV L protein.

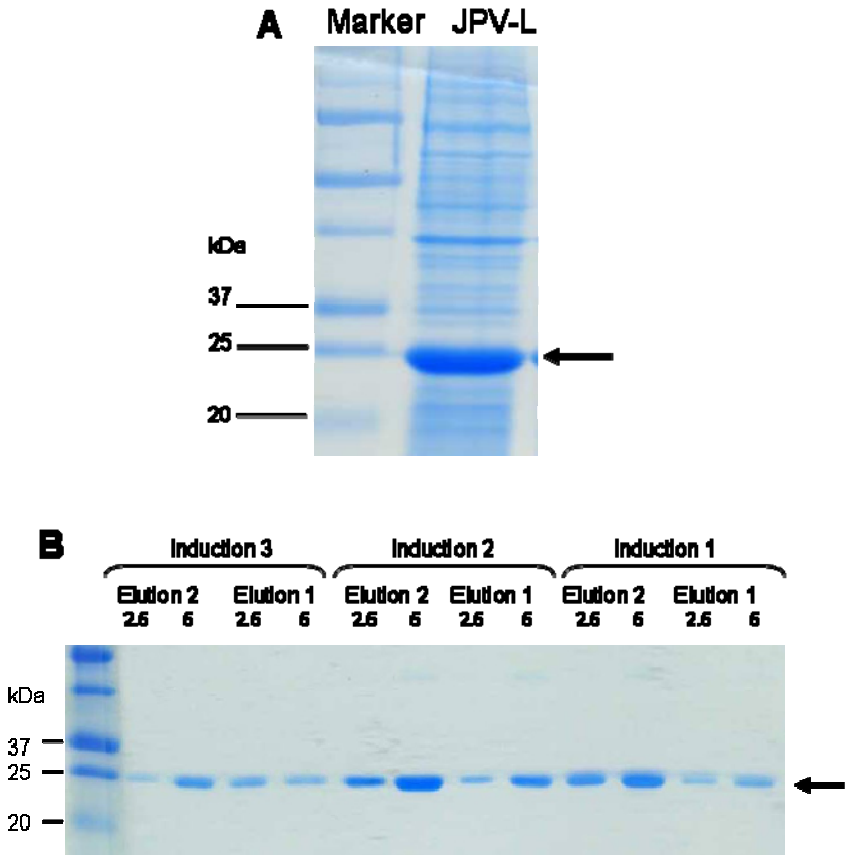


Figure 2.3- Expression and Purification of Recombinant JPV L Protein

The insoluble fraction of the BL21(DE3), pHMN-JVP-L recombinant bacterial cell lysate was separated by SDS-PAGE (A). The 23 kDa JVP L band was excised and eluted from the gel in several fractions in order to determine the purity of the protein (B). The numbering (2.5 and 5) indicates the amount of protein (μL) loaded onto the gel. The arrows indicate the position of the predicted JVP L protein. The Precision Plus marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

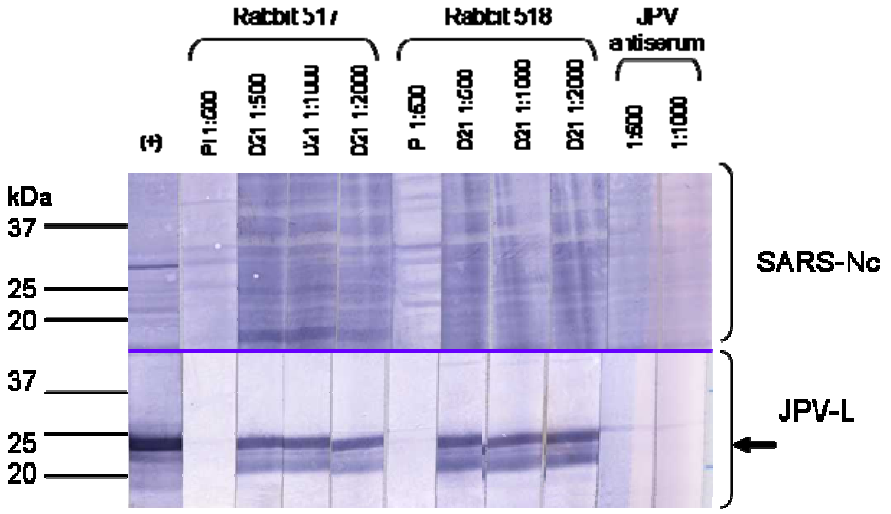


Figure 2.4- Evaluation of JPV L Antibody Production by Western Blot

Sera samples collected from rabbits on day 21 were monitored against the $(His)_6$-tagged JPV L protein, and also a non-related viral protein, the $(His)_6$-tagged SARS Nc protein on Western blot membrane strips. Dilutions of sera are indicated above each membrane strip. The anti-RGS-His_6 MAb (+) was used as a positive control and pre-immune (PI) sera were used as a negative control. The goat anti-mouse (Chemicon) and goat anti-rabbit (Silenus) alkaline phosphatase conjugates were used for the detection of MAbs and rabbit sera, respectively. The arrow indicates the position of the predicted JVP L protein. The Precision Plus marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

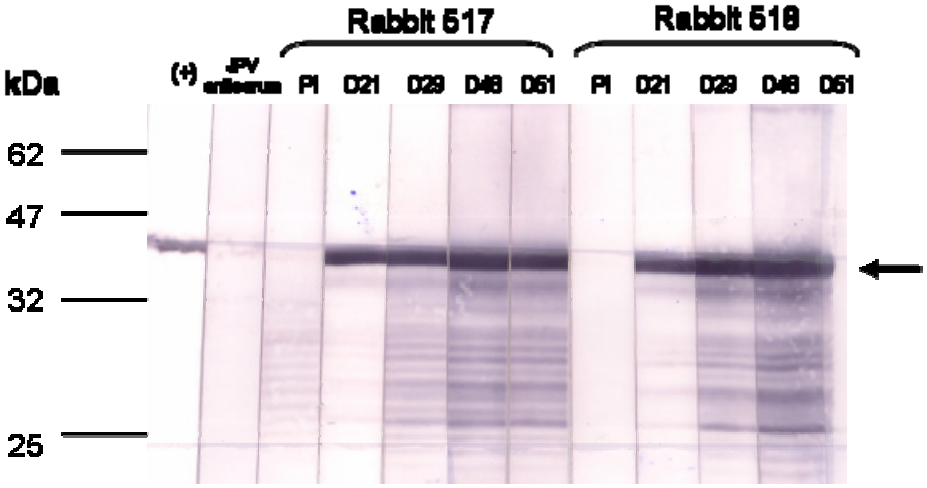


Figure 2.5- Evaluation of the Specificity of the JPV L Antiserum by Western Blot

A Western blot containing the GST-tagged, JPV L protein was cut into strips and incubated with antisera collected from rabbits 517 and 518 on days (D) 21, 29, 48 and 51 of the immunisation protocol. For each rabbit, pre-immune sera (PI) were included as a negative control and the anti-GST MAb as a positive control. One strip was incubated with rabbit JPV antiserum. The goat anti-mouse (Chemicon) and goat anti-rabbit (Silenus, Australia) alkaline phosphatase conjugates were used for the detection of MAbs and rabbit sera, respectively. The arrow indicates the position of the JPV GST-tagged protein. The Kaleidoscope marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

2.3.2 Expression of recombinant JPV polymerase proteins

JPV N, P and L polymerase genes were cloned into the pCIneo mammalian expression vector and transfected into Vero cells. Transfected cells were lysed and recombinant proteins were analysed by Western blot. To ensure that fowlpox virus expressing the T7 promotor did not interfere with the expression of JPV polymerase genes, JPV N transfected cells were also infected with FWPV-T7 and analysed.
The rabbit JPV antiserum detected the recombinant JPV N (Figure 2.6) and JPV P (Figure 2.7) proteins. The JPV L protein was detected with the monospecific JPV L antiserum (Figure 2.8), from above.

2.3.3 Construction of minireplicon systems

Construction of minigenomes

In each minigenome, the coding regions for all viral genes were replaced with a negative-sense copy of the GFP gene (Refer to Figure 2.2). The 5' end of the GFP gene was flanked by the N 3' UTR, gene start signal, viral leader, HDV cleavage sequence and two T7 terminator sequences. The 3' end of the GFP gene was flanked by the L 5' UTR, gene stop sequence, viral trailer and the T7 promoter. Transcription of the minigenome plasmids by the T7 polymerase generated RNA containing the GFP coding region, flanked by the viral genome terminal regions. The precise initiation site of the T7 promoter, and the self-cleavage of the RNA at the 3' end by the HDV ribozyme, ensured that the minigenome RNA produced had the identical genome termini as the complete viral genomic RNA.

Minigenome plasmids were constructed for NiV, JPV and BeiPV and designated pNiV-GFP, pJPV-GFP and pBeiPV-GFP, respectively. Sequence analysis showed that the pNiV-GFP contained a PCR induced mutation in NiV N UTR at position 92 of the NiV genome (a92g change), but this mutation seemed to have no effect on NiV minireplicon function (see Section 2.3.4). No mutations were generated in the construction of either JPV or BeiPV minigenomes.

Construction of support plasmids

The NiV support plasmids consisted of the NiV N, P and L genes, cloned individually into the pTM1 vector (pTM1-NiV-N, pTM1-NiV-P and pTM1-NiV-L, respectively) as previously described (Halpin *et al.* 2004). The NiV-P plasmid contained two mutations that eliminated both predicted translation start sites of the C ORF. A wild-type NiV-P construct was not generated.

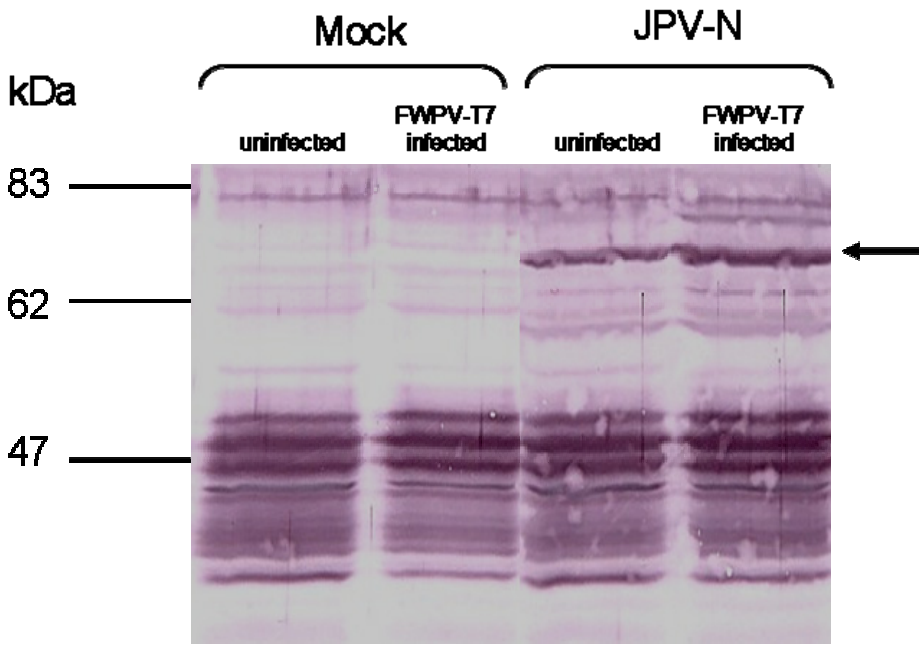


Figure 2.6- Analysis of JPV N Expression in Mammalian Cells by Western Blot

The Western blot was incubated with rabbit JPV antiserum and a goat anti-rabbit (Silenus) alkaline phosphatase conjugate was used for the detection of the rabbit serum. The arrow indicates the position of the predicted JPV N protein. The Kaleidoscope marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

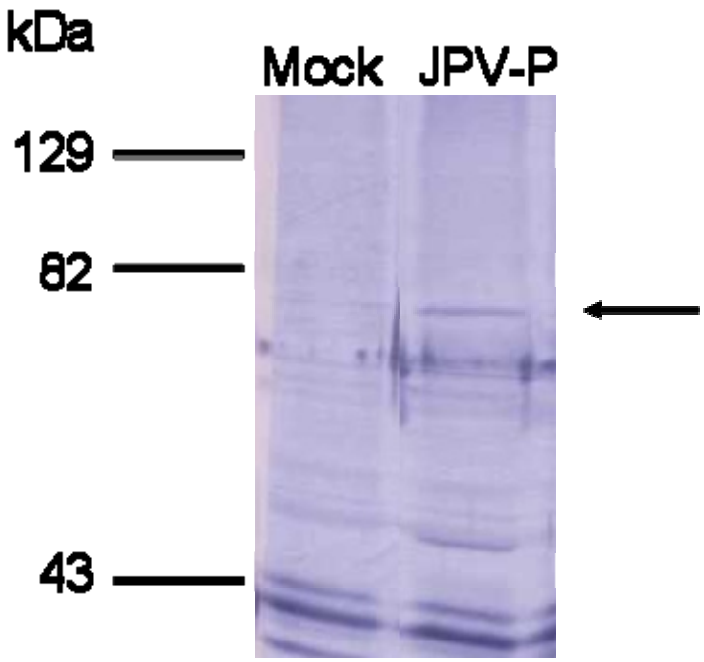


Figure 2.7- Analysis of JPV P Expression in Mammalian Cells by Western Blot

The Western blot was incubated with rabbit JPV antiserum and a goat anti-rabbit (Silenus) alkaline phosphatase conjugate was used for the detection of the rabbit serum. The arrow indicates the predicted position of the JPV P protein. The Kaleidoscope marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

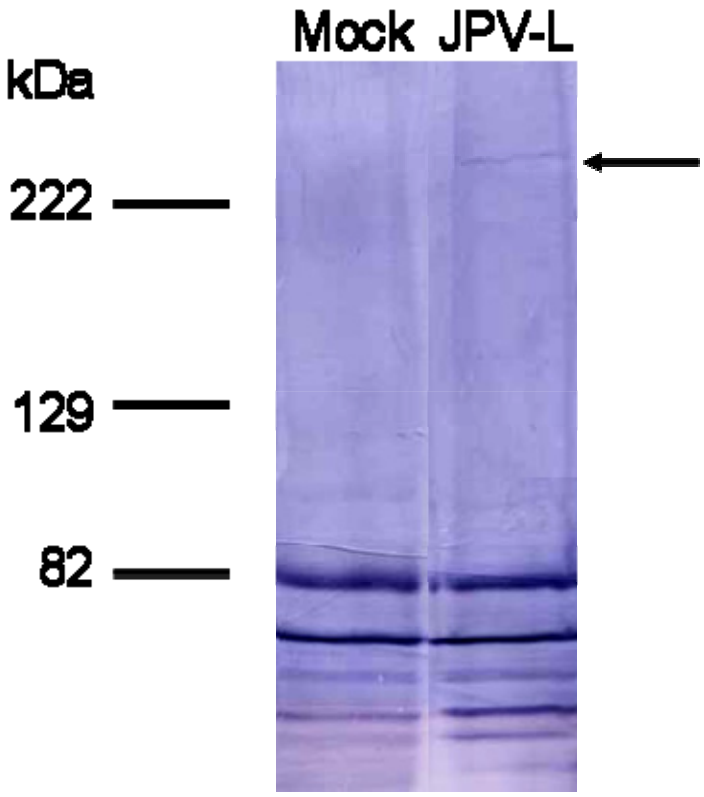


Figure 2.8- Analysis of JPV L Expression in Mammalian Cells by Western Blot

The Western blot was incubated with rabbit JPV antiserum and a goat anti-rabbit (Silenus) alkaline phosphatase conjugate was used for the detection of the rabbit serum. The arrow indicates the predicted position of the JPV L protein. The Kaleidoscope marker (Bio-Rad) was used to determine the MW (kDa) of proteins.

The N, P and L genes of JPV and BeiPV were amplified from cDNA and cloned into the pTM1 expression vector. The sequences of the cloned N, P and L genes of JPV and BeiPV were compared to the published genome sequences for each virus. The JPV N and P, PdC and C gene sequences were both identical to the wild-type sequences. The JPV L gene contained a silent mutation (c to t) at position 4275 of the L ORF.

Two mutations were present in the BeiPV N ORF. The BeiPV N gene contained a mutation that altered an amino acid in the N protein from an alanine to a proline (g424c), and a silent (c999t) mutation. The BeiPV P ORF also contained two mutations. One mutation altered an amino acid in the P protein from a threonine to a methionine (c404t), and the other mutation was silent (c1395t). As the BeiPV PdC gene was constructed from pTM1-BeiPV-P, the same two mutations that were present in the BeiPV P ORF were also present in the BeiPV PdC ORF. The BeiPV C gene contained a silent mutation (t234c) in the C ORF. There were four mutations generated in the cloning of the BeiPV L gene. Three of the mutations (a1422g, g1842a and t1491c) in the BeiPV L ORF were silent, and one (t5548a) substituted the amino acid threonine with a serine. Since the mutations in the BeiPV support plasmids were either silent or located in non-conserved regions of the BeiPV genes, these mutations were not corrected, given that all minireplicon systems seemed to function as expected (see Section 2.3.4).

2.3.4 Functional minireplicon assays

NiV, JPV and BeiPV minireplicon systems

GFP expression was observed 72 hr after transfection of pNiV-GFP into Vero cells that had been previously infected with FWPV-T7 and co-transfected with the NiV N, P and L support plasmids (Figure 2.9A). This result demonstrated that transfection of the pNiV-GFP minigenome, in conjunction with the NiV N, P and L genes produced a functional minireplicon system. Each of the NiV N, P and L plasmids was required for the formation of the vRdRp, and GFP was not synthesised when any of these plasmids were omitted from the NiV minireplicon system (Figure 2.9B). As with the homotypic combination of the pNiV-GFP minigenome and NiV support plasmids, GFP was synthesised in FWPV-T7 infected cells, 72 hr after transfection of the pJPV-GFP minigenome and the JPV N, P and L support plasmids (Figure 2.9C). The JPV minireplicon system was not functional when any of the JPV support plasmids were omitted from the transfection mix (Figure 2.9D).

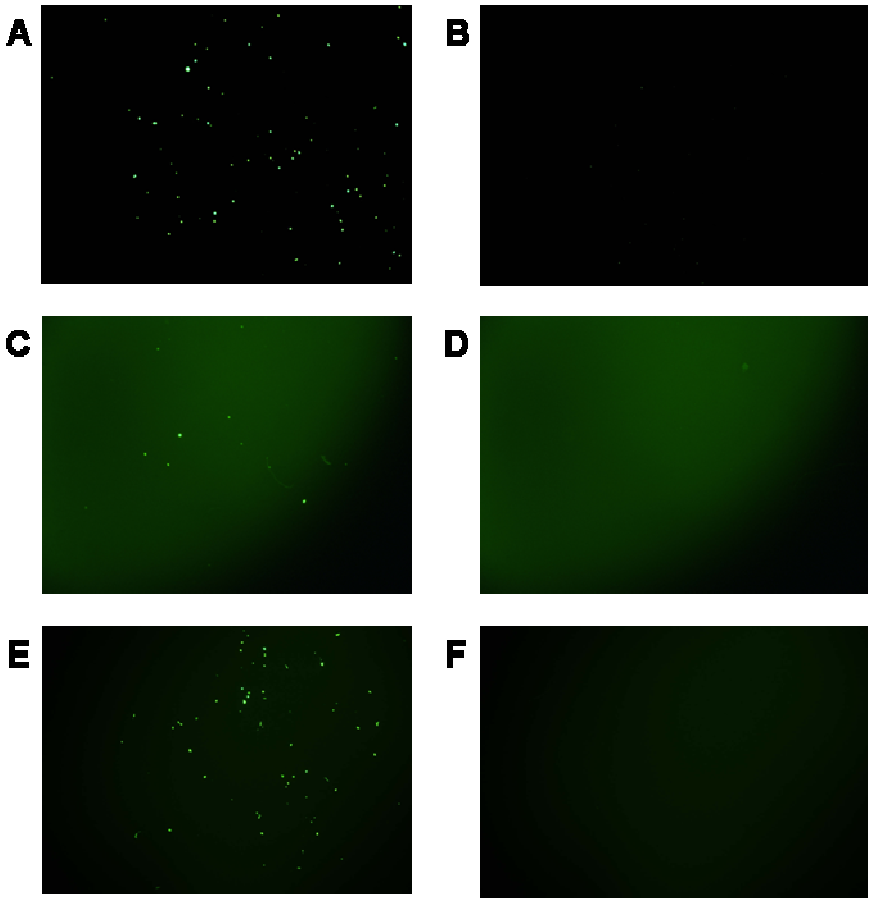


Figure 2.9- GFP Expression in NiV, JPV and BeiPV Minireplicon Systems

Vero cells in 96 well plates were used for transfection. The pNiV-GFP plasmid was transfected with the NiV N, P and L support plasmids (A), and with the NiV N and P support plasmids, without the L support plasmid (B). The pJPV-GFP plasmid was transfected with the JPV N, P and L support plasmids (C), with the JPV N and P support plasmids, without the L support plasmid (D). The pBeiPV-GFP plasmid was transfected with the BeiPV N, P and L support plasmids (E), with the BeiPV N and P support plasmids, without the L support plasmid (F). Photographs were taken at 72 hr post-transfection, at a magnification of 40X.

Although the JPV minireplicon system was functional, the level of GFP expression was lower than that of NiV minireplicon system. Similar results were obtained for the BeiPV minireplicon system (Figure 2.9E and F). These results indicated that the minireplicon systems for NiV, JPV and BeiPV were functional and when the minigenome plasmid was provided with homotypic support plasmids, synthesis of GFP occurred, although not at equivalent levels.

Examination of the 'rule of six' for JPV and BeiPV

JPV and BeiPV minigenomes of differing lengths were constructed. The minigenomes which contained an additional four nucleotides in the N UTR, (pJPV-GFP(+4) and pBeiPV-GFP(+4)) were transfected with homotypic support plasmids in order to determine if the minireplicon systems were functional when the minigenome was not evenly divisible by six. In these cases, GFP was not produced in either JPV or BeiPV minireplicon system (data not shown), demonstrating that both JPV and BeiPV minigenomes must be divisible by six, suggesting that these viruses obey the 'rule of six'.

Effect of the C protein on the replication of the JPV and BeiPV minireplicon

There is evidence that C protein expression from the P gene is inhibitory in minireplicon ststems. There is also evidence that the inhibiton is dose dependent (Malur *et al.* 2004). To determine the effect of the C protein on the JPV and BeiPV minireplicon systems, either the pJPV-GFP or pBeiPV-GFP plasmid was transfected with homotypic support plasmids, including the P gene or the P gene with the C ORF silenced (PdC). Transfection studies with the two different versions of the P genes showed that although GFP was expressed in both JPV and BeiPV minireplicon systems, the level of GFP expression was lower when the 'wild-type' P support plasmid was used. To further analyse the effect of the C protein on replication, the minireplicon systems were supplemented with the C gene in a dose dependant manner, with the C plasmid supplied at concentrations ranging from 0.02 ng to 2000 ng. Results demonstrated that as the level of C plasmid increased, the level of GFP expression significantly decreased. In both JPV and BeiPV minireplicon systems, the C protein had an inhibitory effect at plasmid levels of 100 ng and above. At plasmid levels of 0.02 ng to 100 ng, the level of GFP expression was variable. GFP expression, normalised to mean fluorescence for the minireplicon containing the PdC support plasmid, which was set at 100%, is given in Figure 2.10A for JPV and Figure 2.10B for BeiPV.

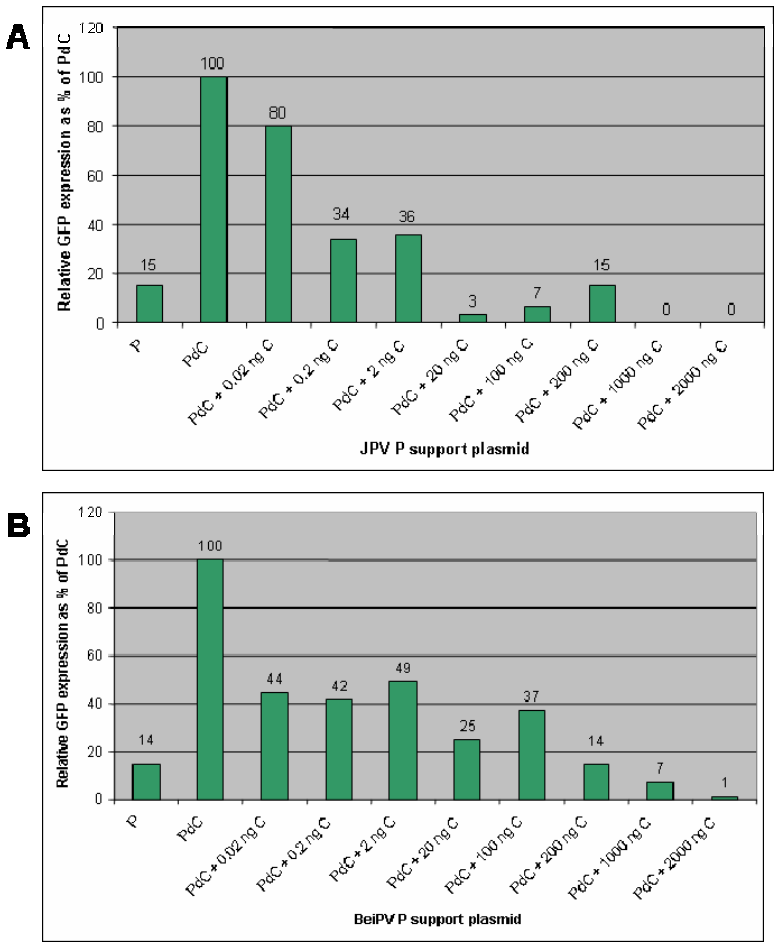


Figure 2.10- Effect of the C Protein on JPV and BeiPV Minireplicon Systems

The pJPV-GFP and pBeiPV-GFP plasmids were transfected with homotypic JPV and BeiPV support plasmids, including the P gene or the P gene with the C ORF silenced (PdC). The JPV and BeiPV minireplicon systems were supplemented with the C plasmid at a range of 0.02 ng to 2000 ng. Levels of GFP expression, normalised to mean fluorescence for the minireplicon containing the PdC support plasmid (set at 100%) are shown for the JPV (A) and BeiPV (B) minireplicon systems.

The levels of GFP expression in both JPV and BeiPV minireplicon systems, where 200 ng of C plasmids were added with the PdC support plasmids, were similar to GFP expression level in the minireplicon systems with the 'wild-type' P support plasmids. As the P support plasmid was fixed at a concentration of 200 ng in the minireplicon system, this result indicated that the C protein was expressed from both the P plasmid and the C plasmid, at comparable levels.

2.4 Discussion

The gene expression strategy utilised by NNSV involves replication of the ribonucleoprotein complex and sequential synthesis of mRNA. Neither the genomic nor antigenomic RNA alone is capable of initiating an infectious cycle. Reverse genetics allows genetic manipulation of the viral genome and rescue infectious virus particles from entirely engineered clones. Minireplicon systems are generated by replacing the entire protein coding region of a viral genome with a reporter gene. Minireplicon systems allow the function of *cis* and *trans*-acting sequences at the terminal ends of the viral genome to be determined. Function of the proteins that form the polymerase complex (derived from the N, P and L genes) may also be studied using a minireplicon system.

Minireplicon systems utilising GFP as a reporter gene were created for NiV, JPV and BeiPV. In all minigenomes, the coding regions of the virus were replaced with the GFP gene, but the viral genomic termini remained intact. Insertion of either the genome or antigenome into a full-length infectious clone leads to the production of infectious virus (Kato *et al.* 1996). The expression of a reporter gene in a minireplicon system is also possible when the minigenome is engineered in negative or positive polarity, but the polarity of foreign genes determines the efficiency of gene expression. Hybridisation of negative-sense, genomic RNA, and positive-sense RNA from protein-encoding plasmids, produced simultaneously during transcription, is detrimental (Epstein *et al.* 2006), may evoke an IFN response in the host cell, (Conzelmann 1998) and should be avoided. For these reasons, the GFP gene was engineered in the reverse orientation in the NiV, JPV and BeiPV minigenomes.

Reporter genes in minireplicon systems function in the quantitation of rescued synthetic RNA. Vast arrays of reporter genes are available and the CAT gene has historically been the gene of choice in the construction of minireplicon systems since this reporter gene was first expressed in a SeV minigenome (Park *et*

al. 1991). Protocols involving construction of CAT plasmids have been modified in attempts to optimise the rescue of this foreign gene via reverse genetics techniques. High background levels of CAT expression in conventional assays have been avoided by using radioactive methods in the detection of CAT, rather than enzyme-linked immunosorbent assay (ELISA) (Khromykh & Westaway 1997). Green fluorescent protein (Watanabe *et al.* 2004), β-glucuronidase (Izeta *et al.* 1999) and fire fly luciferase (Kato *et al.* 1996) are all reporter molecules that are detected by chemiluminescence. Secreted placental alkaline phosphatase (SAEP) (Barretto *et al.* 2003) and β-galactosidase (Tang *et al.* 2001) also function as reporters. The GFP reporter gene was chosen for the NiV, JPV and BeiPV minireplicon systems as GFP is able to be visualised in live cells, hence allowing real-time observation of experiments, without disrupting cells. Background fluorescence is not observed in cells where GFP is not expressed, making experimental results easier to quantify.

Support plasmids (N, P and L) were required to form the vRdRp, and the genes encoding the polymerase proteins were cloned individually into the pTM1 expression vector (or supplied, in the case of NiV). Polymerase genes were under the control of a T7 promotor. The bacteriophage T7 RNA polymerase has been integrated into the FWPV genome by Britton *et al.* (1996), under the control of the VACV early/late promotor. The recombinant virus, fpEFLT7pol, able to stably express T7 RNA polymerase in avian and mammalian cells, undergoes abortive replication in mammalian cells, and infectious virus is not produced. FWPV-T7 was chosen as the source of the T7 polymerase, as FWPV is less cytopathic than VACV, and a stable cell line expressing T7 was not readily available.

The 'rule of six' states that the replication of paramyxoviruses is more efficient when the total number of nucleotides in the viral genome is divisible by six (Calain & Roux 1993). This is because the RNA polymerase initiates more efficiently when *cis*-acting promotor sequences are found in the correct context with relation to N subunits, determined by the length of the entire genome (Hausmann *et al.* 1996). The 'rule of six' not only applies to genome replication, but also transcription of a minigenome. The sequence corresponding to the viral 3' terminal region, representing the end of a paramyxovirus genome must also be present for the genome to be functional. This criterion, in conjunction with the requirement for the genome/reporter to obey the 'rule of six', was able to be fulfilled by inserting a ribozyme sequence, providing a template for autocatalytic cleavage of the genome. The HDV ribozyme sequence is more efficient than the hammerhead ribozyme (Fearns *et al.* 1997), with a higher rate of cleavage. The HDV ribozyme sequence

mediates efficient self-cleavage of the 3' terminus of the minigenome RNA, and was engineered to ensure that the minigenomes were divisible by six.

In a NiV-CAT replication system, CAT was only produced when the minigenome was divisible by six (Halpin *et al.* 2004). In the same study, CAT was produced when a HeV minigenome divisible by six was transfected with the NiV support plasmids. This phenomenon was also observed for SeV (Tapparel *et al.* 1997) and NDV (Barretto *et al.* 2003). Minigenomes of JPV and BeiPV, with four additional nucleotides, were not divisible by six, and subsequently failed to express GFP. These results indicated that in order to maintain efficient replication, both JPV and BeiPV comply with the 'rule of six'. The length of the genomes of NiV, JPV and BeiPV are multiples of six. The conclusion that a functional reverse genetics system for paramyxoviruses must obey the 'rule of six' is supported.

The majority of studies indicate that the expression of the C protein inhibits the transcription of a minigenome, and results from JPV and BeiPV minireplicon experiments confer with these previous studies. Silencing of the C gene from the JPV and BeiPV P support plasmids resulted in increased GFP expression from the minigenomes. When the C protein from either JPV or BeiPV was incorporated into the JPV and BeiPV minireplicon assay, respectively, by expression from a separate plasmid, GFP expression decreased. The decrease in GFP expression, in both JPV and BeiPV minireplicon systems, occurred in a dose dependent manner. Similarly, the decrease in luciferase activity when either the HPIV3 or SeV C gene was included in a HPIV3 minireplicon system was also dose dependent (Malur *et al.* 2004).

There is overwhelming support for the conclusion that the C protein of paramyxoviruses is inhibitory to transcription in reverse genetics systems. Although inhibitory, the non-structural C protein may have an essential role in paramyxovirus replication. The formation of virus-like particles (VLPs) can be used as a model for virus budding. The SeV C protein interacts with AIP1/Alix, a host protein involved in apoptosis and endosomal membrane trafficking, and this interaction facilitates budding of SeV VLPs (Sakaguchi *et al.* 2005). Co-expression of the SeV C gene with the M, N, F and HN genes resulted in enhanced VLP release, suggesting that the SeV C protein has a role in virus budding (Sugahara *et al.* 2004). The SeV C protein co-localises with the M protein in the cytoplasm and may act as a chaperon to convert newly synthesised M proteins into assembly initiating forms (Hasan *et al.* 2000). Taken together, these studies with the SeV C protein support the hypothesis that the C protein is essential in virus assembly. There is a possibility that the C protein is only inhibitory once expression levels reach a threshold, and in a viral

infection this expression level is likely to be regulated. In order to draw any conclusion about the effect of C protein expression on viral replication of JPV or BeiPV, it would be necessary to study the protein in the context of a full-length infectious clone, as research on paramyxovirus C proteins varies depending on reverse genetics system or particular virus used.

The studies described in this chapter aimed to generate functional reverse genetics systems for JPV and BeiPV, two recently characterised rodent paramyxoviruses. The successful establishment of these reverse genetics systems have allowed JPV and BeiPV to be further characterised and have aided in the classification in these rodent viruses, as described in subsequent chapters.

CHAPTER 3
Use of Minireplicon Systems to Study Virus Evolution and Classification

3.1 Introduction

The family *Paramyxoviridae* is taxonomically placed within the order *Mononegavirales* together with three other families- *Filoviridae, Bornaviridae,* and *Rhabdoviridae.* All members of these viral families contain a NNS genome. The family *Paramyxoviridae* is divided into two subfamilies- *Paramyxovirinae* and *Pneumovirinae*. Classification of viruses at the subfamily level is based on morphological appearance, genome organisation, biological activity of proteins and the sequence relationship between encoded proteins (Lamb *et al.* 2000). At present, the subfamily *Paramyxovirinae* is divided into five genera- *Morbillivirus*, *Respirovirus*, *Rubulavirus*, *Avulavirus* and *Henipavirus* (Pringle 2005).

At least four novel paramyxoviruses, NarPV (Tikasingh *et al.* 1966), MosPV (Campbell *et al.* 1977), JPV (Jun *et al.* 1977) and BeiPV (Li *et al.* 2006) have been isolated from rodents since the 1960s. JPV was isolated on four separate occasions via kidney autoculture of trapped wild mice (*Mus musculus*), during an investigation into the pathology of feral rodents in northern Queensland, Australia, in 1972 (Jun *et al.* 1977; Mesina *et al.* 1974). The entire genome of JPV has been sequenced and contains 18,954 nucleotides (Jack *et al.* 2005). BeiPV was initially identified in a human mesangial cell line, but was later isolated from a rat mesangial cell line, which is believed to be the original source of the virus (Li *et al.* 2006). The genome of BeiPV has been completely sequenced and the genome organisation is similar to JPV. At 19,212 nucleotides in length the BeiPV genome is the largest in the family *Paramyxoviridae* sequenced to date. In fact, the BeiPV genome is also the largest among all known NNSV in the order *Mononegavirales.*

NiV and HeV both belong to the genus *Henipavirus*. Recently, the suggestion was made that BeiPV is closely related to the henipaviruses, and should be classified as a member of this genus (Schomacker *et al.* 2004). Previous studies have demonstrated that in a reverse genetics system, replication of a viral minigenome can be driven by the polymerase complex of another closely related virus within the same genus (Pelet *et al.* 1996).

Reverse genetics systems for NiV, JPV and BeiPV were established as detailed in Chapter 2. Using these reverse genetics systems, the evolutionary

relatedness of NiV, JPV and BeiPV was investigated by exchanging individual viral minigenomes and polymerase complexes. The relatedness between JPV and BeiPV was further investigated by exchanging individual viral polymerase proteins and examining the effect on polymerase complex formation. The NiV and JPV reverse genetics systems were also used to examine the function of the conserved GDNE/GDNQ motif of the NiV and JPV L polymerase proteins.

3.2 Materials and Methodology

3.2.1 Construction of minireplicon systems

Construction of NiV, JPV and BeiPV minireplicon systems

Minireplicon systems were constructed for NiV, JPV and BeiPV, as detailed in Chapter 2, Sections 2.2.7 (NiV), 2.2.8 (JPV) and 2.2.9 (BeiPV), respectively.

Construction of JPV and BeiPV hybrid minigenomes

Hybrid minigenomes were generated, where the leader and trailer regions of JPV and BeiPV were exchanged. The JPV minigenome, pJPV-GFP, was used as a template for the construction of both hybrid minigenomes. As the leader and trailer region of JPV contain the same number of nucleotides as the respective leader and trailer region of BeiPV, both hybrid minigenomes were divisible by six.

The first hybrid minigenome was constructed by replacing the 3' N gene UTR of JPV with the BeiPV 3' N gene UTR. The BeiPV 3' N UTR was amplified from cDNA with the DM10 and DM11 (*Nco*I) primers. This PCR product was used as a template in a subsequent PCR reaction, with the DM03 (*Nar*I) and DM11 (*Nco*I) primers. The DM03 primer extended the BeiPV genome specific PCR into the HDV region and added the *Nar*I restriction site to the product. The PCR product containing the 3' terminus of the BeiPV minigenome was cloned into the *Nar*I and *Nco*I sites of the pJPV-GFP minigenome, hence replacing the 3' JPV terminus with the 3' BeiPV terminus. The hybrid GFP minigenome, pBle-GFP-Jtr, contained the BeiPV 3' terminus, and the JPV 5' terminus (Figure 3.1A).

The second hybrid minigenome was constructed by replacing the L 5' UTR of JPV with the BeiPV L 5' UTR. The BeiPV L 5' UTR was amplified from cDNA with DM12 (*Hind*III) and DM13 (*Not*I) primers. The PCR product containing the 5' terminus of the BeiPV minigenome was cloned into the *Hind*III and *Not*I sites of the pJPV-GFP minigenome, consequently replacing the 5' JPV terminus with the 5' BeiPV terminus. The hybrid GFP minigenome, pJle-GFP-Jtr, contained the JPV 3' termini and the BeiPV 5' terminus (Figure 3.1B).

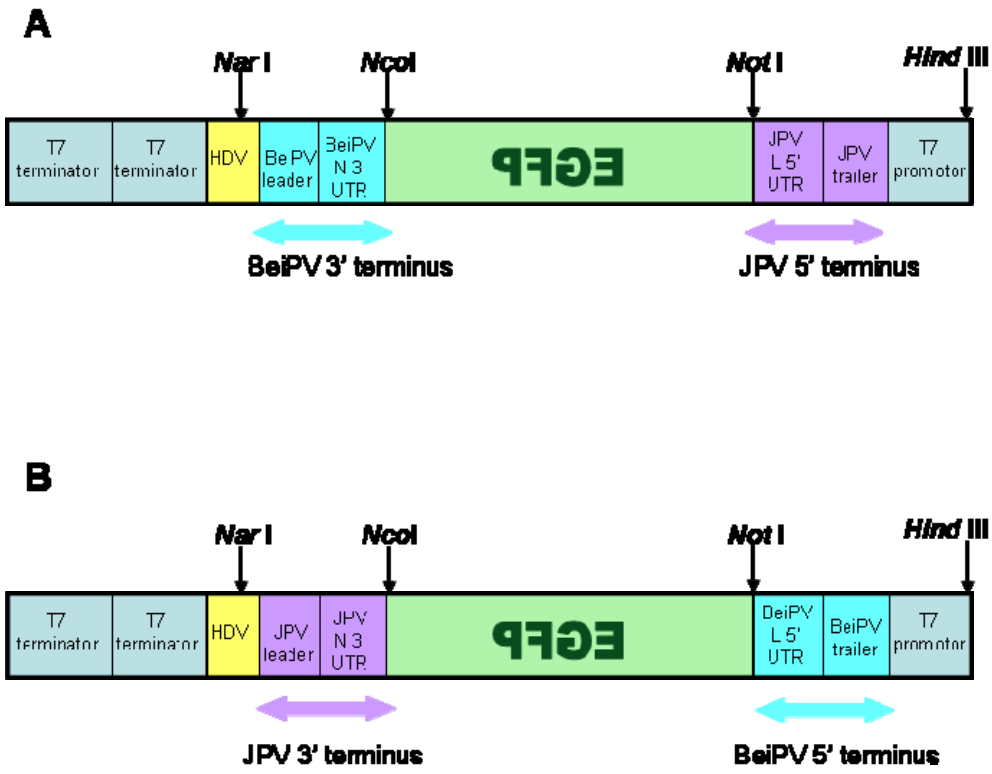


Figure 3.1- JPV and BeiPV Hybrid Minigenomes

Hybrid minigenomes were generated where the leader and trailer regions of JPV and BeiPV were exchanged. The hybrid GFP minigenome, pBle-GFP-Jtr, contained the BeiPV 3' terminus, with the JPV 5' terminus (A). The hybrid GFP minigenome, pJle-GFP-Btr, contained the JPV 3' terminus, with the BeiPV 5' terminus (B).

3.2.2 Mutagenesis of the NiV and JPV L genes

Mutagenesis of the NiV L gene

To obtain the GDNQ mutation in the NiV L gene, site directed mutagenesis was performed. The pTM1-NiV-L plasmid was used as a template for PCR, with the primers DM28 (*Bsi*WI) and DM29 (*Bst*BI). These primers amplified a 1616 bp fragment, spanning from the BstBI restriction site to the *Bsi*WI restriction site of the L gene. A second PCR of 217 bp was amplified with the DM29 (*Bst*BI) reverse primer and DM30 (*Sty*I) forward primer containing point mutations to alter the E residue of the conserved GDNE sequence into a Q residue. Each of the forward primers in the mutagenesis PCR reactions contained a *Sty*I restriction site. The GDNE sequence contained a *Sty*I site that was unique between the *Bst*BI and *Bsi*WI sites of the NiV L gene. The two PCR products were digested with *Sty*I, ligated together, and the ligation product was used as a template for a third PCR, with the primers DM28 (*Bsi*WI) and DM29 (*Bst*BI). Both the pTM1-NiV-L vector and the GDNQ PCR products were digested with *Bst*BI and *Bsi*WI, and ligated. The resulting pTM1-NiV-L clone contained the GDNQ motif, as a replacement for the GDNE motif.

Using the strategy above employed to create the GDNQ mutant, NiV L plasmids were constructed to contain GDND, GDNA, GDNN, GDNI and GDNG motifs. Forward primers used for the mutagenesis of the GDNE motif were DM37 (*Sty*I), DM38 (*Sty*I), DM39 (*Sty*I), DM40 (*Sty*I) and DM41 (*Sty*I) for GDND, GDNA, GDNN, GDNI and GDNG, respectively.

Mutagenesis of JPV L gene

To obtain the GDNE mutation in the JPV L gene, site directed mutagenesis was performed. The pHMN-JPV-L plasmid was used as a template for PCR with the primers PJ53 and PJ138. These primers amplified a 499 bp fragment, spanning from the *Pci*I restriction site to the *Blp*I restriction site of the JPV L gene. A second fragment of 459 bp was amplified with the DM27 (*Pst*I) and PJ138 primers. Primer DM27 contained point mutations to alter the Q residue of the conserved GDNQ sequence into an E residue. The two PCR products were digested with *Pst*I, ligated together and the ligation product was used as a template for a third PCR with the primers PJ53 and PJ138. Both the pCIneo-JPV-L construct, and GDNE PCR product, were digested with *Pci*I and *Blp*I and ligated. The resulting pCIneo-JPV-L clone contained the GDNE motif as a replacement for the GDNQ motif. The JPV-L

fragment containing the GDNE motif was then removed from the pCIneo plasmid and cloned into the pTM1 plasmid, using the *Mlu*I and *Not*I restriction sites.

3.2.3 Functional studies using JPV, NiV and BeiPV minigenomes

Functional studies were carried out with NiV, JPV and BeiPV minireplicon systems. All minireplicon assays were performed under the same conditions as described in Chapter 2, Section 2.2.10, unless otherwise stated.

3.2.4 Detection of the NiV L protein by radioimmunoprecipitation

Antibodies

The NiV rabbit antiserum, supplied by Chris Morrissy, was obtained from rabbits immunised at AAHL.

Transfection and infection of Vero cells

Vero cells were seeded into 6 well plates at 5 X 10^5 cells per well in 3 mL EMEM-10 and incubated overnight at 37°C, with 5% CO_2. Cells were transfected with 2 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen), as described in Section 2.2.4. Following a 6 hr transfection, cells were infected with VACV-T7 (Fuerst *et al.* 1986), at an m.o.i. of 10, in EMEM containing 2.5% FCS. After allowing infection to proceed for 6 hr, the NiV proteins were metabolically labelled.

^{35}S labelling of transfected Vero cells

Transfected NiV proteins were metabolically labelled with [^{35}S] Methionine-Cysteine (Amersham Pharmacia Biotech, USA). For ^{35}S metabolic labelling, the virus and media were removed from transfected cells and replaced with L-methionine and L-cysteine free EMEM (MP Biomedicals), containing 2.5% FCS and 100 μCi of ^{35}S-Met/Cys. Cells were incubated overnight at 37°C, with 5% CO_2. Radioactive media was removed and replaced with EMEM-10 for a 2 hr chase.

Lysis of ^{35}S metabolically labelled Vero cells

Cells were harvested by centrifugation and lysed in ice-cold lysis buffer containing 100 mM Tris-HCL (pH 8.0), 100 mM NaCl and 1% Triton-X-100. Nuclei were removed by centrifugation and supernatant was incubated with Protein G–Sepharose (Amersham Biosciences) for 45 min to pre-clear. Following the 45 min pre-clear, 2 μL of rabbit NiV antiserum was added to each immunoprecipitation mixture, and incubated at 4°C for 1 hr. Following the incubation, Protein G–Sepharose (Amersham Biosciences) was added and incubated for a further 45 min.

Bead complexes were washed three times with lysis buffer, and boiled at 100°C in reducing buffer (0.04% (w/v) bromophenol blue, 6% (w/v) SDS, 190 mM Tris-HCl pH 6.5, 45% (v/v) glycerol, 15% (v/v) 2-β mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE on a 4-12% NuPage Novex Bis-Tris gradient gel (Invitrogen). Following SDS-PAGE, gels were fixed overnight, in a solution containing 40% methanol and 10% acetic acid.

Autoradiography

Fixed, radioactive gels were dried onto filter paper using a vacuum gel drier (Labconco, USA) at 80°C, for 60 min, and then exposed to Blue XB-1 Kodak Scientific Imaging film (Kodak, USA) for autoradiography. Autoradiographs were developed using Ilford Phenisol High Contrast Film Developer and Ilford Rapid Fixer (Ilford, UK).

3.3 Results

3.3.1 Exchange of leader and trailer regions of JPV and BeiPV

In order to ascertain the degree of functional similarity between the non-coding regions of JPV and BeiPV, hybrid minigenomes were generated where the leader or trailer region was exchanged between the two viruses. The leader region of the BeiPV minigenome was replaced with the JPV leader region in the pJle-GFP-Btr minigenome, and the leader region of the JPV minigenome was replaced with the BeiPV leader region in the pBle-GFP-Jtr minigenome. Hybrid minigenomes were transfected with JPV support plasmids, and transfected cells were observed 72 hr post transfection. Both hybrid minigenomes were efficiently transcribed by the JPV support plasmids. There was no significant difference in level of GFP expression between the hybrid minigenomes, or between each hybrid minigenome and the JPV minigenome (data not shown).

Comparative analysis of the minigenomes at the nucleotide level revealed the combined leader region and N gene UTR of JPV and BeiPV were the same length, and share 64% homology. In contrast, the combined trailer region and L gene UTR of JPV and BeiPV, although the same length, were only 55% identical.

3.3.2 Exchange of NiV, JPV and BeiPV minigenomes and polymerase proteins

To determine the relatedness between NiV, JPV and BeiPV, the functionality of each minigenome was analysed when support plasmids were

supplied in heterotypic combinations in the minireplicon systems. Results from Chapter 2, Section 2.3.4 demonstrated that the minireplicon systems for NiV, JPV and BeiPV were functional. When the NiV, JPV or BeiPV minigenome plasmids were provided with homotypic support plasmids, synthesis of GFP occurred, although not at equivalent levels (Figure 3.2A, E and I for NiV, JPV and BeiPV, respectively). When the pJPV-GFP minigenome was co-transfected with the BeiPV N, P and L support plasmids, GPF expression was observed, indicating that the BeiPV N, P and L plasmids formed a polymerase complex capable of transcribing the minigenome of JPV (Figure 3.2H). To prove that the JPV polymerase complex could transcribe the BeiPV minigenome, the JPV N, P and L support plasmids were co-transfected with the pBeiPV-GFP minigenome. Cells were analysed 72 hr after transfection, and GFP expression was observed (Figure 3.2F). The level of GFP expression for the heterotypic minireplicon combinations was generally higher than that of the homotypic combinations. These results indicated that the polymerase complexes of JPV and BeiPV were interchangeable, suggesting that the relatedness of these viruses was high enough for them to share replication machinery.

In contrast, when the pNiV-GFP minigenome was co-transfected with either BeiPV or JPV N, P and L support plasmids, GFP expression was not detected (Figure 3.2D and G). The same was true when the NiV N, P and L support plasmids were co-transfected with either JPV or BeiPV minigenomes (Figure 3.2B and C). These results indicated that the NiV polymerase complex was not capable of supporting the replication of either the BeiPV or JPV minigenomes.

3.3.3 Exchange of individual JPV and BeiPV polymerase proteins

The exchange of JPV and BeiPV polymerase proteins did not impinge on the functionality of either the JPV, or the BeiPV minireplicon system. In order to determine whether a functional polymerase could be produced through heterotypic protein-protein interactions between individual JPV and BeiPV polymerase proteins, support plasmids were exchanged in JPV and BeiPV minireplicon systems. There were six possible support plasmid combinations to be used in conjunction with the JPV and BeiPV minigenomes, and these combinations are listed in Table 3.1. GFP expression was not evident in any of the twelve combinations (data not shown). These results indicated that although the JPV and BeiPV polymerase complexes could be exchanged, the individual N, P and L proteins were not interchangeable between the viral minireplicon systems. The results suggested that that the JPV and BeiPV polymerase proteins were unable to interact to form a functional polymerase complex.

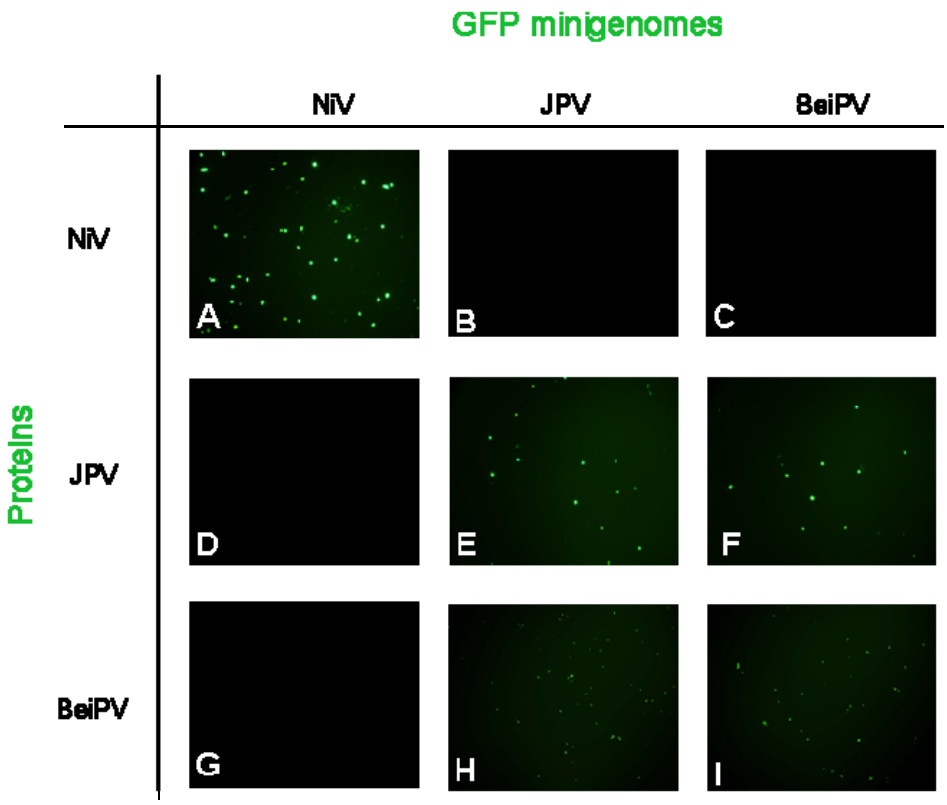


Figure 3.2- Exchange of NiV, JPV and BeiPV Minigenomes and Polymerase Proteins

The pNiV-GFP minigenome plasmid was transfected with the NiV, JPV and BeiPV support plasmids (A, D and G, respectively). The pJPV-GFP minigenome plasmid was co-transfected with the NiV, JPV and BeiPV support plasmids (B, E and H, respectively). The pBeiPV-GFP minigenome plasmid was co-transfected with the NiV, JPV and BeiPV support plasmids (C, F and I, respectively).

N plasmid	P plasmid	L plasmid
JPV-N	BeiPV-P	BeiPV-L
BeiPV-N	JPV-P	BeiPV-L
BeiPV-N	BeiPV-P	JPV-L
BeiPV-N	JPV-P	JPV-L
JPV-N	BeiPV-P	JPV-L
JPV-N	JPV-P	BeiPV-L

Table 3.1- Minireplicon Support Plasmid Combinations

3.3.4 Mutagenesis of NiV and JPV L gene

Mutagenesis of the E residue in the conserved GDNE motif of the NiV L protein

Replacement of the E residue of the NiV L GDNE motif with a Q residue was achieved by site directed mutagenesis. The activity of NiV L-GDNE (wild-type) was compared with that of NiV L-GDNQ, the sequence motif common to most other NNSV. Replacing the NiV-L plasmid with NiV-L-GDNQ did not result in a loss of GFP production from the minigenome in Vero cells. There was no significant difference in the GFP expression level between the minireplicon system containing the NiV L-GDNE plasmid (Figure 3.3A) and the minireplicon system containing the NiV L-GDNQ plasmid (Figure 3.3B). GFP expression was not evident in the negative control, where the L plasmid was omitted from the minireplicon system (Figure 3.3C).

To establish whether activity of the NiV polymerase could be affected by introducing a bulky, positively charged residue, or a residue known to disrupt protein secondary structure, two additional L gene mutants, GDNK and GDNP, were constructed. These amino acid substitutions abolished GFP expression, demonstrating that the K and P residues were not tolerated at this position (Figure 3.4).

To further determine the tolerance range of amino acid substitutions of the E residue in the GDNE motif of the NiV L protein, five additional L gene mutants were constructed. NiV L plasmids were constructed to contain GDND, GDNA, GDNN, GDNI and GDNG motifs. The mutant L plasmids replaced the wild-type L plasmid in the NiV minigenome assay, and although GFP expression was evident, the level of GFP produced was significantly lower ($P < 0.01$). GFP fluorescence of these minireplicon assays are displayed in Figure 3.4. The mean number of fluorescent cells per field of view for the GDND, GDNA, GDNN, GDNI and GDNG minigenome assays were 29%, 8%, 4%, 40% and 2% of wild-type, respectively (Figure 3.5). Although actual levels of GFP expression were considerably variable between minireplicon assays, these results were representative of the general trend observed across experiments.

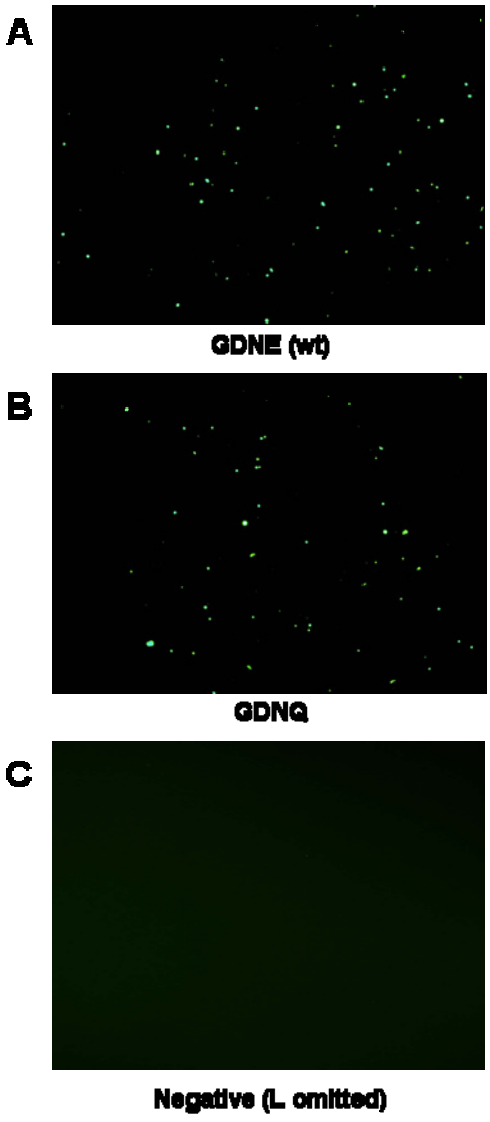


Figure 3.3- Exchange of the E Residue with a Q Residue in the Conserved NiV GDNE Motif

The NiV minigenome plasmid and the NiV N and P support plasmids were transfected with the NiV L-GDNE plasmid (A) and the NiV L-GDNQ plasmid (B), or the L plasmid was omitted from the minireplicon system (C).

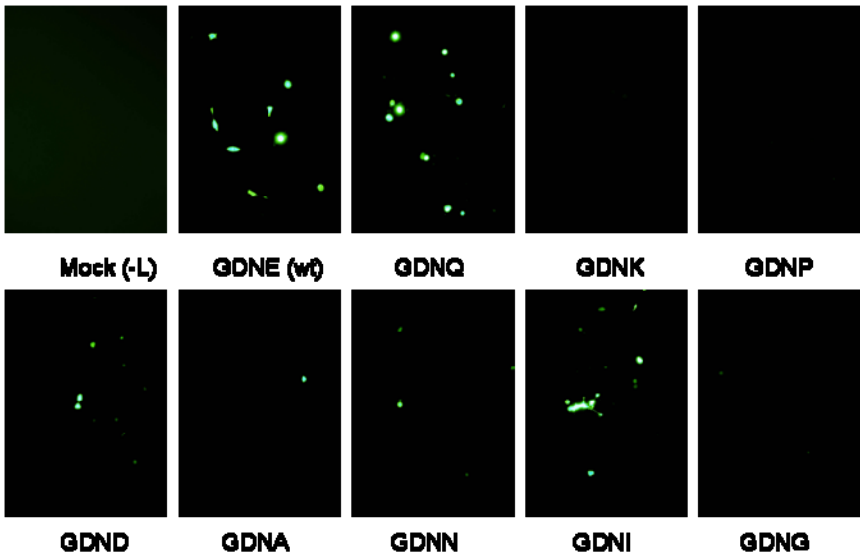


Figure 3.4- Amino Acid Substitutions of the E Residue in the Conserved NiV GDNE Motif

The NiV minigenome plasmid and the NiV N and P support plasmids were transfected without the NiV L plasmid (-L) or with the NiV L plasmids containing the GDNE, GDNQ, GDNK, GDNP, GDND, GDNA, GDNN, GDNI and GDNG motifs.

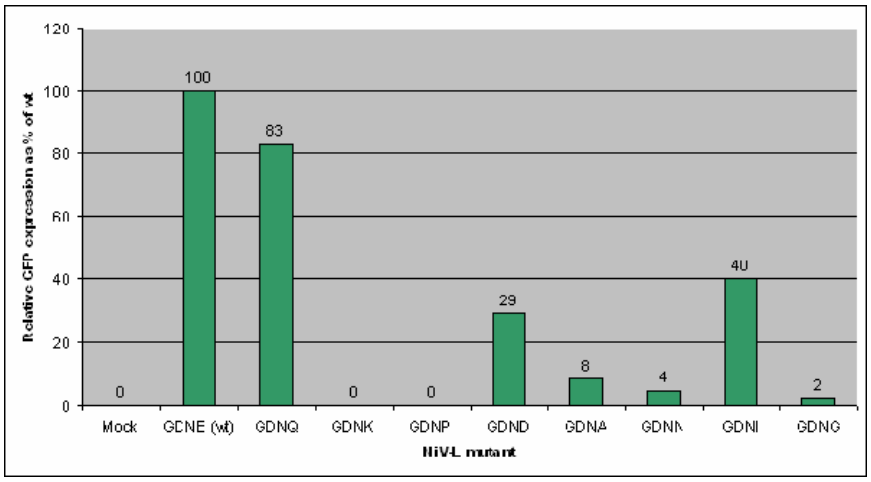


Figure 3.5- Quantitation of GFP in NiV Minireplicon Assays with Modified GDNE Motif

The NiV minigenome plasmids were provided with mutant NiV L support plasmids, in minireplicon assays. The mean number of fluorescent cells per field of view for GDNE, GDNQ, GDNK, GDNP, GDND, GDNA, GDNN, GDNI and GDNG minireplicon assays are given.

The results from the NiV minireplicon assays demonstrated that the E residue within the GDNE motif was interchangeable with a Q residue. Furthermore, although the E residue could be replaced with D, A, N, I and G residues, albeit with considerable reduction in activity, replacement with K or P residues resulted in complete abrogation of polymerase activity in the NiV minireplicon system.

Attempted quantitation of expression of the mutant NiV L proteins

A radioimmunoprecipitation assay was performed using a rabbit anti-NiV antibody to compare the expression of the mutant NiV L proteins. The NiV L genes were transiently transfected into Vero cells, and gene expression was driven by VACV T7 polymerase. Proteins were metabolically labelled with [^{35}S] Methionine-Cysteine and precipitated with the anti-NiV antibody. Proteins were separated by SDS-PAGE and visualised by autoradiography. The predicted molecular mass of the NiV L proteins was 257 kDa. The NiV L protein was not visible on the autoradiograph (data not shown).

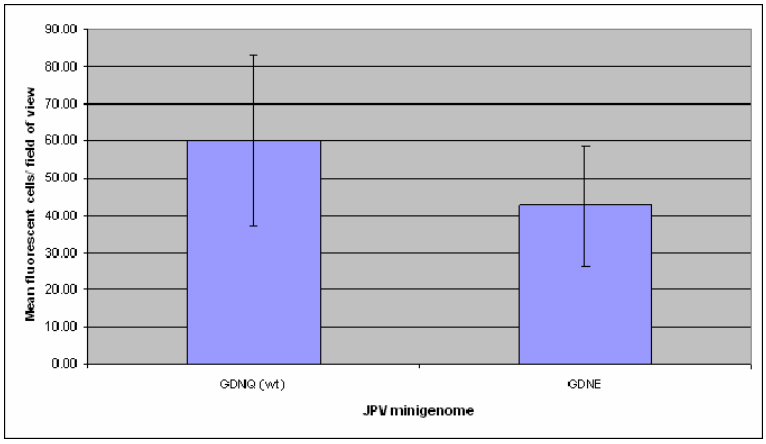
Mutagenesis of the Q residue in the conserved GDNQ motif of the JPV L protein

The Q residue of the JPV L GDNQ motif was replaced with an E residue by site directed mutagenesis. The level of GFP expression in a minireplicon system containing the JPV L GDNQ (wild-type) plasmid was compared with a minireplicon system containing the JPV L GDNE plasmid. There was no significant difference in the GFP expression level between both minireplicon systems across several experiments. The mean number of fluorescent cells per field of view in a representative minireplicon assay is given in Figure 3.6A. This data, where the mean fluorescence of the JPV L GDNE minireplicon is expressed as a percentage of the wild-type JPV L GDNQ minireplicon is given in Figure 3.6B. These results demonstrated that the JPV L GDNQ and GDNE residues were interchangeable.

3.4 Discussion

The replication of a minigenome from one virus can be driven by the support plasmids of another virus, providing that the support plasmids belong to a closely related virus in the same genus (Pelet *et al.* 1996). The replication of a HeV minigenome has been shown to be able to be maintained by support plasmids of NiV, validating the inclusion of these viruses in the same genus (Halpin *et al.* 2004).

A



B

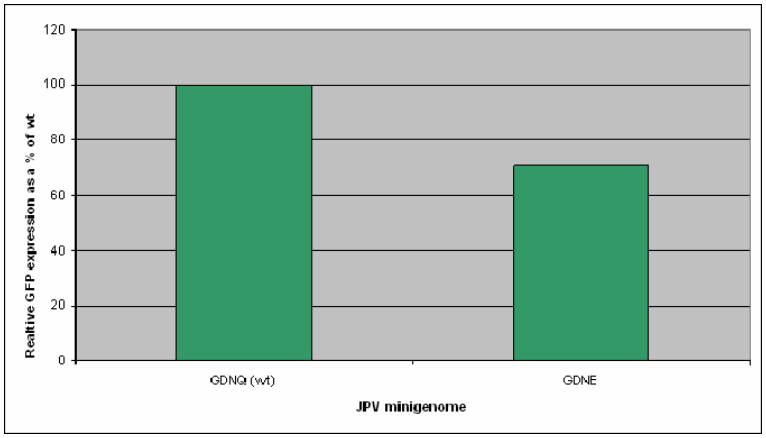


Figure 3.6- Exchange of the Q Residue with an E Residue in the Conserved JPV GDNQ Motif

The level of GFP expression in a minireplicon system containing the JPV L GDNQ (wild-type) plasmid was compared with a minireplicon system containing the JPV L GDNE plasmid. The mean number of fluorescent cells per field of view in a representative minireplicon assay is given (A). The data, where the mean fluorescence of the JPV L GDNE minireplicon is expressed as a percentage of the wild-type JPV L GDNQ minireplicon, is given (B).

To determine the relatedness between NiV, JPV and BeiPV, the functionality of each minigenome was analysed when support plasmids were supplied in heterotypic combinations in the minireplicon systems.

To exclude JPV and BeiPV from the genus *Henipavirus* based on sequence disparity, heterotypic combinations of minireplicon systems using NiV were assembled. When either the NiV minigenome was transfected with JPV or BeiPV support plasmids, or, JPV or BeiPV minigenomes were transfected with NiV support plasmids, GFP expression was not evident. These results indicated that JPV and BeiPV were not structurally similar to NiV, as the replication machinery of these viruses could not be interchanged. On the other hand, when the JPV minigenome was transfected with the support plasmids of BeiPV, or where the BeiPV minigenome was transfected with the support plasmids of JPV, expression of GFP occurred. These results indicated that JPV and BeiPV were structurally similar, and adequately divergent from NiV to be excluded from the genus *Henipavirus*.

GFP expression in Vero cells, after transfection with a heterotypic plasmid combination using JPV and BeiPV, was generally higher than when a homotypic plasmid combination was transfected. Although the exact mechanism of this heterotypic enhancement was not clear, similar results were observed when a HeV minigenome was driven by NiV support plasmids (Halpin *et al.* 2004). Also using a CAT minireplicon system, the Reston strain of EBOV produced a higher level of CAT expression when the minigenome was driven by support plasmids from the more pathogenic Zaire strain, and even the more distantly related *Marburg virus* (Groseth *et al.* 2005). EBOV and Marburg are both viruses that belong to the *Filoviridae* family, another family in the order *Mononegavirale*s. As an explanation for these results, the authors suggest that pathogenic members of the *Filovirus* family may be more transcriptionally active than their apathogenic counterparts (Groseth *et al.* 2005). Similarly, although not quantified, MeV produced syncytia earlier, and in higher numbers, when support plasmids from RPV were supplied in the reverse genetics system, than when MeV support plasmids were supplied (Brown *et al.* 2005a). The authors suggest that dominant-negative effects, such as the generation of non-physiological levels of N protein, may occur when homotypic support plasmids are supplied in reverse genetics rescue systems (Brown *et al.* 2005a). When support plasmids from closely related viruses are supplied in a reverse genetics system, negative effects may not be as pronounced as when homotypic support plasmids are supplied. The heterotypic proteins may not compete

with viral proteins produced from the rescued genome and hence would not interfere with the balance between transcription and replication.

In order to determine the level of structural similarity that exists between JPV and BeiPV, the leader and trailer regions of the minigenomes were exchanged. Research with RSV indicated that residues in the first 13 nucleotides of the leader region, in conjunction with the 3' terminus of the N gene, were responsible for the recruitment of the polymerase (Cowton & Fearns 2005). As both JPV and BeiPV minigenomes were capable of functioning with heterotypic polymerase complexes, and as polymerase recruitment signals were seemingly present in the leader region, the hybrid JPV/BeiPV minigenomes should also be efficiently transcribed. Transfection of polymerase complexes consisting of JPV polymerase proteins, along with hybrid JPV/BeiPV minigenomes, resulted in GFP expression from both minireplicon systems containing hybrid minigenomes.

Previous studies with EBOV indicated that *cis*-acting signals can be exchanged between the Reston and Zaire strains as the 3' and 5' promotor regions of these viral strains display a high degree of structural similarity (Boehmann *et al.* 2005). These results were in accordance with published research indicating replication complexes are specific for autologous RNA, but can occasionally transcribe RNA from closely related viruses. Further studies revealed that the EBOV replication promotor is bipartite and the viral leader does not interact with the trailer (Weik *et al.* 2005). Taken together, the generation of functional JPV and BeiPV minireplicon systems, with heterotypic support proteins and also hybrid minigenomes, provides evidence for the lack of interaction between the viral leader and trailer regions.

The ability to successfully exchange support plasmids in the JPV and BeiPV minireplicon systems provided evidence that polymerase complexes of the viruses were functionally similar. Additional evidence for the functional similarity of the JPV and BeiPV polymerase complexes was provided by the successful replication of both JPV/BeiPV hybrid minigenomes, by the JPV polymerase proteins. To examine the structural compatibility between JPV and BeiPV polymerase proteins, the JPV and BeiPV support plasmids were interchanged in an attempt to produce a functional heterogeneous polymerase complex. The lack of GFP expression in the JPV and BeiPV minireplicon systems, utilising all possible combinations of support plasmids, suggested that JPV and BeiPV polymerase proteins were not structurally compatible.

The JPV and BeiPV polymerase proteins share variable levels of amino acid homology. The sequence identity between the JPV and BeiPV N, P and L

proteins is 48%, 47% and 73%, respectively (Li *et al.* 2006). Although the JPV and BeiPV proteins share some sequence homology, minireplicon results indicated that these proteins were unable to interact to form a functional polymerase complex. Similar results were observed when two morbillivirus minigenomes, MeV and RPV, were transfected with combinations of MeV, CDV and RPV support plasmids (Brown *et al.* 2005a). In this study, low levels of CAT expression were detected when the RPV minigenome was transfected with the CDV N plasmid and RPV P and L plasmids, but the result could not be consistently repeated across experiments. Higher and more constant levels of CAT expression were observed when the MeV minigenome was transfected with MeV N and P plasmids in conjunction with the RPV L plasmid. Although low levels of CAT expression were detected from minireplicon systems where support plasmids combinations from different viruses were used, rescue of infectious virus was not possible (Brown *et al.* 2005a). Together, these results provide evidence that although the N, P and L support plasmids are able to maintain minigenome expression from closely related viruses, they are not, with the exception of one morbillivirus minireplicon, able to form heterogeneous polymerase complexes.

Consequently, based on the fact that replication machinery was interchangeable between JPV and BeiPV, but not with NiV, the conclusion was reached that these viruses were evolutionarily closely related, and significantly divergent from the henipaviruses. This data provided further support for the classification of JPV and BeiPV into a new genus.

The L genes of NNSV have a high level of amino acid sequence conservation. For paramyxoviruses, sequence comparison of the L genes of SeV, NDV and MeV identified six conserved domains (I-VI), separated by variable regions, suggesting that the structure consisted of concatenated functional domains (Poch *et al.* 1990). Insertion of the EGFP gene between domains II and III of the L protein of RPV (Brown *et al.* 2005b) and MeV (Duprex *et al.* 2002) did not disrupt polymerase function, suggesting that the polymerase was a multi-domain protein. Domain II is essential for RNA recognition and domain III contains a pentapeptide motif, QGDNQ, which has been hypothesised to be an active site for phosphodiester bond formation. The QGDNQ sequence is located within the C motif of domain III and contains a β-turn-β structure critical for polymerase activity (Poch *et al.* 1989). Specific locations of amino acid residues in these tight turns may be critical for correct orientation, cation binding, template specificity and catalytic processes.

Prior to the discovery of HeV in 1994, all known NNSV contained the highly conserved GDNQ sequence motif in domain III of their respective L proteins. For the

henipaviruses, the Q residue has been replaced by an E residue, giving rise to a GDNE sequence motif. This alternate motif has also been found in two recently characterised paramyxoviruses, TuPV (Wang *et al.* 2001) an MosPV (Miller *et al.* 2003). This amino acid change introduces a second negatively charged residue into a predicted functionally important area of the L protein. The GDNE motif was initially thought to be associated with paramyxoviruses with a relatively large genome size, but this now seems unlikely, as JPV and BeiPV, with genome sizes of approximately 19,000 nucleotides, contain the conserved GDNQ motif in their L proteins (Jack *et al.* 2005; Li *et al.* 2006).

The role of the Q residue in the conserved GDNQ motif in the function of the L protein was examined. The E residue in the GDNE motif of the NiV L protein was replaced with either a Q residue, or other amino acid residues, and the activity of the L protein was assessed using the NiV-GFP minigenome replication assay. The Q residue of the GDNQ motif in the JPV L protein was replaced with an E residue, and the activity of the L protein was assessed using the JPV-GFP minigenome replication assay. NiV and JPV minireplicon assay results demonstrated that the Q residue within the GDNQ motif was interchangeable with an E residue.

The tolerance for amino acid substitutions at the Q residue position of the GDNQ motif appears to be virus dependent. Results in the JPV minireplicon system demonstrated that the Q residue in the GDNQ motif was interchangeable with an E residue. However, when the Q residue of RPV L protein was mutated to an E residue, activity in the minigenome assay decreased to 33% of wild-type (Chattopadhyay *et al.* 2004). The same Q to E alteration in the GDNQ motif of RABV resulted in a drastic reduction of activity to 0.4% of wild-type GDNQ in a minigenome assay (Schnell & Conzelmann 1995). While RPV was able to retain a functional minireplicon system with an E residue in the GDNQ motif, the same mutation in RABV, a rhabdovirus, dramatically reduced minigenome functionality. Hence, the ability of the polymerase to tolerate an E residue in the GDNQ motif may be unique to paramyxoviruses.

Although the E residue of the NiV L GDNE motif could be replaced with D, A, N, I and G residues, albeit with considerable reduction in activity, replacement with K or P residues resulted in complete abrogation of polymerase activity in the NiV minireplicon system. A radioimmunoprecipitation assay was performed to ensure that differences observed between NiV the minireplicon systems were not due to differences in L gene expression. The radioimmunoprecipitation was unsuccessful, with the quality of the NiV antiserum most likely the basis of the

failure. As the rabbit NiV antiserum was unlikely to contain sufficient antibodies directed against the NiV L protein, the recombinant NiV L proteins were not able to be immunoprecipitated. The GFP expression levels were analysed comparatively to the wild-type NiV minireplicon system. The introduction of the A, N or G residue reduced the polymerase activity by more than 90%. On the other hand, the functionality of the NiV minireplicon system was retained when the E residue was substituted with D or I residues. When the Q residues of the VSV and RABV GDNQ motif were substituted with an N residue, the RABV minigenome lost all activity (Schnell & Conzelmann 1995) and the VSV minigenome only retained 5% activity (Sleat & Banerjee 1993). Conversely, the same Q to N substitution in the RPV GDNQ motif resulted in 42% activity in the minigenome (Chattopadhyay *et al.* 2004). Taken together, the retention of minireplicon function in RPV with a Q to N mutation in the GDNQ motif further highlights a difference between viral L proteins, within the same or different viral families, in their ability to tolerate point mutations at this critical site.

The results presented suggested that the NiV L protein functioned similarly with either an E or Q residue in the GDNE motif. Results also proved that the same was true for JPV, where the JPV L protein functioned equally well with either an E or Q residue in the GDNQ motif. Considering that the E residue carries a negative charge in comparison to the non-charged Q residue, it appears unlikely that the E/Q residue is directly involved in catalysis. This was further supported by the fact that replacement of the E residue with D, A, N, I or G did not abolish L protein function in the NiV minireplicon system. However, when residues known to disrupt protein secondary structures (P and K) were introduced at this site, the NiV L polymerase activity was abolished. Together these results suggested that the amino acid at the E position played an important role in maintaining structure, not catalytic activity.

CHAPTER 5
Development of Assays for the Detection of Rodent Paramyxoviruses

5.1 Introduction

In addition to the well characterised SeV, at least four other paramyxoviruses have been isolated from rodents in the past fifty years, including NarPV, MosPV, JPV and BeiPV. NarPV was isolated from the brains of forest rodents, *Zygodontomys b. brevicauda*, trapped in the Nariva swamp of Eastern Trinidad in 1962 and 1963 (Tikasingh *et al.* 1966). MosPV was isolated in Queensland, Australia, on three occasions from 1970-1971 from pooled organs of a female rat (*Rattus leucopus*), a male rat (*Rattus fuscipes*) and a pool of mites (*Laelaps echidninus*) (Campbell *et al.* 1977). JPV was isolated on four separate occasions from wild mice (*Mus musculus*) in Queensland, Australia, in 1972 (Jun *et al.* 1977). BeiPV was isolated from a human mesangial cell line in 2003, but a rat mesangial cell line from the same laboratory was believed to be the original source of the virus (Li *et al.* 2006). There have been no reported disease outbreaks associated with any of these rodent paramyxoviruses, and for JPV, no disease was demonstrated upon experimental infection of mice (Miller 2004).

Currently, there is no evidence of human infection with any rodent paramyxoviruses, however, this does not rule out the possibility that these viruses or viruses closely related to them may still pose a threat as zoonotic agents. In recent years several paramyxoviruses have emerged, including NiV and HeV, the only zoonotic paramyxoviruses fatal to humans, and NiV in particular caused significant disease in humans and pigs (Eaton *et al.* 2006). Both asymptomatic and symptomatic rodent populations have also been associated with transmission of disease to humans. *Cowpox virus*, an orthopoxvirus, does not induce any obvious clinical disease in rodents, yet is zoonotic (Begon *et al.* 2003). Members of the *Arenaviridae* family are associated with rodent-transmitted disease and, recently, the arenavirus *Lymphocytic choriomeningitis virus* (LCMV) was transmitted through organ transplantation. Organs from one person who was infected with LCMV were transplanted into four people, and three of the four organ recipients subsequently acquired the LCMV infection and died (Fischer *et al.* 2006). Hantaviruses are regularly transmitted from rodents to humans, and *Puumala virus*, a rodent hantavirus, was shown to be infectious for up to fifteen days in urine, faeces, saliva

and nesting material (Kallio *et al.* 2006). If in the future, rodent paramyxoviruses adapt to infect humans, the availability of rapid, specific and sensitive detection assays would greatly aid in disease diagnosis and outbreak control.

The prevalence and distribution of paramyxovirus infection in wild rodent populations is not known. To monitor the seroprevalence of JPV, BeiPV and MosPV in Australian and Malaysian wild rodent populations, a multiplex microsphere assay for measuring virus-specific antibodies in sera from potentially infected animals was developed. Specifically, JPV and MosPV RNP, and recombinant BeiPV P protein, were purified and coupled to different microspheres for use on the Bio-Plex platform. To assess viral tropism in tissue from animals experimentally infected with JPV, a real-time PCR assay was developed. Specifically, a TaqMan^{TM}-based fluorogenic assay for the detection of JPV RNA was established. As MosPV, NarPV, and BeiPV experimental animal infections have not been attempted, similar assays were not developed for all rodent paramyxoviruses. With further refinement and validation, the multiplex microsphere assay, as well as the real-time PCR assay, may be useful for serology and agent identification purposes, respectively.

5.2 Materials and Methodology

5.2.1 Rodent tissue samples

Experimental infection of mice with JPV was performed at AAHL by Philippa Jack (Miller 2004). Briefly, 21-day-old specific pathogen free BALB/c inbred mice were exposed to an aerosol containing 7 x 10^7 $TCID_{50}$ of JPV in normal saline. Control mice were exposed to an aerosol containing only normal saline. Mice were euthanised at various time points and blood samples were taken prior to euthanasia. At autopsy the brain, lungs, liver, kidneys and spleen were removed, and tissue samples were stored at -80°C, in RNA*later* (Ambion, USA). Sera obtained from these animals were also used in the multiplex microsphere assay (see below).

5.2.2 Isolation of total RNA from rodent tissue

The MagNA Pure LC RNA isolation kit III [Tissue] (Roche, Germany) was used for the extraction of total RNA from mouse tissue that had been stored in RNA*later* (Ambion, USA). Tissues from which RNA was extracted were lung, liver, kidney, spleen and brain. Tissue samples were weighed and 10 mg sections were removed, diced and added to MagNA Lyser Green Bead tubes (Roche). A volume of 400 μL of Tissue Lysis Buffer from the RNA Isolation Kit III [Tissue] (Roche) was added to each sample. The tissues were homogenised using the MagNA Lyser

(Roche) at 6500 rpm for 60 sec, cooled on ice for 2 min, then homogenised for a further 60 sec, at 6500 rpm. The homogenised lysates were incubated at room temperature for 30 min, before centrifuging at 13000 rpm, in a Biofuge pico bench top centrifuge (Heraeus, Germany) for 2 min to clarify the lysate.

After centrifugation, 350 µL of each homogenate was transferred to a sample cartridge well and placed in the MagNA Pure LC (Roche) instrument where RNA isolation was performed, as outlined in the Roche MagNA Pure LC RNA Isolation Kit III [Tissue] instruction manual. RNA was eluted into a final volume of 50 µL of elution buffer.

RNA concentrations of samples were measured using the GeneQuant II RNA/DNA calculator (Pharmacia Biotech, USA).

See Chapter 2, Section 2.2.1 for cDNA synthesis method.

5.2.3 Design of TaqMan primers and probe for JPV assay

The primers and probes were designed using ABI PRISM Primer Express™ Version 1.5 software (Applied Biosystems, USA), taking into account the guidelines outlined in the Applied Biosystems publication "Sequence Detection Systems Quantitative Assay Design and Optimization".

The target region for viral amplification was in the N gene of JPV (GenBank Accession number AY900001). The 18S ribosomal RNA (rRNA) was utilised for normalisation, for which the primers and probe were supplied in the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems). The JPV probe contained the fluorescent reporter dye, 6-carboxy-fluorescein (FAM) located at the 5' end and the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) located at the 3'end. The 18S rRNA probe contained a proprietary fluorescent reporter dye, VIC, located at the 5' end and the quencher TAMRA located at the 3'end. Primer and probe sequences are given in Table 5.1.

5.2.4 TaqMan real-time PCR assay

The TaqMan® real-time PCR reactions were carried out in 96 well Optical Reaction Plates (Applied Biosystems). Each 25 µL reaction contained 4.375 µL H_2O, 12.5 µL TaqMan® Universal PCR Master mix (2X), 2.25 µL JPV-N F primer (10 µM), 2.25 µL JPV-N R primer (10 µM), 1.25 µL FAM labelled JPV-N probe (5 µM), 0.125 µL 18S rRNA F primer (10 µM), 0.125 µL 18S rRNA R primer (10 µM) and 0.125 µL VIC labelled 18S rRNA probe (40 µM). 2 µL of JPV cDNA was added to each sample reaction, 2 µL of SARS cDNA was added to each 'No Amplification Control' reaction and 2 µL of H_2O was added to each 'No Template Control' reaction.

Table 5.1- JPV and 18S rRNA Primer and Probe Sequences

Primer/Probe	Reporter	5'→3' Sequence	Quencher
JPV-N F	-	CTT CTG CAG GTC CGT CGA A	-
JPV-N R	-	GGG CAC AGC TCT TGA TAC TCT TG	-
JPV-N Probe	FAM	TGC CTC TCC CTA CCC AT	TAMRA
18S F	-	CGG CTA CCA CAT CCA AGG A	-
18S R	-	GCT GGA ATT ACC GCG GCT	-
18S Probe	VIC	TGC TGG CAC CAG ACT TGC CCT C	TAMRA

Reactions were run on the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems) to detect fluorescence emitted during amplification of the cDNA template. Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and then 45 cycles of 15 sec at 95°C followed by 1 min at 60°C.

5.2.5 Generation of real-time PCR efficiency curve

A dilution series of JPV cDNA (see Chapter 2, Section 2.2.1, for cDNA synthesis) was analysed to determine the efficiency of JPV amplification, compared with the amplification of 18S rRNA. The slope of the curves were given in the equation y=mx+c, where m is the slope. The slope of the curve was used to calculate the efficiency of the real-time PCR reaction, according to the efficiency equation (Pfaffl 2001):

$$E \text{ (Efficiency)} = 10^{(-1/\text{slope})}$$

5.2.6 Normalisation of real-time PCR data by Q-Gene

Upon completion of all real-time PCR assays, results were analysed using the Q-Gene program (Muller *et al.* 2002). Q-Gene was downloaded from www.BioTechniques.com and consisted of three Microsoft® Excel® based documents. The three documents were the Q-Gene Core Module (qgene96.xls, a template for analysing 96 well plates and qgene384.xls, a template for analysing 384 well plates), Q-Gene Database (qgenedb.xls, a database to compile experimental results obtained from Q-Gene Core Modules) and Q-Gene Statistics Add-in (qstats.xla, a collection of frequently used statistics programs).

As the JPV real-time PCR assays were performed in 96 well plates, the Q-Gene Core Module used for analysis of data was qgene96.xls. This Excel® document contained four worksheets. The first worksheet completed was the 'Plate Setup' worksheet, where all sample names were entered into the spreadsheet, along with primer and probe names. The 'Amplification Efficiency Plot' worksheet was completed by inputting values obtained from the slope of the JPV (target) and 18S rRNA (reference) plots, obtained from the efficiency curve (see Section 5.3.1). Using the data provided from the slopes of the efficiency curves, the spreadsheet also denoted a value for the amplification efficiency (E) of the target and reference samples. These values were found to match those manually calculated, hence the E_{target}=2.0893 and $E_{reference}$=2.0732. Sample labelling information, efficiency

calculations and standard curves were exported by the program into the 'Data Analysis' worksheet. The 'Data Analysis' worksheet, once Ct values of JPV samples and their 18S rRNA counterparts were entered, calculated the 'Mean Normalised Expression' (MNE) of the samples. This information was used to compare levels of JPV RNA, relative to 18S rRNA within a sample, and then to determine differences between samples. It is important to note that the mean values can be calculated many different ways and the calculation chosen to derive the mean must be kept constant when comparing experiments.

The calculation chosen to determine the mean was taken from Muller *et al* (2002) as follows:

$$\text{MNE} = \frac{\dfrac{(E_{target})^{Ct\ target,well1}}{(E_{ref})^{Ct\ ref,well1}} + \dfrac{(E_{target})^{Ct\ target,well2}}{(E_{ref})^{Ct\ ref,well2}} + \dfrac{(E_{target})^{Ct\ target,well3}}{(E_{ref})^{Ct\ ref,well3}}}{3}$$

A Q-Gene Core Module file was created for each real-time PCR assay. Once data from each experiment was entered into a module, results were manually evaluated. All results derived from positive samples were compiled in a Q-Gene Database spreadsheet. The database worksheet calculated the mean normalised expression of a sample relative to a calibrator. A calibrator was a sample against which expression levels of the target gene RNA were compared. The mean normalised expression of each sample was divided by the mean normalised expression of the calibrator.

5.2.7 Animal sera

Rodent field sera samples

Rodent sera were sourced from laboratories located in Australia and Malaysia. The rodent sera, kindly provided by Greg Smith and Ina Smith of the Queensland Health Scientific Services (Public Health Virology department), were obtained from rodents captured in Queensland, Australia. A list of the sera, which includes details about rodent species and capture location, is given in Appendix 2. The rodent sera kindly provided by Kaw Bing Chua and Sazaly Abu Baker of the University of Malaya (Department of Medical Microbiology) were obtained from rodents captured in two fishing settlements in Pulau Ketam and Port Klang, Malaysia.

Control sera

The JPV rabbit antiserum is described in Chapter 2, Section 2.2.1. The BeiPV P antiserum was raised in rabbits and antibodies were targeted against a recombinant N-terminal fragment of the BeiPV P protein (Liang *et al.* 2003). The rodent negative control sera, SARS rat and mouse antisera, were provided by Meng Yu, and produced at AAHL.

5.2.8 Purification of JPV and MosPV RNP

For the purification of JPV RNP, two 840 cm^2 roller bottles containing monolayers of Vero cells were infected with 9.5 x 10^5 $TCID_{50}$ of JPV. Roller bottles were incubated at 37°C, until syncytia were observed. After the observation of syncytia, the media was decanted (see below for virus purification from supernatant) from the infected cells, and cells were washed with ice-cold PBS-A. After washing, 50 mL of ice-cold PBS-A was added to the roller bottles and cells were scraped into the solution. Cells were centrifuged at 1500 rpm in an IEC Centra-7R centrifuge (210 rotor) for 20 min, at 4°C, and the supernatant was discarded. The cell pellet was resuspended in ice-cold TNM buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.5) and 10% NP-40 was added to a final concentration of 1%. The cell solution was homogenised in a Dounce homogeniser and then the nuclei were removed by centrifugation at 13,000 rpm, for 1 min, at 4°C. After centrifugation, EDTA (pH 7.2) and sodium deoxycholate were added, to a final concentration of 10 mM and 1%, respectively.

A sucrose and caesium chloride (CsCl) gradient column was prepared, containing (from bottom to top) 40% CsCl, 30% CsCl, 20% CsCl and 5% sucrose. The RNP virus suspension was added to the top of the gradient, tubes were placed into a SW41 Ti rotor (Beckman) and centrifuged at 25,000 rpm, overnight (18-24 hr), at 10°C, under vacuum, in a Beckman L8-80M ultracentrifuge. After overnight centrifugation, the visible RNP bands were collected, diluted in TNE buffer, placed into a 55.2 Ti rotor (Beckman) and centrifuged at 40,000 rpm for 90 min, at 10°C, under vacuum, in a Beckman L8-80M ultracentrifuge. The JPV RNP was resuspended in TNE buffer.

JPV was purified from cell culture supernatant (from above). Once decanted from cells, the supernatant was centrifuged at 1500 rpm, in JA 25.5 rotor (Beckman) for 20 min, at 4°C, to remove cell debris. A 50% (w/v) solution of polyethylene glycol (PEG, MW 6000) was prepared in TNE buffer. The PEG solution was added to virus supernatant at a final concentration of 10% and the solution was

gently stirred overnight, at 4°C. Following overnight stirring, the PEG solution was centrifuged at 1500 rpm, in JA 25.5 rotor (Beckman), for 20 min, at 4°C, and the supernatant was discarded.

The viral pellet was resuspended in ice-cold TNE buffer and added to the top of a 20% sucrose cushion. The tubes were placed into a 55.2 Ti rotor (Beckman) and centrifuged at 40,000 rpm, for 90 min, at 10°C, under vacuum, in a Beckman L8-80M ultracentrifuge. The viral pellet was resuspended in TNE buffer, and then added to the top of a 20-50% sucrose gradient. The tubes were placed into a 55.2 Ti rotor (Beckman) and centrifuged at 35,000 rpm, for 90 min, at 10°C, under vacuum, in a Beckman L8-80M ultracentrifuge. The visible bands were removed from the gradient and stored at -20°C.

MosPV RNP was provided by Gary Crameri (Stocks prepared in 1999 and 2000).

5.2.9 Purification of recombinant BeiPV P protein

Recombinant BeiPV P protein expressed from the pHMN-BeiPV-P construct was purified. See Chapter 2, Section 2.2.3, for the method for expression of proteins in bacterial cells, and Chapter 2, Section 2.2.5, for the method for analysis of protein by Western blot and protein purification.

5.2.10 Multiplex microsphere assay equipment, software and calibration

Multiplex microsphere assays were performed on a Bio-Plex Protein Array System integrated with Bio-Plex Manager Software (v 3.0) (Bio-Rad). The high setting was used for the reporter target channel (RP1) and fluorescent identification of microspheres. A Bio-Plex calibration kit (Bio-Rad) was used to maintain reporter conjugate emission wavelengths. A Bio-Plex validation kit (v 3.0) (Bio-Rad), was used to assure consistent optical alignment, fluidics performance, doublet discrimination and identification of individual bead signatures. The coefficient of variation for bead discrimination and reporter channel identification did not exceed 7.0% and 8.0%, respectively. The Bio-Plex equipment was maintained at AAHL, and routinely calibrated by John White, Gary Crameri and Vicky Boyd.

5.2.11 Coupling of purified JPV and MosPV RNP and BeiPV P protein to microspheres

The methods for protein coupling to microspheres and multiplex microsphere assays were adapted from protocols previously established at AAHL

(Katharine Bossart, personal communication). Katharine Bossart and Jennifer McEachern assisted with protein coupling and bead activation.

Purified JPV RNP, MosPV RNP and BeiPV P recombinant protein were coupled to $1x10^6$ carboxylated (COOH) beads (Bio-Rad), on bead sets #46, #43 and #42, respectively. Prior to protein coupling, beads were sonicated, vortexed and activated. For bead activation, each bead set was added to a well of a MultiScreen-BV 1.2 µm hydrophilic, low protein binding, 96-well filter plate (Millipore, Australia), pre-wet with PBS-A. Liquid was removed using a vacuum manifold and the beads were washed twice in the bead activation buffer, supplied in the Amine Coupling Kit (Bio-Rad). Beads were incubated in the dark for a further 20 min, in activation buffer containing 5 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Pierce, USA) and 5 mg/mL N-hydroxysulfosuccinimide (Pierce), at room temperature, with shaking.

Liquid was removed using a vacuum manifold, and JPV RNP, MosPV RNP and BeiPV P recombinant protein were added to bead sets #46, #43 and #42, respectively, in 100 µL of PBS-A. Beads and antigen were incubated for 2 hr, at room temperature, with shaking, in the dark. Beads were washed twice with 0.05% Tween-20 in PBS-A, and removed from the filter plate by resuspension in storage buffer (1% BSA/PBS-A/0.05% sodium azide containing protease inhibitor cocktail) (Roche Diagnostics, Australia).

5.2.12 Multiplex microsphere assay

Test sera, biotinylated rodent conjugates and streptavidin-phycoerythrin (QIAGEN) were diluted in PBS-A. Wells of 96-well filter plates were pre-wet with PBS-A. Activated, protein-coupled beads were vortexed and sonicated (Ultrasonic cleaner, Model K42-752, Ultrasonics, Australia) for 1 min prior to use. PBS-A was removed from filter plates using a vacuum manifold and 100 µL of PBS-A, containing approximately 1500 microspheres of each bead set, were added per well. Buffer was removed from the microspheres using a vacuum manifold. For detection of JPV, MosPV or BeiPV antibodies, 100 µL of sera (at a 1:50 dilution) was added to appropriate wells and incubated with the beads for 30 min, at room temperature, with shaking, in the dark. Liquid was removed using the vacuum manifold and 100 µL of either biotinylated goat anti-mouse (Pierce), or biotinylated goat anti-rat (Pierce) conjugate, at a 1:500 dilution in PBS-A, was added to each well, and incubated for 30 min as described above. Liquid was removed using a vacuum manifold and 100 µL of streptavidin-phycoerythrin (QIAGEN,) at a 1:1000 dilution, was added to each well and incubated for 30 min, as described above. Following

incubation, 30 μL of PBS-A was added to each well. Samples were read for median fluorescence intensities (M.F.I.) using a protocol template for bead sets #46, #43 and #42 on the Bio-Plex Protein Array System.

5.3 Results

5.3.1 JPV real-time PCR optimisation

JPV Primer optimisation

Primer optimisation was performed according to the manufacturer's instructions. Variable forward and reverse primer concentrations were analysed. The primer concentrations that gave the lowest Ct (threshold cycle) value and highest ΔRn were determined. A probe concentration of 250 nM was used in the primer optimisation assay and a 1:10,000 dilution of JPV cDNA was selected as the template for amplification. Standard real-time PCR cycling conditions were used for the reaction. Results were analysed after a baseline of 3-15, and a threshold of 0.2 were manually set. Samples were analysed in triplicate, and the average Ct values of the primer concentrations are shown in Table 5.2. The primer concentrations that gave the lowest average Ct values were 300F/900R (23.30), 300F/300R (23.40) and 900F/900R (23.44). Although the Ct values were similar, the ΔRn differed between samples. The primer concentration with the highest ΔRn was 900F/900R (Figure 5.1). This primer concentration was used in all subsequent experiments. The primer optimisation assay ensured optimal performance of the primers in a real-time PCR assay where a probe concentration of 250 nM was used.

JPV probe optimisation

A probe optimisation was carried out to determine if the real-time PCR assay could be run with a probe concentration under 250 nM. This was done not to improve the assay, but to reduce running costs associated with the experiment. The probe optimisation analysed the differences in probe concentrations, ranging from 50 to 250 nM.

A primer concentration of 900 nM was used for both forward and reverse primers in the assay, and the template for amplification was JPV cDNA, at a dilution of 1:10,000. Standard real-time PCR cycling conditions were used for the reaction. Results were analysed after a baseline of 3-13 and a threshold of 0.1 were manually set.

Table 5.2- Average Ct Values from the JPV N Primer Optimisation

JPV N Reverse Primer (nM)	JPV N Forward Primer (nM)		
	50	300	900
50	25.91	24.50	25.59
300	25.08	23.40	23.62
900	25.41	23.30	23.44

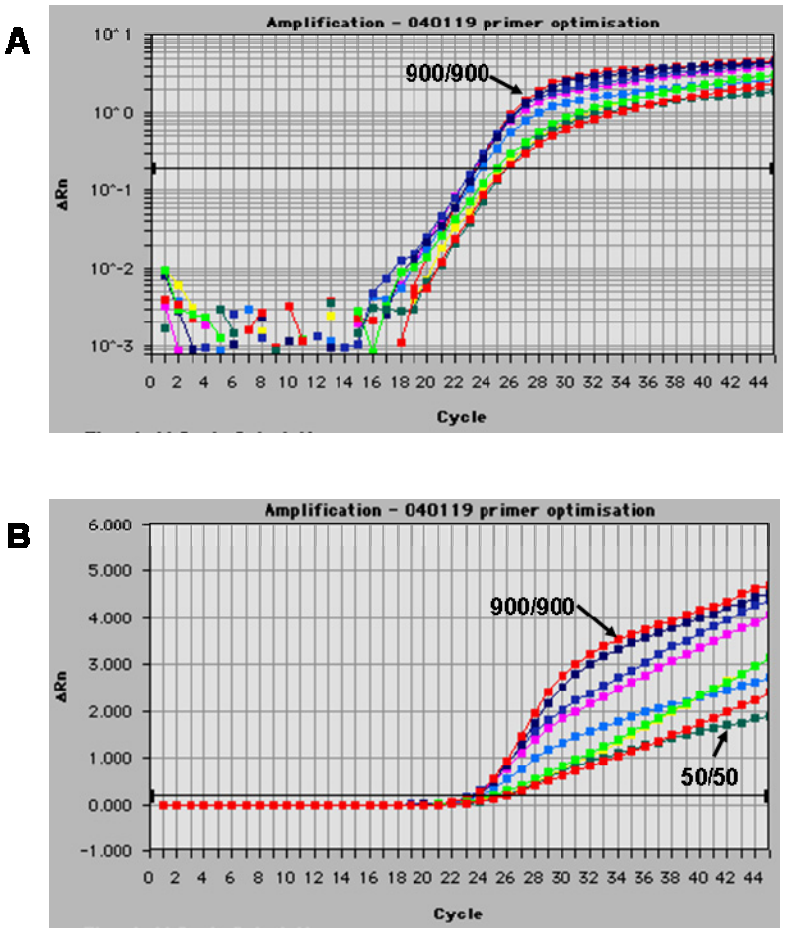


Figure 5.1- Primer Optimisation Assay

The JPV N primer optimisation was performed according to the manufacturer's instructions. Results from the primer optimisation assay display the Ct values, and ΔRn of the samples in the primer matrix. The primer optimisation assay is displayed on a logarithmic scale (A) and a linear scale (B), with Ct values on the x-axis, and ΔRn on the y-axis.

Samples were analysed in triplicate and average Ct values obtained for the different probe concentrations are shown in Table 5.3. As observed in the primer optimisation experiment, the Ct values between assays with different probe concentrations did not vary greatly, but the ΔRn differed between samples. The Ct values and ΔRn values are displayed in Figure 5.2.

These results demonstrated that a JPV real-time PCR assay could be successfully performed with the JPV probe at concentrations lower than 250 nM, but higher than 50 nM. Using a lower probe concentration would reduce the cost of the assay, however as the probe concentration must never be limiting, and a high ΔRn must be achieved, the decision was made that all subsequent reactions would be carried out with a probe concentration of 250 nM.

Determination of efficiency curve for JPV real-time PCR assay

For a highly sensitive and reproducible real-time PCR assay with cDNA as the substrate for amplification, Applied Biosystems recommends a primer concentration of 900 nM and a probe concentration of 250 nM. These recommendations were verified by the primer and probe optimisation experiments. It should be noted that in both optimisation experiments 18S rRNA primers and probe were included in the PCR reactions. The 18S rRNA primers and probe were duplexed to ensure experimental results would be comparable to optimisations.

In addition to primer and probe optimisations, the real-time PCR reactions were analysed to ensure that the housekeeping gene and target sample nucleic acid were amplified with equal efficiencies. The efficiency, at which the sample and reference real-time PCR reactions occurred, relative to each other, determined how the data were normalised.

A standard curve was generated for both the JPV N gene and 18S rRNA amplification. Reactions were carried out in the same tube, using standard real-time PCR conditions. Dilutions of JPV cDNA, ranging from 10^{-2} to 10^{-8}, were prepared for amplification. The 10^{-8} dilution of JPV cDNA was omitted from the standard curve as the Ct values obtained varied significantly between replicates, due to the low amount of template nucleic acid present in the reaction. The Ct values obtained from the JPV N and 18S RNA assays were plotted against the relative amount of JPV cDNA present in the different dilutions (Figure 5.3).

Table 5.3- Average Ct values from the JPV N Probe Optimisation

Probe Concentration (nM)	Average Ct Value
50	22.56
100	22.17
150	21.82
200	21.98
250	21.83

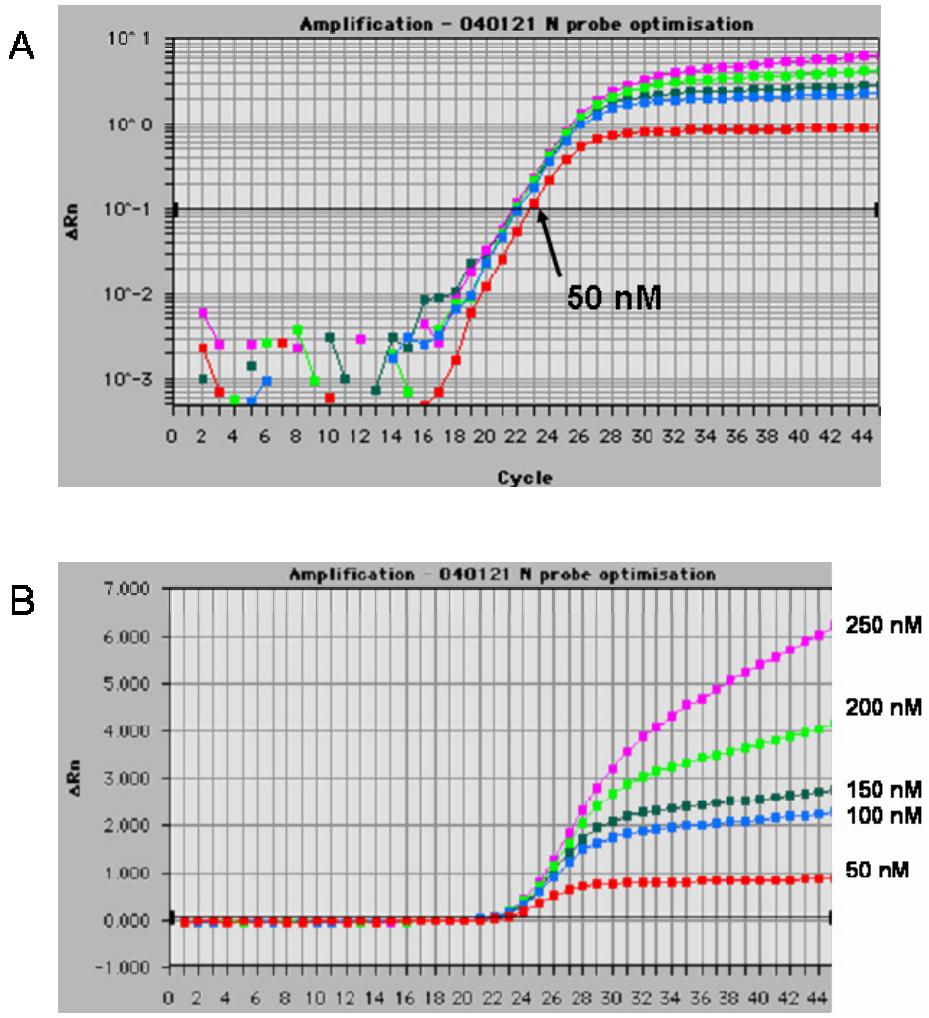


Figure 5.2- Probe Optimisation Assay

The JPV N probe optimisation assay was performed according to the manufacturer's instructions. Results from the probe optimisation assay display the Ct values, and ΔRn of the samples on a logarithmic scale (A) and a linear scale (B), with Ct values on the x-axis, and ΔRn on the y-axis.

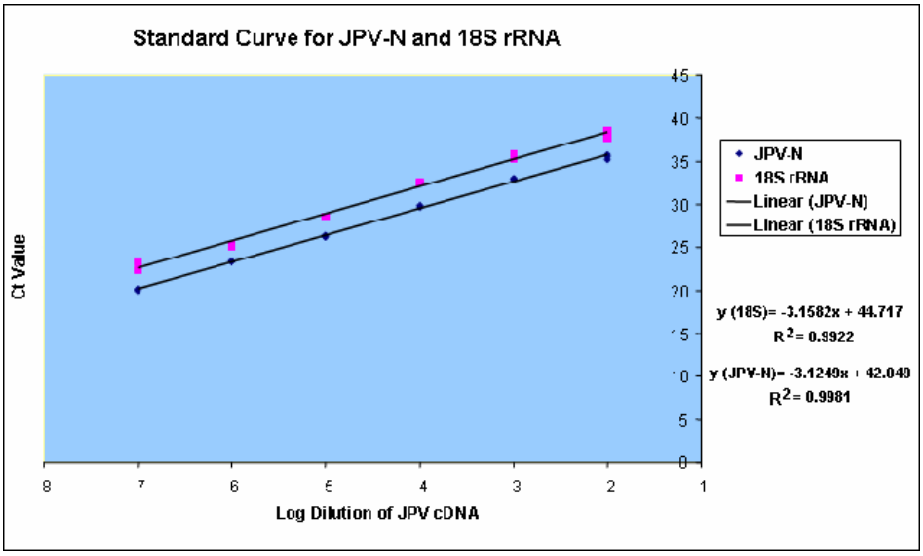


Figure 5.3- JPV N and 18S rRNA Efficiency Curve

A dilution series of JPV cDNA was analysed to determine the efficiency of JPV and18S rRNA amplification. The slope of the curves were given in the equation y=mx+c, where m was the slope. The efficiency of template amplification in the real-time PCR reactions was calculated using the equation E (Efficiency) = $10^{(-1/slope)}$.

The efficiencies of JPV N and 18S rRNA amplification were 2.089 and 2.073 for JPV N and 18S rRNA, respectively. These calculations demonstrated that JPV and 18S rRNA were amplified in the real-time PCR reaction with equal efficiency. An efficiency value of 2.0 signifies that a decrease in Ct value by 1 leads to an increase of viral genetic material by a factor of 2.0.

5.3.2 Detection of JPV in rodent tissue by real-time PCR

RNA was obtained from tissue samples removed from JPV infected mice, and subsequently converted to cDNA. A summary of tissues removed from mice is given in Table 5.4. Real-time PCR assays were performed on all tissues, but brain samples were not included in the analysis as there was a possibility that cross-contamination between brain and lung samples may have occurred during excision of tissue from mice (Philippa Jack, personal communication). All real-time PCR assays utilised standard cycling conditions. Prior to data analysis, the baseline and threshold were manually set in order to display the real-time PCR amplification plot. For analysis of JPV N amplification, the baseline was set at 3-15 and the threshold at 0.2. To view 18S rRNA amplification, the baseline was set at 3-8 and the threshold at 0.15. These parameters remained constant throughout experiments to eliminate variation between samples and reaction plates. An example of real-time PCR results, for different mouse tissues, is given in Figure 5.4. The JPV amplification curves are given in Figure 5.4A, and the corresponding 18S rRNA amplification curves are given in Figure 5.4B.

A Q-Gene Core Module file was created for each real-time PCR experiment. Once the data from each experiment was entered into a module, results were manually evaluated. The negative control samples were negative for viral RNA amplification in each experiment. . All data pertaining to brain samples (which may have been contaminated), controls (SARS cDNA, negative H_20 and positive JPV cDNA) and negative samples (triplicates with Ct values of 45) were removed to facilitate analysis of positive results.

All results derived from positive samples were compiled in a Q-Gene Database spreadsheet. The JPV RNA amplification data were normalised against 18S rRNA amplification, then the amplification of each sample was compared, relative to a calibrator. The calibrator chosen was the tissue sample to which amplification of the JPV N RNA, for each of the experimental samples, were compared against.

Mouse ID	Day	Date	Lung	Liver	Spleen	Kidney	Brain
K1	-1	20 1.03	+	+	+	+	+
K2	-1	20 1.03	-	+	-	+	+
TT1	0	21 5.03	+	-	-	-	-
TT2	0	21 5.03	+	-	-	-	-
CC1	1	22 5.03	-	+	-	+	+
TT3	1	22 5.03	+	+	+	+	+
TT4	1	22 5.03	-	+	-	+	+
TT5	1	22 5.03	+	+	+	+	+
TT6	2	23 5.03	-	+	-	+	+
TT7	2	23 5.03	+	+	+	+	+
TT8	2	23 5.03	+	+	+	+	+
TT9	3	24 5.03	+	+	+	+	+
TT10	3	24 5.03	+	+	+	+	+
TT11	3	24 5.03	-	+	-	+	+
CC2	4	25 5.03	-	I	-	I	I
TT12	4	25 5.03	+	+	+	+	+
TT13	4	25 5.03	-	+	-	+	+
TT14	4	25 5.03	+	+	+	+	+
TT15	5	26 5.03	+	+	+	+	+
TT16	5	26 5.03	-	+	-	+	+
TT17	5	26 5.03	+	+	+	+	+
CC3	7	28 5 03	+	+	+	+	+
TT18	7	28 5.03	+	+	+	+	+
TT19	7	28 5.03	+	+	+	+	+
TT20	7	28 5.03	+	+	+	+	+
TT21	21	11 6.03	+	+	+	+	+
TT22	21	11 6.03	I	I	-	I	I
TT23	21	11 6.03	+	+	+	+	+

Table 5.4- Mouse Tissue Samples

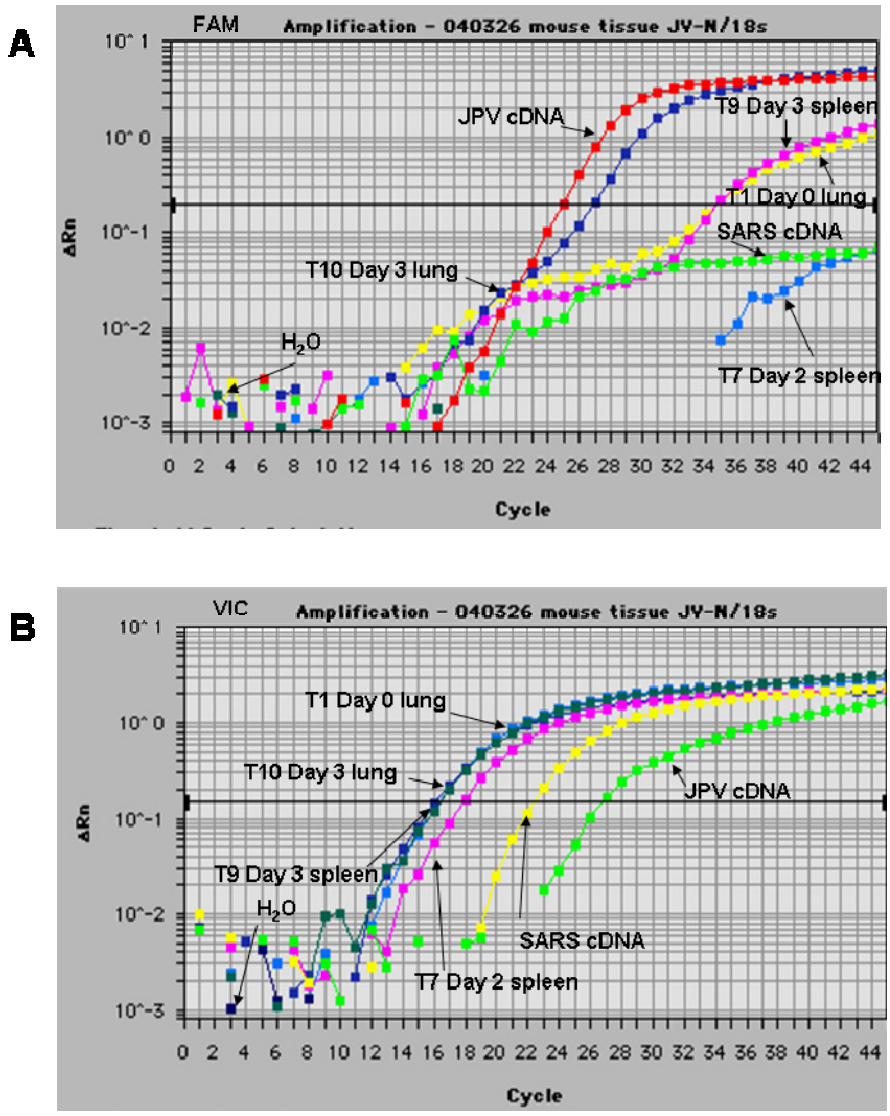


Figure 5.4- JPV N and 18S rRNA Real-Time PCR

An example of real-time PCR results for different mouse tissues displaying JPV amplification curves (A) and the corresponding 18S rRNA amplification curves (B) are shown.

Lung tissue from Mouse 1 at Day 0 (TT1) was chosen as the calibrator for analysis, as this was the earliest sample from which JPV was isolated (Miller 2004). The mean normalised expression of each tissue sample was divided by the mean normalised expression of the calibrator. Numerical values obtained for tissue samples were expressed as 'Mean Normalised Expression Relative to Calibrator', which indicated amount of viral RNA in the sample compared with the amount of viral RNA present in the lung sample of Mouse 1 at Day 0. A value higher than 1.0 indicated more viral RNA was present in the sample than the lung tissue of Mouse 1 at Day 0. For example, lung tissue of Mouse 9 at Day 3 had a value of 148, indicating that Mouse 9 had approximately 148 times the amount of viral nucleic acid in the lung at time of sacrifice than Mouse 1. A value below 1.0 indicated less viral nucleic acid was present in the sample than the lung tissue of Mouse 1 at Day 0. The 'Mean Normalised Expression Relative to Calibrator' of all positive samples is displayed graphically in Figure 5.5.

5.3.3 Purification of antigens for multiplex microsphere assay

Purification of RNP

JPV RNP was purified from JPV infected cells. The JPV RNP band was removed from the sucrose, CsCl gradient and resuspended in TNE buffer. The JPV RNP purification was performed in duplicate and these RNP samples were analysed by Western blot. The intensity of proteins in purified JPV, crude JPV and JPV RNP were compared when detected by JPV antiserum (Figure 5.6). Although the protein level in the JPV RNP samples was less than that of the purified JPV samples, only the JPV RNP was coupled to microspheres, considering that purity was most important in this context. The RNP samples were combined, before coupling to bead set #46. The MosPV RNP was purified by Gary Crameri and provided as five aliquots, which were combined before coupling to bead set #43.

Purification of recombinant BeiPV P protein

The BeiPV P gene was cloned into the pHMN vector and the recombinant P protein was expressed in *E. coli*. Six colonies were selected for IPTG induction. Proteins from cell lysates were separated by SDS-PAGE and a His-tagged protein with a molecular mass of approximately 58 kDa was detected by Western blot (Figure 5.7A), and also on a Coomassie stained gel (Figure 5.7B). The recombinant BeiPV P protein was expressed at equivalent levels in bacterial cells with, and without, IPTG induction.

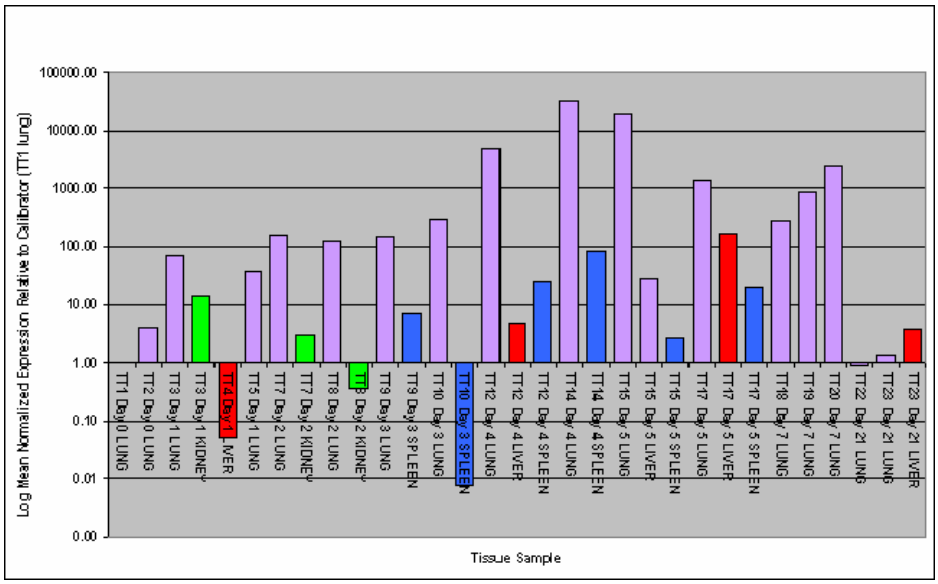


Figure 5.5- Graphical Presentation of Mouse Tissue Containing JPV RNA

Results derived from positive samples were compiled in a Q-Gene Database spreadsheet. Lung tissue from Mouse 1 at Day 0 (TT1) was chosen as the calibrator for analysis. Numerical values were expressed as 'Mean Normalised Expression Relative to Calibrator', which indicated amount of viral RNA present in the sample, compared with the amount of viral RNA present in the lung sample of Mouse 1 at Day 0. Purple bars represent lung samples, green bars represent kidney samples, red bars represent liver samples and blue bars represent spleen samples.

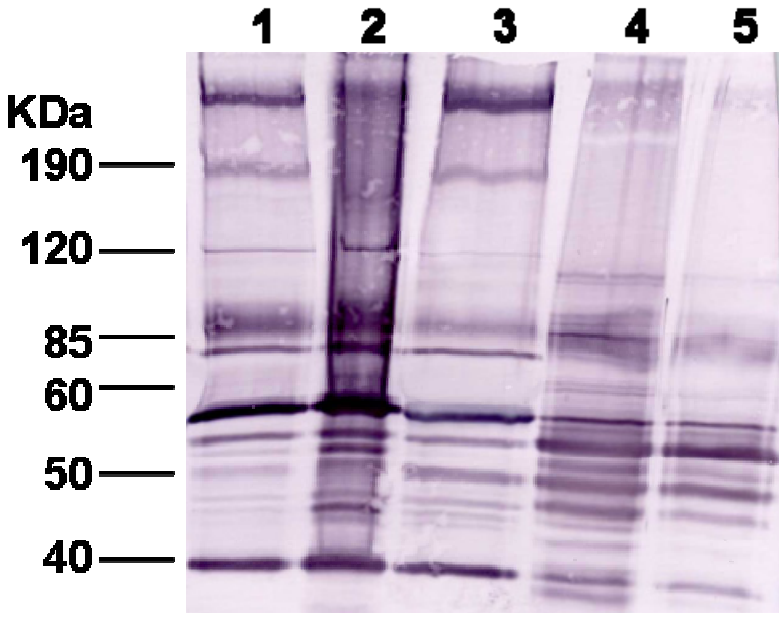


Figure 5.6- Analysis of JPV RNP by Western Blot

The Western blot was incubated with rabbit JPV antiserum and a goat anti-rabbit (Silenus) alkaline phosphatase conjugate was used for the detection of the rabbit serum. The JPV samples in lanes 1 and 2 were purified from tissue culture supernatant. The crude JPV in lane 3 was harvested by ultracentrifugation. Two samples of purified JPV RNP are shown in lanes 4 and 5. The Benchmark marker (Invitrogen) was used to determine the MW (kDa) of proteins.

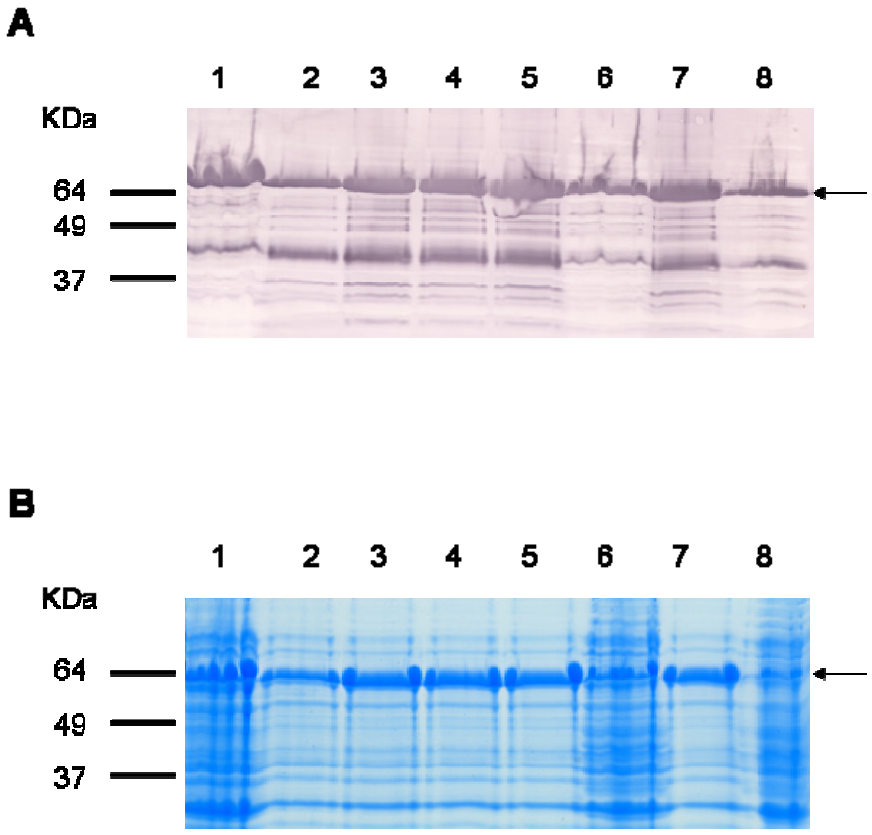


Figure 5.7- Analysis of Recombinant BeiPV P Protein Expression

The recombinant BeiPV P protein was analysed by Western blot after separation by SDS-PAGE (12% gel). The Western blot was incubated with an anti-His MAb (Qiagen) and the MAb was detected with a goat anti-mouse (Chemicon) alkaline phosphatase conjugate (A). The same samples were analysed after Coomassie staining (B). Six colonies were selected for IPTG induction. A sample of the overnight bacterial culture, from colony 2 culture, was harvested (lane 1) as was a pre-induction sample (lane 2). Lanes 3-8 contained protein samples from inductions from colony 1-6 cultures, respectively. The arrows indicate the position of the predicted BeiPV P protein. The Benchmark marker (Invitrogen) was used to determine the MW (kDa) of proteins.

The 58 kDa BeiPV P protein was excised and eluted from the SDS-PAGE gel. After several elutions, sufficient protein was purified for coupling to microspheres. The BeiPV P protein was coupled to bead set #42.

5.3.4 Detection of JPV, MosPV and BeiPV antibodies using a multiplex microsphere assay

Spectrally distinct microsphere subsets that excite at one wavelength but emit at slightly different wavelengths allow each microsphere subset to be individually identified, gated and quantified using Luminex® technology (Luminex Corporation, USA). Multiplexed assays rely upon the incorporation of different microsphere subsets within an individual test. Purified JPV RNP, MosPV RNP and BeiPV P protein were coupled to three different bead sets under identical conditions. Samples were measured and analysed using the Bio-Plex Protein Array System and the median fluorescence intensities (M.F.I.) were calculated for each sample.

A total of 384 rodent field serum samples were analysed. Of these, 80 were from rats captured in Malaysia and 216 were from rats captured in Australia. The remaining 88 serum samples were from mice captured in Australia. Initially, the 80 rat serum samples from Malaysia were analysed to assess the multiplex assay. In preliminary assays, two antibodies were used sequentially to detect rodent serum; the first was a mouse anti-rat antibody and the second was a biotinylated goat anti-mouse antibody. Through incubation with the mouse anti-rat antibody, and subsequent incubation with biotinylated goat anti-mouse antibody, serum from either rats or mice would be detectable. The 80 rat samples were screened in the multiplex assay. Serum samples with M.F.I. values higher than 2000 were chosen for further analysis. In all subsequent assays, only one antibody, (either biotinylated anti-rat or biotinylated anti-mouse), was used for species-specific detection of antibodies.

Rodent serum samples were systematically screened in the multiplex microsphere assay. An arbitrary M.F.I. value of 1000 was selected as the lower limit where rat serum samples were putatively positive for JPV, BeiPV or MosPV antibodies. Rat serum samples where the M.F.I. were above 1000 and the number of microsphere events was above 50 for one or more of the bead subsets are listed in Table 5.5 and these results are displayed graphically in Figure 5.8. In the Malaysian rat samples, there were four samples putatively positive for BeiPV antibodies and four samples putatively positive for MosPV. In the Australian rat samples there were five, six and twelve samples putatively positive for JPV, BeiPV and MosPV antibodies, respectively.

Description	BeV P (42)	J-virus (46)	MoV (43)
Queensland Health 6001 #14	1889.0 (167)	81.0 (55)	189.0 (96)
Queensland Health 6001 #23	199.0 (134)	777.0 (51)	1455.0 (68)
Queensland Health 6001 #25	208.0 (125)	560.0 (46)	1437.0 (99)
Queensland Health 6001 #31	345.0 (125)	1741.0 (53)	2080.0 (100)
Queensland Health 6001 #38	1032.0 (101)	680.0 (51)	579.0 (77)
Queensland Health 6001 #44	1309.0 (119)	2035.0 (48)	2221.0 (74)
Queensland Health 6024 #03	846.0 (178)	130.0 (92)	1608.0 (125)
Queensland Health 6024 #12	468.0 (171)	633.0 (81)	1124.0 (98)
Queensland Health 6024 #17	470.0 (129)	1120.0 (41)	1553.0 (75)
Queensland Health 6024 #43	1019.0 (165)	80.0 (96)	59.0 (124)
Queensland Health 6024 #49	3394.0 (142)	10547.0 (90)	13197.0 (89)
Queensland Health 6024 #55	632.0 (134)	847.0 (72)	1650.0 (76)
Queensland Health 6026 #01	510.0 (140)	1131.0 (69)	1317.0 (107)
Queensland Health 6026 #13	647.0 (109)	164.0 (51)	1019.0 (76)
Queensland Health 6035 #22	1052.0 (110)	1141.0 (51)	1569.0 (75)
University of Malaysia #01	258.0 (139)	599.0 (69)	1082.0 (102)
University of Malaysia #10	4593.0 (159)	135.0 (52)	212.0 (126)
University of Malaysia #11	989.0 (144)	401.0 (55)	1767.0 (128)
University of Malaysia #17	6060.0 (95)	185.0 (50)	446.0 (75)
University of Malaysia #28	377.0 (98)	665.0 (70)	1320.0 (75)
University of Malaysia #34	377.0 (129)	315.0 (59)	1337.0 (96)
University of Malaysia #35	2260.0 (132)	212.0 (77)	909.0 (102)
University of Malaysia #77	3582.0 (126)	137.0 (58)	349.0 (77)

Table 5.5- Multiplexed Detection of JPV, MosPV and BeiPV in Rat Serum

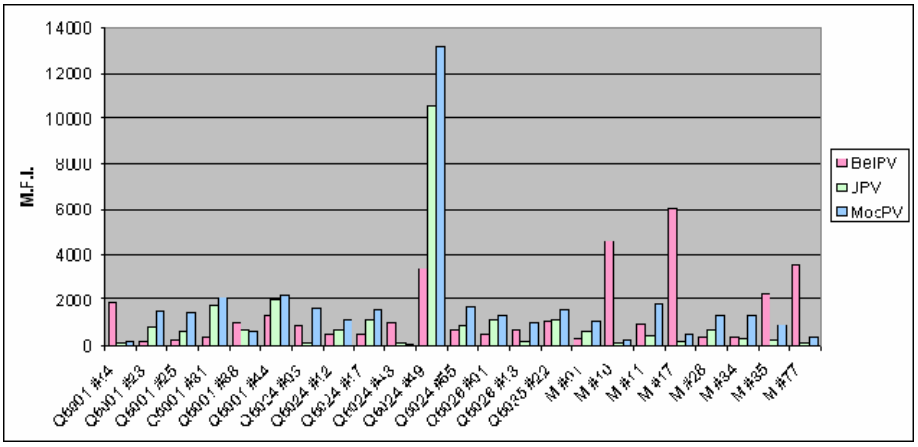


Figure 5.8- Multiplexed Detection of JPV, MosPV and BeiPV in Rat Serum

Serum samples from rats captured in Australia and Malaysia were mixed with JPV RNP, MosPV RNP and BeiPV P protein coupled microspheres. Biotinylated goat anti-rat and streptavidin-phycoerythrin were added to each sample and samples were analysed using the Bio-Plex Protein Array System. Only samples where the Median fluorescence intensities (M.F.I.) were above 1000 and the number of microsphere events were above 50 for at least one microsphere population are displayed. Sera from Queensland and Malaysia are abbreviated Q and M, respectively.

The M.F.I. values obtained were generally higher in multiplex assays containing mouse sera as compared to those containing rat sera. An arbitrary M.F.I. value of 3000 was selected as the lower limit where mouse serum samples were putatively positive for JPV, BeiPV or MosPV antibodies. Mouse serum samples where the M.F.I. were above 3000 and the number of microsphere events was above 50 for one or more of the bead subsets are listed in Table 5.6 and these results are displayed graphically in Figure 5.9. In the Australian mouse serum samples there were nine samples putatively positive for BeiPV antibodies and six samples putatively positive for MosPV antibodies. These data suggested that the rodent paramyxoviruses JPV, BeiPV and MosPV may be circulating in rodent populations throughout Australia and southern Asia.

5.4 Discussion

Molecular and serological assays are required for a wide variety of applications, including disease diagnosis and basic research such as pathogenesis studies. Ideally these assays are sensitive, specific, rapid and cost effective. In the case of new or emerging infectious diseases, there are many unknown aspects of the pathogen, such as genetic sequence, which can cause significant delays in the generation of reagents required for disease diagnosis. In the past fifty years, several rodent paramyxoviruses have been isolated. The real-time PCR assay for the detection of JPV and multiplex microsphere assay for the detection of JPV, MosPV and BeiPV were developed to facilitate future diagnosis and research pertaining to these rodent paramyxoviruses and potential new viruses related to them.

The stop/start model of paramyxovirus transcription (see Chapter 1, Section 1.6.1) results in a gradient of mRNA production inversely proportional to the distance of the gene from the 3' end of the genome (Lamb & Kolakofsky 2001). The N gene was chosen as the target for JPV real-time PCR, as mRNA from this gene is likely to be the most abundant in infected tissues. The N protein produced in MeV has been shown to aggregate and bind with cellular or MeV RNA (Kühne *et al.* 2006), suggesting that assays requiring RNA may be inhibited. Under these conditions, real-time PCR reactions can be treated with proteinase K to increase the quantity of RNA free from viral protein (Rzezutka & Mizak 2002). The JPV real-time PCR assay established within was functional as protease treatment of tissues occurred during extraction of RNA using the MagNA Pure LC RNA isolation kit.

Description	BeV P (42)	J-virus (46)	MoV (43)
Queensland Health 6002 #26	6243.0 (172)	461.0 (87)	7456.0 (112)
Queensland Health 6002 #30	2700.0 (171)	1025.0 (48)	5509.0 (132)
Queensland Health 6002 #31	4460.0 (164)	1920.0 (52)	5146.0 (91)
Queensland Health 6002 #32	3979.0 (147)	643.0 (67)	875.0 (94)
Queensland Health 6002 #36	1213.0 (129)	749.0 (51)	3043.0 (103)
Queensland Health 6002 #56	808.0 (164)	678.0 (77)	4653.0 (92)
Queensland Health 6002 #63	6909.0 (138)	973.0 (51)	3142.0 (105)
Queensland Health 6002 #65	4182.0 (142)	552.0 (59)	1608.0 (131)
Queensland Health 6002 #73	4098.0 (158)	193.0 (46)	762.0 (123)
Queensland Health 6002 #74	3334.0 (152)	677.0 (43)	2192.0 (118)
Queensland Health 6002 #78	4350.0 (144)	799.0 (36)	1594.0 (94)
Queensland Health 6005 #10	4122.0 (140)	553.0 (49)	2503.0 (98)
Queensland Health 6005 #18	2590.0 (129)	913.0 (31)	5730.0 (101)

Table 5.6- Multiplexed Detection of JPV, MosPV and BeiPV in Mouse Serum

Serum samples from rodents captured in Australia and Malaysia were mixed with JPV RNP, MosPV RNP and BeiPV P protein coupled microspheres. Biotinylated goat anti-mouse and streptavidin-phycoerythrin were added to each sample and samples were analysed using the Bio-Plex Protein Array System. Median fluorescence intensities (M.F.I.) are shown for each microsphere population and numbers in parentheses represent number of microsphere events. Serum samples where the MFI was above 3000 and the number of microsphere events was above 50 are shaded in pink and blue, for BeiPV and MosPV, respectively.

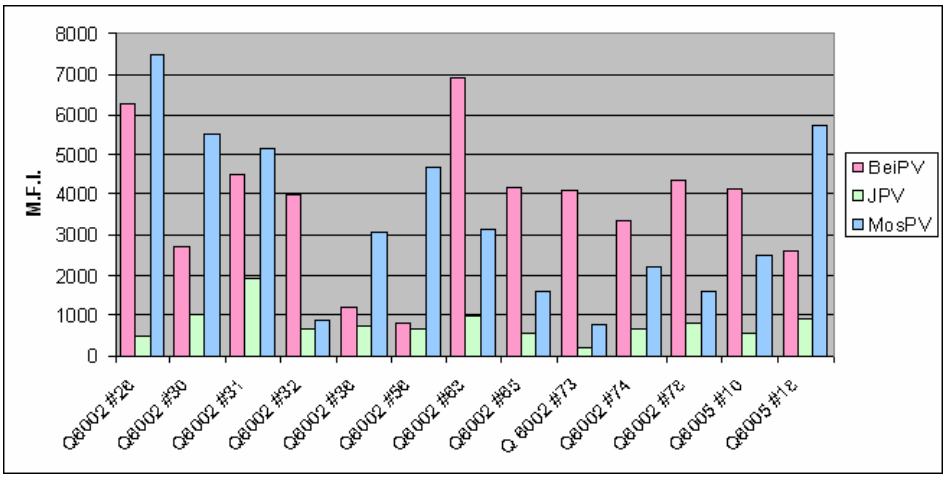


Figure 5.9- Multiplexed Detection of JPV, MosPV and BeiPV in Mouse Serum

Serum samples from mice captured in Australia were mixed with JPV RNP, MosPV RNP and BeiPV P protein coupled microspheres. Biotinylated goat anti-mouse and streptavidin-phycoerythrin were added to each sample and samples were analysed using the Bio-Plex Protein Array System. Only samples where the Median fluorescence intensities (M.F.I.) were above 3000 and the number of microsphere events were above 50 for at least one microsphere population are displayed. Sera from Queensland and Malaysia are abbreviated Q and M, respectively.

Normalisation of data is an integral part of the analysis process when using real-time PCR, as there may be differences in efficiencies of primer and probe affinities to cDNA, and these differences could be sample or tissue specific. Normalisation of data can help compensate for experimental variation. The JPV real-time PCR assay was designed in such a way that there were three possible means to normalise data. The first method in which data could be normalised was the correlation of PCR amplification results with initial amount of tissue (10 mg) used for each RNA extraction. This method was the least reliable of the three as tissue weight can vary slightly, depending on the precision of the scales used to weigh samples. Also, the weight of tissue does not necessarily correspond to the number of cells present in the tissue. In the second method, RNA concentrations could be normalised to each other. As each cDNA synthesis reaction contains 200 ng of template RNA, theoretically each real-time PCR reaction would contain the same amount of cDNA template for amplification. This method assumes that all cDNA reactions are equally efficient and real-time PCR amplification does not vary between samples.

The method of normalisation whereby data were eventually analysed was the third method. Here, data were normalised by comparison of amplification of viral RNA in samples with the gene expression of a housekeeping gene within the same sample. The presumption was made that the expression of housekeeping genes remained constant throughout different tissues, stages of cell cycle and experimental conditions.

The selection of a housekeeping gene for normalisation purposes was important as control genes should be constantly expressed, independent of experimental conditions. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene is widely used as in internal control and was considered for use in the JPV real-time PCR assay. Although widely used, GAPDH expression is variable across tissue types and levels of expression have been shown to vary with experimental conditions. In many instances, GAPDH has been reported as an unsuitable reference gene for expression studies. For example, research on neutrophils (Zhang *et al.* 2005), Atlantic salmon tissue (Olsvik *et al.* 2005), chicken muscle (Mozdziak *et al.* 2003) and normal human tissue (Barber *et al.* 2005) demonstrated that GAPDH was not an acceptable reference gene. The expression level of GAPDH was directly compared with β-actin, cyclophilin and 28S rRNA. Of these control genes, 28S rRNA displayed negligible variation across the four cell lines tested, in both hypoxic and normoxic experiments, but the use of GAPDH as a control was deemed unsuitable

(Zhong & Simons 1999). The 18S rRNA is the most abundant RNA in the cell and, although reported as variable in some studies, 18S rRNA expression was also reported as consistent, and use of 18S rRNA as a control demonstrated as reliable. For example, as reference genes, 18S and 25S rRNA exhibited stable expression in plants grown under various environmental conditions (Jain *et al.* 2006) and expression level of 18S rRNA in rice correlated with total RNA (Kim *et al.* 2003). During gene expression analysis in islet grafts, only 18S gene expression remained stable in all conditions (Rodriguez-Mulero & Montanya 2005). In order to measure expression levels accurately, normalisation by multiple housekeeping genes has been suggested (Vandesompele *et al.* 2002). Although normalisation with multiple housekeeping genes may permit a more accurate analysis of experimental data, the cost of the experiment would significantly increase. With these considerations in mind, 18S rRNA was chosen as the reference for the JPV real-time PCR assay. The amplification of JPV RNA was compared to that of 18S rRNA using a Microsoft® Excel®-based software application called Q-Gene (Muller *et al.* 2002). Q-Gene normalised RNA amplification was directly proportional to the amount of target RNA, relative to the amount of RNA of the housekeeping gene. Results were analysed by Q-Gene and the efficiencies of JPV N and 18S rRNA amplification in real-time PCR assays were found to be equivalent.

As described in Section 5.3.1, the Q-Gene worksheet calculated the mean normalised expression of each sample relative to a calibrator. Lung tissue from Mouse 1 at Day 0 (TT1) was chosen as the calibrator for analysis. Mouse TT1 was inoculated with JPV and immediately euthanised. The lung tissue from this mouse represented the earliest sample from which JPV was detected by virus isolation (Miller 2004). Although JPV was isolated from the lungs of this mouse, the amount of virus detected does not represent infection in the animal, but the virus from the aerosolised inoculum.

Numerical values obtained for tissue samples were expressed as 'Mean Normalised Expression Relative to Calibrator'. A value higher than 1.0 indicated more viral RNA was present in the sample than the lung tissue of Mouse TT1 and a value below 1.0 indicated less viral RNA was present in the sample than the lung tissue of Mouse TT1. When the results were displayed graphically in Figure 5.5, four samples (T4 Day 1 liver, T8 Day 2 kidney, T10 Day 3 spleen and T22 Day 21 lung) had MNE values of less than 1.0. These values were not negative, but demonstrated that these four samples contained less viral nucleic acid than the lungs of mouse TT1 at day 0. As mentioned above, the amount of viral RNA in the

lung of mouse TT1 at day 0 represented initial viral inoculum, explaining why subsequent samples contained lower levels of viral RNA.

Lung (days 1-7) was the only tissue from which JPV was detected by virus isolation (Miller 2004). Conversely, through analysis by real-time PCR, JPV was detected in not only lung tissue, but also liver, spleen and kidney. JPV RNA was present in both lung and liver samples from days 1 through 21. Detection of JPV RNA only occurred in spleen tissue between days 3 and 5, and JPV was not detectable in kidney tissue after day 2. The highest level of JPV RNA was detected in the lung of mouse TT14, on day 4, where the level of viral RNA was almost 32 thousand times that of the amount detected in mouse TT1. Although JPV was detected in the tissues of infected mice by real-time PCR and virus isolation, these animals did not show any clinical signs of disease (Miller 2004), contrasting original studies by Jun (Jun *et al.* 1977), where extensive haemorrhagic lesions were present in lungs of infected mice. Taken together, this work demonstrated that the real-time PCR assay was more sensitive than virus isolation in detecting the presence of virus in tissue.

Diagnostic real-time PCR assays have been developed for many paramyxoviruses, such as MuV (Kubar *et al.* 2004), RSV (Hu *et al.* 2003) and CDV (Elia *et al.* 2006). Rapid, specific and sensitive results from real-time PCR assays allow diseases to be detected earlier, making quarantine or treatment options available sooner. Studies on NDV demonstrated that detection of virus by real-time PCR correlates well with virus isolation, but has the advantage of being quicker (Wise *et al.* 2004). Real-time PCR assays have been shown to be more sensitive and quicker than convention PCR based assays, a factor which is especially important when diagnosing infection with zoonotic agents, such as RABV (Nagaraj *et al.* 2006) and HeV (Smith *et al.* 2001). Although further validation is required before the JPV real-time PCR assay can be used as a diagnostic assay, the test is currently useful as a tool for the detection of virus, for research purposes. The development of a real-time PCR assay for BeiPV, or any other rodent paramyxovirus, should be straightforward, using the same parameters as those used for the design of the JPV assay.

Multiplexed assays can deliver rapid, economical, and accurate results. The microsphere assay for the detection of rodent paramyxovirus antibodies was established to determine the seroprevalence of JPV, BeiPV and MosPV in rodents in Australia and Malaysia. The preliminary multiplex microsphere assay for the detection of JPV, BeiPV and MosPV antibodies was performed with an additional incubation step. Incubation with a mouse anti-rat antibody was included prior to the

incubation with the biotinylated goat anti-mouse antibody. This method would theoretically allow sera from either rats or mice to be detectable. This procedure was initially adopted to allow analysis of rodent sera, without the need to distinguish whether the sample originated from a rat or a mouse. There are many reasons why this approach would be advantageous. Diagnostic specimens may be submitted for analysis and the origin of the sample may not be known. An animal may have been incorrectly identified; hence incorrect reagents may be used in a diagnostic assay. The multiplex assay may be completed more rapidly if species from which serum samples were obtained does not have to be determined. Although there were valid reasons for developing the multiplex microsphere assay in a manner in which differentiation of rodent samples was not required, this approach was not pursued. There was a possibility that the addition of the mouse anti-rat antibody interfered with the kinetics of the assay. As specificity and sensitivity were important, the decision was made to omit the mouse anti-rat antibody from subsequent assays.

The data obtained from the multiplex microsphere assay suggested that the rodent paramyxoviruses, JPV, BeiPV and MosPV, may be circulating in rodent populations throughout Australia and southern Asia. It must be noted that the data presented is preliminary and further validation of this assay would be required to definitively determine the presence of antibodies to JPV, BeiPV and MosPV in the Australian and southern Asian rodent population. Adequate rodent positive controls are also required to determine the specificity of the multiplex assay. Ideally, recombinant proteins, instead of RNP, would be more suitable JPV and MosPV antigens to couple to each bead set, and may offer a higher level of specificity within the multiplexed assay. Furthermore, the level of protein expression can be easily quantified and, once induction conditions are optimised, recombinant protein is easier to produce and purify than RNP.

Several multiplex microsphere assays for use on the Bio-Plex Luminex® platform have been developed for a variety of applications. Foot and mouth disease is an economically important disease affecting cloven hoofed animals. A serological assay has been developed to enable the differentiation between animals infected or vaccinated with foot and mouth disease virus (Clavijo *et al.* 2006). Microsphere assays have been used for the detection of donor-specific antibodies following organ transplantation. This assay may assist in identifying patients that require immune monitoring or immunosuppressive therapy following transplantation (Gibney *et al.* 2006).

Bead-based molecular technology has been utilised to develop a molecular assay to detect and differentiate different animal pestivirus strains (Deregt *et al.*

2006). In a highly specific and sensitive molecular assay, all 22 types of human papillomavirus were detected (Schmitt *et al.* 2006). Multiplex microsphere assays are not only useful in the detection of disease. This technology has been used to examine microRNA profiles to classify human cancer (Lu *et al.* 2005).

In conclusion, two assay platforms were established, one for the molecular detection of JPV, and another multiplexed serological assay to test rodent species for antibodies to JPV, BeiPV and MosPV. The JPV real-time PCR assay was utilised to monitor the experimental infection of mice with JPV. The multiplex microsphere assay was established to determine the seroprevalence of JPV, BeiPV and MosPV in Australian and southern Asian rodent populations. Both assays require further validation, but may be valuable tools for research involving rodent paramyxoviruses.

CHAPTER 6

General Discussion and Future Directions

In the past fifty years there have been many instances where new viruses have either emerged or been discovered, often accompanied by spectacular disease outbreaks. Several of these emerging infectious diseases were caused by paramyxoviruses and the emergence of HeV and NiV, both zoonotic, highlighted the severity of disease that could be caused by infection with viruses from this family. In addition to causing disease outbreaks, several newly discovered paramyxoviruses were found to have unique genetic features or novel genes. JPV, a newly characterised rodent paramyxovirus, together with BeiPV, a recently discovered rodent paramyxovirus, represent a new lineage of paramyxoviruses. Although there is no evidence to date that JPV and BeiPV cause disease, the unique genome characteristics of these viruses provided an excellent opportunity to research the genetic diversity within the paramyxovirus family.

JPV was originally isolated from wild mice captured in northern Queensland, Australia, in 1972 (Jun *et al.* 1977). Apart from initial studies, JPV remained uncharacterised for two decades until the emergence of HeV and NiV. Both HeV and NiV exhibited unique genetic and biological features, unlike other previously characterised paramyxoviruses, and renewed interest in the unclassified rodent paramyxovirus. When the genome of JPV was sequenced, two novel genes, SH and TM, and a second ORF within the attachment protein gene were revealed (Jack *et al.* 2005). Following the characterisation of the JPV genome, another rodent paramyxovirus, BeiPV, was isolated and sequencing of the BeiPV genome revealed that this virus also contained two novel genes, SH and TM, and an extended attachment protein gene (Li *et al.* 2006).

JPV and BeiPV are unique within the family *Paramyxoviridae*, and BeiPV contains the largest genome, which is, in fact, larger than that of any virus within the order *Mononegavirales*. The research presented within initially outlined the establishment of reverse genetics systems for JPV, BeiPV and NiV. These GFP minireplicon systems were constructed to aid in the taxonomic classification of JPV and BeiPV, and to determine the relationship between these viruses and the paramyxoviruses formerly containing the largest genomes, the *Henipaviruses*.

Prior to complete genetic characterisation of the virus, the suggestion was made that BeiPV should be classified within the genus *Henipavirus* (Schomacker *et al.* 2004). In order to provide more evidence for taxonomic classification,

experiments were conducted using the newly constructed BeiPV and JPV minireplicon systems. Replication of the BeiPV minigenome was possible when either BeiPV or JPV polymerase proteins were co-transfected, but replication did not occur upon co-transfection of NiV polymerase proteins. The same results were observed when a JPV minigenome was co-transfected with JPV, BeiPV or NiV polymerase proteins. Replication of the JPV minigenome only occurred when JPV or BeiPV polymerase proteins were supplied, not NiV polymerase proteins. The NiV minigenome was not functional when either JPV or BeiPV polymerase proteins were co-transfected, demonstrating that the replication machinery was not interchangeable between NiV, a henipavirus and either JPV or BeiPV. These results provided support for the classification of BeiPV and JPV into a new genus. Importantly, these data also demonstrated that the BeiPV and JPV minireplicon systems were constructed correctly and were functional.

Once established, practical applications of the JPV, BeiPV and NiV minireplicon systems were used to conduct more detailed molecular and functional analyses. These minireplicon systems were utilised to demonstrate that both JPV and BeiPV were compliant with the 'rule of six' (Calain & Roux 1993). Previous studies demonstrated that a NiV minireplicon system was only functional when the NiV minigenome was compliant with the 'rule of six' (Halpin *et al.* 2004). By comparison, the addition of four nucleotides to either the JPV or BeiPV minigenomes rendered both JPV and BeiPV minireplicon systems non-functional. Like other paramyxoviruses, both JPV and BeiPV must obey the 'rule of six' to replicate.

The minireplicon systems were used to study the relatedness of the BeiPV and JPV polymerase proteins at the molecular level, specifically how they recognised the genome. A previous study demonstrated that the genomic termini of the Reston and Zaire strains of EBOV were interchangeable (Boehmann *et al.* 2005). The 3' and 5' genomic termini of JPV and BeiPV were interchanged in the minigenome plasmids and under these conditions, the JPV and BeiPV minireplicon systems were fully functional. Both chimeric JPV and BeiPV minigenomes were transcribed by either JPV or BeiPV polymerase proteins, again indicating that these viral polymerases were structurally similar. To examine the structural similarity of the JPV and BeiPV polymerase complexes, the individual N, P and L proteins were combined and co-transfected with both JPV and BeiPV minigenomes. Although the JPV and BeiPV proteins share some sequence homology, individual JPV and BeiPV proteins were unable to interact in heterotypic combinations to form functional polymerase complexes.

The transcription and translation of the P gene is a complex process for paramyxoviruses and can give rise to several different proteins, including P, V, C, and W. Expression of the C protein, produced through RNA editing of the P gene (Thomas *et al.* 1988), has been shown to have a negative effect on minireplicon systems (Curran *et al.* 1992; Cadd *et al.* 1996; Malur *et al.* 2004). Using the minireplicon for BeiPV and JPV, similar results demonstrated that the C proteins expressed from the viral P genes had inhibitory effects on the replication of these minigenomes. Mutation of the start codons within the C genes of both JPV and BeiPV P genes prevented expression of the JPV and BeiPV C proteins. Both JPV and BeiPV minireplicon systems functioned with higher efficiencies when the C deletions were incorporated into the P support plasmids. Upon addition of JPV and BeiPV C-encoding plasmids, the replication efficiencies of the JPV and BeiPV minireplicon systems decreased in a dose dependent manner. Although these results demonstrated the both JPV and BeiPV C proteins were disadvantageous to the functionality of the JPV and BeiPV minireplicon systems, respectively, results may not necessarily correlate with what would be seen in a natural infection. Studies on SeV and MeV have demonstrated that the C protein, previously regarded as an accessory protein, is essential for the replication of virus (Sugahara *et al.* 2004; Sakaguchi *et al.* 2005; Hasan *et al.* 2000; Takeuchi *et al.* 2005). Construction of a full-length infectious clone would be necessary to analyse the effect of C protein expression on viral replication of JPV or BeiPV.

All known NNSV contain the highly conserved GDNQ sequence motif in domain III of their respective L polymerase proteins, with the exception of NiV, HeV, TPMV (Wang *et al.* 2001) and MosPV (Miller *et al.* 2003), which contain a GDNE sequence motif. Like most paramyxoviruses, JPV contains GDNQ in this region. Domain III of the NNSV L protein has been determined as functionally important for catalytic activity of the polymerase protein (Poch *et al.* 1990). To determine the effect mutations have within this region, the sequence motifs of NiV and JPV were interchanged and function was evaluated using minireplicon systems. The substitution of the JPV L protein containing the GDNQ to a JPV L protein containing the GDNE motif did not have a significant effect on the JPV minireplicon system. Similarly, the substitution of the NiV L protein containing the GDNE to a NiV L protein containing the GDNQ motif did not have a negative effect on the NiV minireplicon system. Upon further evaluation, substitution of the E residue in the NiV GDNE motif with K, P, D, A, N, I and G residues all reduced or abolished the functionality of the NiV minireplicon system. Together, these results suggested that

the amino acid at the E position plays an important role in maintaining structure not catalytic activity in the L polymerase protein.

Together, the research outlined thus far has demonstrated the application of reverse genetics systems for the classification of JPV and BeiPV, and for the functional studies of viral polymerase proteins and specific domains within these proteins. Reverse genetics systems have other attractive applications including the capability to generate recombinant viruses for pathogenesis studies. Construction and identification of attenuated recombinant viruses may represent potential vaccine candidates. Although JPV and BeiPV have not caused disease outbreaks thus far, a vaccine candidate would potentially be very beneficial in the event of an outbreak. Reverse genetics has been utilised to engineer NDV and avian influenza virus, creating attenuated recombinant viruses that when used as vaccines protect against both diseases (Veits *et al.* 2006; Park *et al.* 2006). As JPV and BeiPV have not been associated with disease, they could potentially be used as carrier vaccines, encoding other viral proteins. Recombinant HPIV3 was constructed to express the EBOV structural glycoproteins and when used as a vaccine, protected guinea pigs against a lethal-dose EBOV challenge (Bukreyev *et al.* 2006). Recombinant HPIV3 has also been modified to express a SARS envelope glycoprotein and this virus induced a systemic immune response, resulting from mucosal immunisation (Bukreyev *et al.* 2004).

Although several successful examples have been listed, vaccine development is a complicated process and several factors must be taken into account if reverse genetics will be used. The source of the polymerase required to initiate virus replication, such as the FWPV-T7 polymerase used in these studies, adds another virus to the system and may limit downstream applications, such as vaccine development. An alternate method to viral infection providing T7 RNA polymerase for expression of genes under the control of T7 promotors is the utilisation a stably transfected cell line. A BHK cell line stably expressing T7 RNA polymerase, BHK/T7-9, was created to eliminate detrimental effects of viral infection on cells used in reverse genetics experiments (Ito *et al.* 2003). Although BHK cells have been engineered to stably express T7 RNA polymerase, to date, the only live virus vaccine licensed for human use, derived from a continuous cell line, was produced in Vero cells (Rotavirus Vaccine, RotaTeq, Merck & Co., USA, License #0002). An efficient system has been developed for the rescue of recombinant paramyxoviruses from Vero cells, and this system does not rely on helper virus, but on plasmid-expressed T7 RNA polymerase (Witko *et al.* 2006).

Perhaps the most important consideration for vaccines generated with reverse genetics is the construction of a full-length infectious clone for use as the viral backbone. For JPV, the construction of a full-length infectious clone is currently underway and is expected to be completed in the near future. As mentioned above, JPV represents a promising viral backbone for vaccine development as there is currently no evidence that JPV causes disease and it is unlikely that animals, besides rodents, would have neutralising antibodies against JPV. Significantly, incorporation of foreign genetic material into the JPV attachment protein gene has been demonstrated without affecting the incorporation of this new fusion protein into the mature JPV virions. Specifically, the incorporation of a fragment of the SARS spike gene into the JPV G gene did not have a negative effect on G protein expression or virus particle formation. Together these data demonstrated that JPV had the potential to express foreign genes and be used as a potential vaccine candidate. A complete understanding of the novel genetic characteristics of the JPV genome and all encoded proteins will aid any attempts of vaccine development using this virus. Such studies will also help to understand the new lineage of paramyxoviruses that JPV and BeiPV represent.

As mentioned previously, the genomes of JPV and BeiPV were found to contain two genes, SH and TM, not common to other viruses within the paramyxovirus family, as well as extended attachment protein genes. The presence of an SH gene is only found in JPV, BeiPV, MuV and SV-5 and no sequence homology between these SH proteins has been identified. The deletion of the SH gene of RSV, a member of the subfamily *Pneumovirinae*, did not affect virus growth (Bukreyev *et al.* 1997), suggesting the SH protein may have an accessory function. The SH protein of MuV is capable of inhibiting host cytokine signalling pathways (Wilson *et al.* 2006) and appears to function similarly to the SH protein of SV-5, which is capable of blocking apoptosis (He *et al.* 2001). The SH proteins of JPV and BeiPV may also function to inhibit host defence mechanisms upon virus infection.

The epitope tagging studies on the JPV SH and TM proteins did not provide definitive evidence for the localisation of these proteins, but did substantiate evidence that they are surface glycoproteins (Jack *et al.* 2005). Once the construction of the full-length infectious clone for JPV is complete, deletion of the SH and TM genes from the JPV genome may provide insight into the function of the SH and TM proteins. Given the functions of the SH protein of MuV and SV-5 in the inhibition of host cell defences, it seems likely that the SH proteins of JPV and BeiPV will function through a similar mechanism. There is no homolog to the JPV TM gene in any other paramyxovirus, with the exception of BeiPV; hence it is

difficult to speculate on the function of the JPV and BeiPV TM proteins. The only other paramyxovirus containing a unique gene is FDLV, the reptilian paramyxovirus that contains a U gene between the N and P genes (Kurath *et al.* 2004). Although not experimentally tested, a small transmembrane protein was predicted to be expressed from the U gene (Kurath *et al.* 2004). The function of this U gene has not been determined, and it would be interesting to investigate whether there is any similarity in the functionality of the JPV and BeiPV TM proteins with the FDLV U protein, considering they are all predicted transmembrane proteins.

The JPV attachment gene produces a protein containing 709 amino acids, structurally similar to other paramyxovirus attachment proteins, and also contains a long 3' extension of 2,218 nucleotides after the stop codon (Jack *et al.* 2005). The 3' extension of the attachment gene consists of a 2,115 nucleotide second ORF, ORF-X, which begins directly after the stop codon. JPV may utilise an unconventional mechanism to translate a protein from ORF-X, but so far there is no evidence to suggest this is the case. The stop codon separating the two ORFs of the G gene may be a recent mutation, and prior to the mutation event a protein of 1,414 amino acids in length may have been translated from the JPV G gene. If this hypothesis is correct JPV could represent an ancient lineage of paramyxoviruses. The mutagenesis of the stop codon separating ORF-G and ORF-X, and subsequent incorporation of the resulting GX protein into mature virions supports the hypothesis that JPV may have once encoded an attachment protein twice the size of the current version of the attachment protein.

The constitution of the BeiPV attachment gene further supports the hypothesis that JPV and BeiPV represent an ancient lineage of paramyxoviruses. It is tempting to speculate that a paramyxovirus, precursory to JPV, encoded a large attachment protein from the G gene. Subsequently, JPV evolved from this virus when a smaller attachment protein encoded from the G gene made JPV more evolutionarily 'fit' than the precursor. Additional mutations introduced throughout the G gene and the genome may represent the evolution of BeiPV from JPV. Within the BeiPV G gene there are two ORFs and with sufficient time these viruses may further evolve to completely eliminate the excessive RNA contained within the G gene in order to maintain a compact genome organisation, which offers a selective advantage for an RNA virus where the genome is replicated rapidly and frequently (Domingo & Holland 1997). However, as the genome can accommodate a large G gene, this may represent the ideal site to insert a foreign gene for vaccine development.

The above hypotheses rely upon the presumption that the full-length G-X protein produced from the G gene was somehow disadvantageous to the precursor virus. The attachment proteins are the major determinants of tissue tropism for NDV (Huang *et al.* 2004) and TuPV (Springfeld *et al.* 2005), so perhaps the loss of G-X significantly altered tissue tropism somehow enabling increased fitness. Constructing a JPV full-length infectious clone containing the G-X attachment protein may redirect the tissue tropism of JPV, as was the case when the MeV H attachment protein was restructured (Hadac *et al.* 2004). Additionally, the overall fitness of viruses that encode G and G-X attachment proteins can be compared using reverse genetics, including their influence on disease status. Perhaps viruses that encode G-X attachment proteins are more pathogenic in an infected host. Host antibodies directed against surface glycoproteins are critical for maintaining viral immunity. Recently, the pathogenicity of MeV was shown to be enhanced by antibodies directed against the H protein (Iankov *et al.* 2006). Infection of animals with JPV containing the G or G-X genes may demonstrate a similar effect on host immunity. The advent of a reverse genetics system utilising full-length infectious clones for JPV and BeiPV will facilitate studies that shed light on the evolutionary relationship between these viruses and other paramyxoviruses.

Once a JPV reverse genetics systems is constructed, the diagnostic tools developed for the detection of JPV, as outlined within, could be utilised to monitor the success of the system. The level of JPV nucleic acid could be monitored using the JPV real-time PCR assay, and experimental infection of host rodents could be monitored using the multiplex microsphere assay. Although the real-time PCR assay and multiplex microsphere assay were mainly developed to monitor tissue tropism of JPV and seroprevalence of JPV, BeiPV and MosPV in rodents, respectively, the diagnostic tests could also be used to examine the functionality of a JPV reverse genetics system. Full-length reverse genetics systems for JPV and BeiPV may not only be used to study the evolutionary relationship of these paramyxoviruses, or the development of paramyxovirus vaccine vectors, but in many other applications. For example, paramyxoviruses may be engineered by reverse genetics to function as anti-cancer agents (Kirn *et al.* 2001; Grote *et al.* 2001; Suter *et al.* 2005).

In summary, the work presented within, in its entirety, has pioneered new tools and assays which have proved to be valuable for providing answers to many interesting and intriguing questions pertaining to two previously uncharacterised rodent paramyxoviruses. Firstly, the establishment and requirements of functional reverse genetics systems for JPV and BeiPV were described. These reverse genetics systems were primarily utilised to provide evidence for the classification of

JPV and BeiPV into a new genus. Additionally, to better understand the biology of the viruses, novel viral surface proteins from JPV were expressed and evaluated. The attachment gene of JPV provided the strongest evidence in support of the hypothesis that JPV and BeiPV may represent an ancient lineage of viruses within the family *Paramyxoviridae*. In order to understand tissue tropism of JPV *in vivo* and to aid future work relating to JPV pathogenesis, a real-time PCR assay for JPV was developed and utilised. Finally, to address the risk JPV and BeiPV pose in native rodent populations, a multiplex microsphere assay for JPV and BeiPV serology was used to analyse the seroprevalence of these viruses in Australian and Malaysian rodents. Although there is no evidence for disease caused by JPV or BeiPV, this does not preclude the emergence of a zoonotic rodent paramyxovirus related to these viruses. Importantly, if such a situation was to occur, the tools for virus detection and serological monitoring are now established.

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Appendix I: Primer Sequences

	Minigenome Primer (DM) Sequences
DM01	CGGACACGCTGAACTTGTGG
DM02	CCAACGAGAAGCGCGATCAC
DM03	AGCCGGCGCCAGCGAGGAGGCTGGGACCATGCCGG
DM04	TCGCGGATCCATGCGGCCGCTTTAACCTTCCAAATCCA
DM05	ACGCGGATCCTCACCATGGATGATATATTTCTTG
DM06	GCCCAAGCTTAATACGACTCACTATAAACCTAACAAAGTAGGT
DM07	GTAAAGCGGCCGCAGTTGGTGACAACTCG
DM08	CTGGGACCATGCCGGCCACCAAACAAAGAAGTG
DM09	CTCACCATGGTGTGGAGCAACCAAGTC
DM10	CTGGGACCATGCCGGCCACCAAACAAAGATGTA
DM11	CTCACCATGGTTTGGTTATCCCAAACC
DM12	GCCCAAGCTTAATACGACTCACTATAAACCAGACAAAGGATAT
DM13	GTAAAGCGGCCGCATTTTGAGTTAACGGG
DM14	GTCGACGCGTCAATCATGTCTGGGGTGC
DM15	GTTAGCGGCCGCAATTGTAAACAAAGCTA
DM16	GTCGACGCGTCAATCATGTCAGATTATAATCCTGACGTCCTCCA
DM17	ATTACCGGTGTAATCATGATCG
DM18	GTCGACGCGTCAATCATGGCGCACCCGACTA
DM19	GTTAGCGGCCGCCCGTTAACTCAAAATCC
DM20	GTTAGCGGCCGCCCTCACCGAGGAATAGTC
DM21	GTTAGCGGCCGCCTGATGCATAACTTTCTGC
DM22	GTTAGCGGCCGCAGCTGCTAAAGCT
DM23	GTCGACGCGTCAATCATGTCCCGACTTGGA
DM24	GTTAGCGGCCGCAAGTGTGTATTTATGA
DM25	GTCGACGCGTCAATCATGTCAGATTATA
DM26	GTTAGCGGCCGCATCTAATCACTAT
DM27	AGATAGCTGCAGTGGTACAAGGGGACAATGAGGCCATTG
DM28	CTTTCGTACGTTTGGCCATCCTAT
DM29	CATAATGAATTTTCTTCGAATAA
DM30	GCTGCAATTGTCCAAGGAGACAATCAATCAATTGCTATCACT
DM31	ACAGGCTCTTCTAAATCAATTGCTATCACT
DM32	ACAGGCTCTTCATTTATTGTCTCCTTGGAC
DM33	GCTGCAATTGTCCAAGGAGACAATAAATCAATTGCTATCACT
DM34	GCTGCAATTGTCCAAGGAGACAATCCATCAATTGCTATCACT
DM35	GTCGACGCGTCAATCATGGAAACGAATTGT
DM36	GTTAGCGGCCGCATCTAATCACTAT
DM37	GCTGCAATTGTCCAAGGAGACAATGATTCAATTGCTATCACT
DM38	GCTGCAATTGTCCAAGGAGACAATGCATCAATTGCTATCACT
DM39	GCTGCAATTGTCCAAGGAGACAATAATTCAATTGCTATCACT
DM40	GCTGCAATTGTCCAAGGAGACAATATATCAATTGCTATCACT
DM41	GCTGCAATTGTCCAAGGAGACAATGGATCAATTGCTATCACT
DM42	GTTAGCGGCCGCATTTAGCTGCCTTGATTC
DM43	GTCGACGCGTCAATCATGTCCTCCAGCAGCTTGTCA
DM44	TGCGGCCGCATGATTATCTGATTATACTCTCGAGC

JPV Primer (DJ) Sequences	
DJ 01	GCTCGAGACGCGTTGATAATGACATCAACAGTG
DJ 02	CAAGCTTGCGGCCGCTATCACTTGCTTGCTGG
DJ 03	CTCGAGACGCGTCGATCATGGATATCGACG
DJ 04	AAGCTTGCGGCCGCTATATACAGGTTACTA
DJ 05	GACGCGTCAAAAATGGCAAGCAATCAGGAG
DJ 06	TGCGGCCGCTTACCACCATCCCTTCAAGTAA
DJ 07	GACGCGTTGCTCCACAATGTCGAAACTT
DJ 08	TGCGGCCGCATTGTAGGTTTAGCCATT
DJ 09	GAGGCGTCACCATGAACTCCTATAGTGTTCAG
DJ 10	TGCGGCCGCATTGATTTGTTAGTTCTCGT
DJ 11	GACGCGTAAGGTACGATGGCTGGTAA
DJ 12	TGCGGCCGCAAGTAATTTCATTTCA
DJ 13	GACGCGTACAATCATGAAACCTGTAGCTT
DJ 14	TGCGGCCGCATAGGATTGTGTTTCAGCCAAC
DJ 15	GACGCGTCACCATGAATCCCGTTGCCATGTCGA
DJ 16	TGCGGCCGCAGTGGATTAATTGTGCCCTAT
DJ 17	GACGCGACAATCATGAAACCTGTAGCTT
DJ 18	GACGCGTGCTCCACAATGTCGAAACTT
DJ 19	GACGCGTTCACCATGAACTCCTATAGTGTTCAG
DJ 20	GACGCGTTCACCATGAATCCCGTTGCCATGTCGA
DJ 21	GTCGACGCGTCAATCATGAAACCTGTAGCTT
DJ 22	GACGCGTTCACCATGAATCCCGTTGCCATGTC
DJ 23	GTCGACGCGTTCACCATGTCACTCCTTACAATGGAT
DJ 24	GTTAGCGGCCGCTCCAACCCATCTACTGAAC
DJ 25	ATCGTGGATGAGACTCGATCTGC
DJ 26	GTTAGCGGCCGCCTGTTGTTTGGCTAAATC
DJ 27	TAATGTTGCTGGGGCTTGCATC
DJ 28	GTTAGCGGCCGCAACTGATCAATCCTCAGAATAAAG
DJ 29	GACGCGTCCATGGTCAAGGACGAGCATGA
DJ 30	TGCGGCCGCTACTTTATAAAGATTTGTTTA
DJ 31	GACGCGTCCATGGTAGCCCATGAGTCCAACA
DJ 32	TGCGGCCGCTACACGGTGTTCTCAGAACCT
DJ 33	CATCTGAGCTCGGCGCGCCATAAATTATC
DJ 34	CAGAGGATCCAAGCATTGCCCCCATAGGGCACAATTCATCCACT
DJ 35	GTTAGCGGCCGCGTTGGTATTCCTTTC
DJ 36	AGACATTGCTGAGCCGATTC
DJ 37	GGC CAA GAT TGG TAG AAA GG
DJ 38	TGCATGCCAGAGACTGAGAC
DJ 39	CTCTGAGCTCAGGCGCGCCCATGGTAGCCCATGAGT
DJ 40	ATATGAATTCGCGGCCGCTACACGGT
DJ 41	GTTAGCGGCCGCACCGGCTTGTCTACATGGTC
DJ 42	GTTAGCGGCCGCCCCACTAAACCTGTAT
DJ 43	CCTATAGTGTTCAGGCACTAGAACAATTGGTGAACGACGGAATCA
DJ 44	GGTGTCGACCACTCCAGAGA
DJ 45	TGGTCGACACCATCTTGTTC
DJ 46	GAGCTCCTCGAGAGGCGCGTCTGGTGTTATT
DJ 47	GTTAGCGGCCGCTAAGTCAAAGCTGGGTATTGTATACAGGTTACTACTGAT
DJ 48	GTTAGCGGCCGCTAGCGCCAAGCTGGTGTGAGTATACAGGTTACTACTGAT
DJ 49	ATTCACTAGTACGCGTTGATAATGACATCAAC
DJ 50	CTAGCTCGAGCGGCCGCTATCACTTGCTTGC

DJ 51	GTTAGCGGCCGCTAGCGCCAAGCTGGTGTGAGCTTGCTTGCTGGATTGAGAT
DJ 52	GACGCGTTCACCATGGAATCAAAACTGCCCAAT
DJ 53	TGCGGCCGCATGATCAAATATCATCATTTGC
DJ 54	GTTAGCGGCCGCTAGCGCCAAGCTGGTGTGAGGCCATTGATAGCTGAGAGGT
	JPV Primer (PJ) Sequences
PJ1	AGACAGACGCTCCAGAGGATGTCA
PJ2	TAAGGTCTCCAGCATCAACTGTTC
PJ3	GCACATTGGGGATTGCACCACTCC
PJ4	TGTGCACCCATCACTGTTACTCCT
PJ5	CCCTAGTAGTAAGTCAATCTTGCC
PJ6	GAAAGATTACAACATGAGAGCAGG
PJ7	TGACAGTTCGATCAACAAGTTGAA
PJ8	CATGTTCATCTGAATTACACTCCA
PJ9	TTCTTGCACAACTTGTTAGGTTAC
PJ10	CACTGTTTGTNGAGTCWCCRGG
PJ11	GCAGCAACTTGCTCCTCTGAGAA
PJ12	CTACAGACACACCTAAACAG
PJ13	TTGGTAGTCCAATTGCACTT
PJ14	GAGTCATCAATCCTGGAAGA
PJ15	GCTTCTCTTCTATGATTCC
PJ16	AAATGGCTTACAAACTGTCC
PJ17	CACTGTCCGTGTAGTGCATT
PJ18	GTGCGACAGGTAACCTAACA
PJ19	CTCTCATGGAGCCACTCATT
PJ20	CAATGAGTGGCTCCATGAGA
PJ21	CGGGAGGAAACAAGTCAGTG
PJ22	GGCTCTTGGAGGATCTGTAA
PJ23	GAGACGCTCATTCACTCCTA
PJ24	TGTGGGCAAGGTAGCAATGG
PJ25	CTGCTACCCATTGCTACCTT
PJ26	CACTTGCTTGCTGGATTGAG
PJ27	CTCAATCCAGCAAGCAAGTG
PJ28	CTGGTGGGTGTGTCCGACTA
PJ29	CATGGAGGGTTGGCTTCTCA
PJ30	TCATTGTGGGCAAGGTAGCA
PJ31	TTGGTGGTGAGCTCTCTAGT
PJ32	GTATGTAGCCGATCCACACT
PJ33	TGGTAGATTAGGGTGCACTC
PJ34	CGGACAGTCACCTTGTGAAT
PJ35	AAGAACGTGCTCATCGTCTC
PJ36	CCTGAGAGCAATGAGGAGTA
PJ37	TCCCATCTGTTGTGAGTAGG
PJ38	TGGAACCCTCACTTCCTACT
PJ39	CCCTGAAGCTACATCATGTC
PJ40	TCTTGAGCCCTATCCACTTC
PJ41	TGGCACCAACTGATCAATCC
PJ42	GATATGGTGGAAGATTGTTG
PJ43	TCTGAGGATTGATCAGTTGG
PJ44	CGCTAAGAAAAACTTAGGAGT
PJ45	CGCACTCCTAAGTTTTTCTTA
PJ46	TGGTTTACCCTYAARACAGAGATGAG

PJ47	AGACATGGRGGARCWTGGCCTCC
PJ48	GCTTTATCTTTCAKRTACAT
PJ49	CAGGGTCGAATGAGGTATCC
PJ50	ACCAAGACATCCGGGACCTC
PJ51	AGCAGCCATAGCGACCAAGA
PJ52	TCCCAGATCTTCAGCAACAG
PJ53	GGCTTGAAGACAGAGAGGTA
PJ54	TTGTTTCCTCCCGGTGTCTC
PJ55	CCTCTCTAACCGGCTTGTCT
PJ56	GGCACAGCTCTTGATACTCT
PJ57	CTGCAGAAGCCATTCTCATC
PJ58	TCCAGATGGCACTCAGGATA
PJ59	GGGTTCTATCATGGCTACGA
PJ60	GTCCGGGCTCACATGAATAA
PJ61	CTTGCCGAACATATGGGTAG
PJ62	CAGCTTGCCTGTGACTATGG
PJ63	AGGGTCTGGTGCGTAAGTAT
PJ64	CAACCTCCTGCTTTGCCTAT
PJ65	AGTGACATGCACAGGCTTCT
PJ66	GTGCTGTCACTGGCCCTACT
PJ67	GTGGCTGCTTAAGAGGATCG
PJ68	ATGCCGTTAGAGGTGTACCC
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PJ70	AGCTCCTGAAGAAAGGTCTG
PJ71	TGCAGGAGTATCTGCTAAGG
PJ72	TGATCAGTTGGTGACAACTC
PJ73	CCAGACCCAGCAAGGTTCTA
PJ74	AATACCAACGCACCCAGAGG
PJ75	GACCGTTCTCGTCCCAGTAG
PJ76	CTCTCTCGACCTTCCAACTA
PJ77	CTTGTTGTAGGGCTGAGATG
PJ78	GGTAGTGCCTCTGGTAATCA
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PJ80	ACCAGAGGCACTACCCAATC
PJ81	AGCCTCCGTACAGCAGTATT
PJ82	ACTGCCCTTTAGGATATCGG
PJ83	GAAGGGATGGTGGTAAACTC
PJ84	GTTCCCTCCATAATGGTAGG
PJ85	CCCAACCAAAGGAGAACTAC
PJ86	GGAGAACTACTTTGCTCCTAAG
PJ87	CTTGGTTGCTCCACAATGTC
PJ88	TGAGCCGCTATGAGAATGAC
PJ89	TGGCTGGATTCTTCCTAACC
PJ90	CGTCCTCTATGTCCCTTATG
PJ91	CTGGGTTTCGGTCCAGTCAT
PJ92	CTACTTTGCTCCTAAAGGTTGA
PJ93	ACCAAACAAAGAAGTGTGGGAT
PJ94	GTATGGGATGGTCCGAATGT
PJ95	TCCGCGTCCAGTCTTTCTAT
PJ96	CCTGGTGATACACCTGAAGT
PJ97	GTTCCGAGCTGTGTTTCTCA
PJ98	GAGGATCCGTACCGTTATCT

PJ99	CCTAACTCCAGGCACACCAT
PJ100	ACTGGTCTTTGGGCCAAACT
PJ101	CTGATAGCGCTGATTCAGGA
PJ102	GCTTCAATTCCGACCTCTTC
PJ103	AAGGAAGCAGCGAGGATGAA
PJ104	CATGATCCGCAATTCTTCCG
PJ105	CCGGGATGTCAATCTCCAAC
PJ106	GGGTGCGTTGGTATTCCTTT
PJ107	GGTGTTGCCTGGGTCAGAAT
PJ108	CTCAACCCCATTCCTTATCC
PJ109	TGAGGTCTGACTGGAGGTAT
PJ110	TCCTCCAAGCTGGGATGGAA
PJ111	AGACATTGCTGAGCCGATTC
PJ112	AAACCTCCCGCTATAAGACC
PJ113	GAGAACTACTTTGCTCCTAA
PJ114	CATCAACCATGGCAAGCAATCAGGAGAA
PJ115	ACGTTGGAATTCCCTTCAAGATTCAACC
PJ116	ATCAGACCATGGATATCGACGCAGCCAT
PJ117	GATTAGATGCATGAATTCAC
PJ118	CACTGACCATGGCATCAACAGTGTATGAGGA
PJ119	TGGAGTGAATTCTAGGTTGAGGAGTGTATGT
PJ120	GTCATTGACAGGTGCAAGAC
PJ121	AGGTCCTCCAGCGATGCAAG
PJ122	GGTTCCACCACTGAAGAAAG
PJ123	ACCATGCCGAGATGAACAGA
PJ124	AGTCTCTGGCATGCACGATA
PJ125	GATAGAGGCAGCAAAGACAG
PJ126	CCAGAGATGATTCCGTATCG
PJ127	CAGCTTCACCTGAGTGCTAT
PJ128	GGCGGGTCATCAGTGAGAGA
PJ129	GCCACACAAGCACTCAACAG
PJ130	GCTGCGATAAAGCCAGAGTG
PJ131	GCTCGTCCTTGACCATTCCA
PJ132	AACTCCATGGATGTGGGCCCTGATGGAT
PJ133	ACAGAGCCCATGGAAGCAGCGAGGATGAA
PJ134	CGCGGATCCATGGATATCGACGCAGCCAT
PJ135	AGCCCTTGAAGCAGATCGAA
PJ136	TTGCATCGCTGGAGGACCTT
PJ137	GGGCAAAGAGTGTTCCAAGT
PJ138	GAGAATCGGCTCAGCAATGTC
	BeiPV Primer (B) Sequences
B1	GCGCATCAGCGAAGAACAAC
B2	AGACACCGCATTAGCCATCC
B3	CGTAGGGCTTGATGTTACCG
B4	GAGAAGCGAGGGAGCTAAAG
B5	CTGAGTAGACGAGCCCAAAG
B6	GGCTGGAGAAAGAGGGTATC
B7	AAGGTAGATATGGCCGGAATGCTGG
B8	GTTAGCGGCCGCCTTCATGATCTTCCCAGT
B9	AAGGTACATATGGCGGTCATCGGTGGAG
B10	GTTAGCGGCCGCACCCACGAATCCGGAG

B11	GGGTGTCTGACTCCATGAAC
B12	TATAACCGGCACTCTTCCTG
B13	AAGGCACCATACATGGT
B14	CTATGGAGCTATGCAATGGG
B15	CCTTGACAAGCTGCTGG
B16	CTAATTATAACTGCTATGG
B17	AATAGACCCAACTTGAAGG
B18	GAGTGCCATCTGGATAGYCCYAT
B19	TGCATTTAAACCAAAAYARAAA
B20	ACTTTTGAAATGGNGCTAATG
B21	TGCTTTGTCYTTCATRTACAT
B22	TTCTTTTCATGGCTRCAYAAACG
B23	AATGTTGCTRCATGCAGATC
B24	CCTAGAGCTGCACATGARAT
B25	GTTAATTGGGACAAARAACCA
B26	CTATGATTAGCAATGAYAATYT
B27	AGACTGTTGATATCRTCRTC
B28	CGAAGAGGTAYAATYAARCA
B29	GCATTCTGGTTTYCCATTRAA
B30	GCTTTTCTTGAGCCCTAYCC
B31	TAACCAACARYCTTCCACCA
B32	AATCTCTTGGCGTGCACCAG
B33	CGGGTGTTTAACAACCTG
B34	CCGTCTCTGGTAAAGTTC
B35	AGGATAAGGAGGTGGAGAAC
B36	ATCAAAGAGGAGATCAGGAA
B37	TGAAATCCAGGTCTTGAGTC
B38	CCAGGTGGTTGCTAATACAC
B39	AAGTCCCTCTAACCAACAGC
B40	CTTGGAGGATTCAATTA
B41	GACCCAGTGACTGCATC
B42	ATGCATCAAGAACCTGG
B43	GGGCATATGGTGATACTGAT
B44	ATGTTTGGATTGTACTA
B45	TGTTTAATTGTACCTCT
B46	CAGCTCTGGATACTGCT
B47	TTTGCCCCGGGAAATTA
B48	GTCTCGTCGCAGCCTGATTA
B49	CTGTCGAAAACCAGTGTGAA
B50	GCGCAGCATATGTTCTGGGGGGCGGTCATCGGT
B51	GACATGGMGGAGCMTGGCC
B52	CCCAATVACTTGACATGC
B53	CGTTCTTDACWACKGAT
B54	ACGATWGTYTCTGACCA
B55	CTCTTGATCAAGGMTCKCAT
B56	GTGCTAATGTWBTGTGATGT
B57	CCGCTAACAATGAGGCTCT
B58	CTTGAATTGGAATCCCACAA
B59	TTGGCAATAGCCCTCAATAC
B60	GCCAAAAGAAGTTCCTGTGTT
B61	CTAAATGATCCMGAYMTTG
B62	TAAAGTTGGCTDSSAGAGG

B63	TTGTGAGCAGKRTCCAKA
B64	CCCATTGCAWARCTCCA
B65	GGGACAGATCTACCGTGTCG
B66	TGGCAGTGAGAGTTCCTCCT
B67	GCTGTTCTCCAGCCATCTGT
B68	AGCTCACTGAGGGCCAACT
B69	GAGAACACCGGWGTTRTARWA
B70	CCCTTCGTAYAGYAAYCCRG
B71	GAAGAGAACTGCTGCGTGAT
B72	TGCGTGATAGAAATGGAAGA
B73	TCCTTATTTGTCTCTTTCATGT
B74	CCCTGAGTACTTTCCCTCTAA
B75	TCTGACACTTCGGCAATCTG
B76	GCACATCAATGTCAGGGTCA
B77	GTCAGCAAACATCGAGAGCA
B78	AACTGCCATTGACACAGCAC
B79	CAGGTTGTTGACAGCTCGAT
B80	CTTGGACTGACAGGTGAGCA
B81	GCAAATTGCCCAGATCAAAG
B82	AAGTCTTCCGGACTCTGCTG
B83	TCGGGGTTATGTTGGGATTA
B84	TGGAGGAAGCATGGGTAGTC
B85	GAGGTTCAATCATAGCCACCA
B86	CGCGGAGAAGGAGTGAATC
B87	GAAGAGGCTGCAGACAAGGT
B88	GACCCAGTCACAGCCTCATT
B89	AGGGAGCTGATGGACTCATT
B90	CAGCGCAAAAAACYTAGG
B91	CAGCGCTCCTARGTTTTT
B92	GGTCTCCGGTGATTTCTCAA
B93	TGGCTATTAACTGGTCCTTTGA
B94	GCAATGGATAAGCCCATTGTA
B95	GTAGTCGGGCACCGACAGT
B96	CAAAGAGAACGGAATGGTCAA
B97	TTGGTTATCCCAAACCCAGA
B98	GGGTCTTGTCATTCAGTTCTTCT
B99	GACCGGATCATGTACAAACG
B100	GTTTTGGTGAACGGACCAC
B101	TGCTGCCTGAGAGTGTACAAG
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B103	CTACATAATCATCTCCATGG
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B105	AGAATGTCAGAAGGCTGAAC
B106	ATGGAGAGATGTGTCATTAC
B107	GCTCCATCGATGGCTCAATG
B108	AATTACACCAGAGATCTCTC
B109	TATATGATTACACATATACC
B110	ACAGTGTCGCCCACTACAAC
B111	TTATATCTGGAACCATGTCC
B112	TTTCATTATTGCAGGTGTAG
B113	TGTTGATGAACTGTGCTGAG
B114	CGGAATCAGCAGTGGAATCC

B115	GATCTCACTTCTTGTGTAA
B116	TTACGACGACCGCAACTCC
B117	AAGCAACACCAGCCAGGAG
B118	TTGCAAGAGTTGTCTAGAG
B119	GACTGAAGTCTCTGAATCC
B120	ACAGGCATATCACCTAAGG
B121	GATCACAGCAGCAGGTGTT
B122	GATTATCGACTGTCAAGCC
B123	CCAAGAAGGAGAATCTTTGCTC
B124	GATGTCCTCCAGCAGCTTGT
B125	GAGCTTGAGGAGCTGTCACTTT
B126	GGGTGCAACTCAAGATCAATTA
B127	GGGCCAAAGACAAGTGAGATT
B128	AGGATGCTATTATACAGACATTCA
B129	TTCTTTATGTTAACCCACGAATC
B130	GATCCTATATGATTACAC
B131	GCTCAACGCTCTCTCGATAA
B132	CCAGATATAATTATCGGATC
B133	CCTCAGAATAACTATCAAT
B134	TTCTCCTTCTGTGGTACC
B135	GGTTAGAGGGAGAGCATG
B136	TACTTCATTGTGTCCACAC
B137	CACCTCCACTGGTCTGAA
B138	AGCTCCACCACACTGATG
B139	TTATGACATTAATACTTC
B140	CTAGATCAGCTTGCAACTC
B141	ATGAACACCTTGCTCGAA
B142	GCAATACCTACAGATAGG
B143	ATCATTGAGGAAGACCTC
B144	TGCCTCAATGGTATCAGG
B145	AGCACTGTGTTCAACTCC
B146	CACAACTCCATTCAGAATG
B147	GTTGTAAATGCTCATGTCC
B148	TGCTGTTCGTACTTAATCC
B149	CTCGCAGGATTGAATTTGG
B150	CTCAGCTAACTACTATGAG
B151	AATGTTATTGCTTTGGTTCC
B152	GATGATTCACTCCTTCTCCG
B153	GCCTCAATCTATTGAAGTACT

Appendix II: Queensland Rodent Sera

VialNo	Location	Box	Position	Date	Details
16818	S2R4	6001	1	16/11/1998	Rat 1 R.Ratus Everton Park 25/5/98 Female
16660	S2R4	6001	2	16/11/1998	Rat 2 R.Ratus Everton Park 25/5/98 Female
16661	S2R4	6001	3	16/11/1998	Rat 3 R.Ratus Everton Park 25/5/98 Female
16662	S2R4	6001	4	16/11/1998	Rat 4 R.Ratus Everton Park 27/5/98 Female
16663	S2R4	6001	5	16/11/1998	Rat 5 R.Ratus Everton Park 28/5/98
16664	S2R4	6001	6	16/11/1998	Rat 6 R.Ratus Everton Park 3/6/98 Female
16665	S2R4	6001	7	16/11/1998	Rat 7 R.Ratus Everton Park 4/6/98 Female
16666	S2R4	6001	8	16/11/1998	Rat 8 R.Ratus Everton Park 9/6/98 Female
16667	S2R4	6001	9	16/11/1998	Rat 9 R.Ratus Everton Park 9/6/98 Female
16668	S2R4	6001	10	16/11/1998	Rat 10 R.Ratus Everton Park 12/6/98
16669	S2R4	6001	11	16/11/1998	Rat 11 R.Ratus Wacol 23/6/98 Female
16670	S2R4	6001	12	16/11/1998	Rat 12 R.RatusCoopers Plains Female
16805	S2R4	6001	13	16/11/1998	Rat 13 R.Ratus Tennyson 24/6/98 Male
16806	S2R4	6001	14	16/11/1998	Rat 14 R.Ratus Tennyson 24/6/98 Female
16807	S2R4	6001	15	16/11/1998	Rat 15 R.Ratus Redland Bay 25/6/98 Male
16808	S2R4	6001	16	16/11/1998	Rat 16 R.Ratus Redland Bay 26/6/98 Female
16809	S2R4	6001	17	16/11/1998	Rat 17 R.Ratus Redland Bay 26/6/98 Male
16810	S2R4	6001	18	16/11/1998	Rat 18 R.Ratus Redland Bay 26/6/98 Male
16811	S2R4	6001	19	16/11/1998	Rat 19 R.Ratus Redland Bay 29/6/98 Male
16812	S2R4	6001	20	16/11/1998	Rat 20 R.Ratus Pinkemba 29/6/98 Male
16813	S2R4	6001	21	16/11/1998	Rat 21 Durack R.Norwegicus 1/7/98 Female
16819	S2R4	6001	22	16/11/1998	Rat 22 Durack R.Norwegicus 1/7/98 Male
16815	S2R4	6001	23	16/11/1998	Rat 23 R.Ratus Willawong 1/7/98 Female
16816	S2R4	6001	24	16/11/1998	Rat 24 R.Ratus Willawong 1/7/98 Female
16817	S2R4	6001	25	16/11/1998	Rat 25 R.Ratus Pinkenmba 1/7/98 Female
16820	S2R4	6001	26	16/11/1998	Rat 26 Durack R.Norwegicus 2/7/98 Male
16821	S2R4	6001	27	16/11/1998	Rat 27 Durack R.Lutreolus 2/7/98 Female
16822	S2R4	6001	28	16/11/1998	Rat 28 R.Lutreolus Willawong 2/7/98 Male
16823	S2R4	6001	29	16/11/1998	Rat 29 Durack R.Lutreolus 3/7/98 Male
16824	S2R4	6001	30	16/11/1998	Rat 30 Durack R.Norwegicus 3/7/98 Female
16825	S2R4	6001	31	16/11/1998	Rat 31 Durack R.Norwegicus 6/7/98 Female
16826	S2R4	6001	32	16/11/1998	Rat 32 R.RatusDohleys Rocks Rd 8/7/98 Male
16827	S2R4	6001	33	16/11/1998	Rat 33 R.RatusDohleys Rocks Rd 8/7/98 F
16828	S2R4	6001	34	16/11/1998	Rat 34 R.Ratus Durack 8/7/98 F
16829	S2R4	6001	35	16/11/1998	Rat 35 R.Lutreolus Dakabin 8/7/98 F
16830	S2R4	6001	36	16/11/1998	Rat 36 R.RatusDakabin 9/7/98 M
16831	S2R4	6001	37	16/11/1998	Rat 37 R.RatusDohleys Rocks Rd 9/7/98 F
16832	S2R4	6001	38	16/11/1998	Rat 38 R.RatusDohleys Rocks Rd 9/7/98 M
16833	S2R4	6001	39	16/11/1998	Rat 39 R.RatusDakabin 9/7/98 F
16834	S2R4	6001	40	16/11/1998	Rat 40 R.RatusDohleys Rocks Rd 9/7/98 F
16835	S2R4	6001	41	16/11/1998	Rat 41 R.RatusDohleys Rocks Rd 9/7/98 F
16836	S2R4	6001	42	16/11/1998	Rat 42 R.Ratus Dakabin 10/7/98 F
16837	S2R4	6001	43	16/11/1998	Rat 43 R.Ratus Dohleys Rocks Rd 10/7/98 M
16838	S2R4	6001	44	16/11/1998	Rat 44 R.Ratus Dohleys Rocks Rd 10/7/98 M
16839	S2R4	6001	45	16/11/1998	Rat 45 R.Ratus Dohleys Rocks Rd 10/7/98 M
16840	S2R4	6001	46	16/11/1998	Rat 46 R.Ratus Dakabin 16/7/98 M
16841	S2R4	6001	47	16/11/1998	Rat 47 R.Ratus Dakabin 16/7/98 F
16842	S2R4	6001	48	16/11/1998	Rat 48 R.Ratus Dakabin 16/7/98 F
16843	S2R4	6001	49	16/11/1998	Rat 49 R.Ratus Dakabin 16/7/98 M

16844	S2R4	6001	50	16/11/1998	Rat 50 R.Ratus Dakabin 16/7/98 M
16845	S2R4	6001	51	16/11/1998	Rat 51 R.Ratus Dakabin 16/7/98 F
16846	S2R4	6001	52	16/11/1998	Rat 52 R.Ratus Dakabin 16/7/98 F
16847	S2R4	6001	53	16/11/1998	Rat 53 R.Ratus Dakabin 16/7/98 F
16848	S2R4	6001	54	16/11/1998	Rat 54 R.Ratus Dakabin 16/7/98 F
16849	S2R4	6001	55	16/11/1998	Rat 55 R.Ratus Dakabin 29/7/98
16850	S2R4	6001	56	16/11/1998	Rat 56 R.Ratus Dakabin 29/7/98
16851	S2R4	6001	57	16/11/1998	Rat 57 R.Ratus Dakabin 29/7/98
16852	S2R4	6001	58	16/11/1998	Rat 58 R.Ratus Dakabin 29/7/98
16853	S2R4	6001	59	16/11/1998	Rat 59 R.Ratus Dakabin 29/7/98
16854	S2R4	6001	60	16/11/1998	Rat 60 R.Ratus Dakabin 29/7/98
16855	S2R4	6001	61	16/11/1998	Rat 61 R.Ratus Dakabin 29/7/98
16856	S2R4	6001	62	16/11/1998	Rat 62 R.Ratus Dakabin 29/7/98
16857	S2R4	6001	63	16/11/1998	Rat 63 R.Ratus Dakabin 29/7/98
16858	S2R4	6001	64	16/11/1998	Rat 64 R.Ratus Dakabin 29/7/98
16859	S2R4	6001	65	16/11/1998	Rat 65 R.Ratus Dakabin 29/7/98
16860	S2R4	6001	66	16/11/1998	Rat 66 R.Ratus Dakabin 29/7/98
16861	S2R4	6001	67	16/11/1998	Rat 67 R.Ratus Dakabin 29/7/98
16862	S2R4	6001	68	16/11/1998	Rat 68 R.Ratus Dakabin 29/7/98
16863	S2R4	6001	69	16/11/1998	Rat 69 R.Ratus Dakabin 29/7/98
16864	S2R4	6001	70	16/11/1998	Rat 70 R.Ratus Dakabin 29/7/98
16865	S2R4	6001	71	16/11/1998	Rat 71 R.Ratus Dakabin 29/7/98
16997	S2R4	6001	72	17/11/1998	RAT109 R.RATUS EVERTON PARK OCT 98
17140	S2R4	6002	1	17/11/1998	MUS 1 COOPERS PLAINS 16/6/98 F
17135	S2R4	6002	2	17/11/1998	MUS 2 WILLAWONG 23/6/98 F
17136	S2R4	6002	3	17/11/1998	MUS 3 WILLAWONG 23/6/98 F
17137	S2R4	6002	4	17/11/1998	MUS 4 WACOL 23/6/98 F
17138	S2R4	6002	5	17/11/1998	MUS 5 COOPERS PLAINS 24/6/98
17139	S2R4	6002	6	17/11/1998	MUS 6 REDLAND BAY 25/6/98
17141	S2R4	6002	7	17/11/1998	MUS 7 CARBROOK 26/6/98 F
17142	S2R4	6002	8	17/11/1998	MUS 8 ACACIA RIDGE 26/6/98 F
17143	S2R4	6002	9	17/11/1998	MUS 9 ACACIA RIDGE 26/6/98
17144	S2R4	6002	10	17/11/1998	MUS 10 CARBROOK 26/6/98 F
17145	S2R4	6002	11	17/11/1998	MUS 11 CARBROOK 26/6/98 F
17146	S2R4	6002	12	17/11/1998	MUS 12 CARBROOK 26/6/98 M
17147	S2R4	6002	13	17/11/1998	MUS 13 COOPERS PLAINS 26/6/98 F
17148	S2R4	6002	14	17/11/1998	MUS 14 CARBROOK 26/6/98
17149	S2R4	6002	15	17/11/1998	MUS 15 CARBROOK 26/6/98
17150	S2R4	6002	16	17/11/1998	MUS 16 CARBROOK 26/6/98
17167	S2R4	6002	17	18/11/1998	MUS 17 BIRKDALE 26/6/98
17168	S2R4	6002	18	18/11/1998	MUS 18 CARBROOK 26/6/98
17169	S2R4	6002	19	18/11/1998	MUS 19 COOPERS PLAINS 26/6/98 M
17170	S2R4	6002	20	18/11/1998	MUS 20 BEAUDESERT 29/6/98
17171	S2R4	6002	21	18/11/1998	MUS 21 PINKEMBA 30/6/98
17172	S2R4	6002	22	18/11/1998	MUS 22 PINKEMBA 30/6/98
17173	S2R4	6002	23	18/11/1998	MUS 23 PINKEMBA 30/6/98
17174	S2R4	6002	24	18/11/1998	MUS 24 HENDRA 30/6/98
17175	S2R4	6002	25	18/11/1998	MUS 25 PINKEMBA 30/6/98
17176	S2R4	6002	26	18/11/1998	MUS 26 PINKEMBA 30/6/98 M
17177	S2R4	6002	27	18/11/1998	MUS 27 PINKEMBA 30/6/98
17178	S2R4	6002	28	18/11/1998	MUS 28 PINKEMBA 1/7/98
17179	S2R4	6002	29	18/11/1998	MUS 29 PINKEMBA 1/7/98 F

17180	S2R4	6002	30	18/11/1998	MUS 30 PINKEMBA 1/7/98
17181	S2R4	6002	31	18/11/1998	MUS 31 PINKEMBA 1/7/98
17185	S2R4	6002	32	18/11/1998	MUS 32 PINKEMBA 1/7/98
17186	S2R4	6002	33	18/11/1998	MUS 33 WILLAWONG 1/7/98
17184	S2R4	6002	34	18/11/1998	MUS 34 WILLAWONG 1/7/98
17187	S2R4	6002	35	18/11/1998	MUS 35 WILLAWONG 1/7/98
17188	S2R4	6002	36	18/11/1998	MUS 36 WILLAWONG 1/7/98
17189	S2R4	6002	37	18/11/1998	MUS 37 WILLAWONG 1/7/98
17190	S2R4	6002	38	18/11/1998	MUS 38 WILLAWONG 1/7/98
17191	S2R4	6002	39	18/11/1998	MUS 39 WILLAWONG 2/7/98
17192	S2R4	6002	40	18/11/1998	MUS 40 WILLAWONG 2/7/98
17193	S2R4	6002	41	18/11/1998	MUS 41 ARCHERFIELD 2/7/98
17194	S2R4	6002	42	18/11/1998	MUS 42 ARCHERFIELD 2/7/98 F
17195	S2R4	6002	43	18/11/1998	MUS 43 WILLAWONG 2/7/98 F
17196	S2R4	6002	44	18/11/1998	MUS 44 WILLAWONG 2/7/98 F
17197	S2R4	6002	45	18/11/1998	MUS 45 ARCHERFIELD 2/7/98 F
17198	S2R4	6002	46	18/11/1998	MUS 46 RESEARCH RD 2/7/98 F
17199	S2R4	6002	47	18/11/1998	MUS 47 WILLAWONG 2/7/98 F
17200	S2R4	6002	48	18/11/1998	MUS 48 WILLAWONG 2/7/98
17201	S2R4	6002	49	18/11/1998	MUS 49 ACACIA RIDGE 2/7/98
17202	S2R4	6002	50	18/11/1998	MUS 50 ACACIA RIDGE 2/7/98
17203	S2R4	6002	51	18/11/1998	MUS 51 ACACIA RIDGE 2/7/98
17204	S2R4	6002	52	18/11/1998	MUS 52 ACACIA RIDGE 2/7/98 M
17205	S2R4	6002	53	18/11/1998	MUS 53 WILLAWONG 3/7/98 F
17206	S2R4	6002	54	18/11/1998	MUS 54 WILLAWONG 3/7/98 F
17207	S2R4	6002	55	18/11/1998	MUS 55 ARCHERFIELD 3/7/98 F
17208	S2R4	6002	56	18/11/1998	MUS 56 ARCHERFIELD 3/7/98 F
17209	S2R4	6002	57	18/11/1998	MUS 57 ARCHERFIELD 3/7/98
17210	S2R4	6002	58	18/11/1998	MUS 58 WILLAWONG 3/7/98
17211	S2R4	6002	59	18/11/1998	MUS 59 PINKEMBA 7/7/98
17212	S2R4	6002	60	18/11/1998	MUS 60 HENDRA 7/7/98
17213	S2R4	6002	61	18/11/1998	MUS 61 PINKEMBA 7/7/98
17214	S2R4	6002	62	18/11/1998	MUS 62 DEAGON 7/7/98
17215	S2R4	6002	63	18/11/1998	MUS 63 PINKEMBA 7/7/98
17216	S2R4	6002	64	18/11/1998	MUS 64 DOHLEYS ROCKS RD 8/7/98
17217	S2R4	6002	65	18/11/1998	MUS 65 DAKABIN 8/7/98
17218	S2R4	6002	66	18/11/1998	MUS 66 DOHLEYS ROCKS RD 8/7/98
17219	S2R4	6002	67	18/11/1998	MUS 67 CABOULTURE 8/7/98
17220	S2R4	6002	68	18/11/1998	MUS 68 CABOULTURE 8/7/98
17221	S2R4	6002	69	18/11/1998	MUS 69 DOHLEYS ROCKS RD 9/7/98
17222	S2R4	6002	70	18/11/1998	MUS 70 DOHLEYS ROCKS RD 9/7/98
17223	S2R4	6002	71	18/11/1998	MUS 71 DOHLEYS ROCKS RD 9/7/98
17224	S2R4	6002	72	18/11/1998	MUS 72 DOHLEYS ROCKS RD 10/7/98
17225	S2R4	6002	73	18/11/1998	MUS 73 DOHLEYS ROCKS RD 10/7/98 F
17226	S2R4	6002	74	18/11/1998	MUS 74 DOHLEYS ROCKS RD 10/7/98 F
17227	S2R4	6002	75	18/11/1998	MUS 75 DOHLEYS ROCKS RD 10/7/98 M
17228	S2R4	6002	76	18/11/1998	MUS 76 DOHLEYS ROCKS RD 10/7/98 M
17229	S2R4	6002	77	18/11/1998	MUS 77 DOHLEYS ROCKS RD 10/7/98 F
17230	S2R4	6002	78	18/11/1998	MUS 78 DOHLEYS ROCKS RD 10/7/98 F
17231	S2R4	6002	79	18/11/1998	MUS 79 WEST END 13/7/98 F
17232	S2R4	6002	80	18/11/1998	MUS 80 ACACIA RIDGE 13/7/98
11695	S2R4	6005	1	07/08/1998	Serum MOUSE 81

11696	S2R4	6005	2	07/08/1998	Serum MOUSE 82
11697	S2R4	6005	3	07/08/1998	Serum MOUSE 83
11698	S2R4	6005	4	07/08/1998	Serum MOUSE 84
11699	S2R4	6005	5	07/08/1998	Serum MOUSE 85
11700	S2R4	6005	6	07/08/1998	Serum MOUSE 86
11701	S2R4	6005	7	07/08/1998	Serum MOUSE 87
11702	S2R4	6005	8	07/08/1998	Serum MOUSE 88
11703	S2R4	6005	9	07/08/1998	Serum MOUSE 89
11704	S2R4	6005	10	07/08/1998	Serum MOUSE 90
11705	S2R4	6005	11	07/08/1998	Serum MOUSE 91
11706	S2R4	6005	12	07/08/1998	Serum MOUSE 92
11707	S2R4	6005	13	07/08/1998	Serum MOUSE 93
11708	S2R4	6005	14	07/08/1998	Serum MOUSE 94
11709	S2R4	6005	15	07/08/1998	Serum MOUSE 95
11710	S2R4	6005	16	07/08/1998	Serum MOUSE 96
11711	S2R4	6005	17	07/08/1998	Serum MOUSE 97
11712	S2R4	6005	18	07/08/1998	Serum MOUSE 98
11713	S2R4	6005	19	07/08/1998	Serum MOUSE 99
11714	S2R4	6005	20	07/08/1998	Serum MOUSE 100
11715	S2R4	6005	21	07/08/1998	Serum MOUSE 101
11716	S2R4	6005	22	07/08/1998	Serum MOUSE 102
11717	S2R4	6005	23	07/08/1998	Serum MOUSE 103
11718	S2R4	6005	24	07/08/1998	Serum MOUSE 104
17233	S2R4	6024	1	18/11/1998	RAT 1 26/8/97
17234	S2R4	6024	2	18/11/1998	RAT 1 ACT GUNGAHLIN 1998
17235	S2R4	6024	3	18/11/1998	RAT 2 ACT GUNGAHLIN 1998
17236	S2R4	6024	4	18/11/1998	RAT 3 ACT GUNGAHLIN 1998
17237	S2R4	6024	5	18/11/1998	RAT 4 ACT GUNGAHLIN 1998
17238	S2R4	6024	6	18/11/1998	RAT 5 ACT GUNGAHLIN 1998
17239	S2R4	6024	7	18/11/1998	RAT 6 ACT GUNGAHLIN 1998
17240	S2R4	6024	8	18/11/1998	RAT 7 ACT GUNGAHLIN 1998
17241	S2R4	6024	9	18/11/1998	RAT 8 ACT GUNGAHLIN 1998
17242	S2R4	6024	10	18/11/1998	RAT 9 ACT GUNGAHLIN 1998
17243	S2R4	6024	11	18/11/1998	RAT 10 ACT GUNGAHLIN 1998
17244	S2R4	6024	12	18/11/1998	RAT 11 ACT GUNGAHLIN 1998
17245	S2R4	6024	13	18/11/1998	RAT 12 ACT GUNGAHLIN 1998
17246	S2R4	6024	14	18/11/1998	RAT 13 ACT GUNGAHLIN 1998
17247	S2R4	6024	15	18/11/1998	RAT 14 ACT GUNGAHLIN 1998
17248	S2R4	6024	16	18/11/1998	RAT 15 ACT GUNGAHLIN 1998
17249	S2R4	6024	17	18/11/1998	RAT 16 ACT GUNGAHLIN 1998
17250	S2R4	6024	18	18/11/1998	RAT 17 ACT GUNGAHLIN 1998
17251	S2R4	6024	19	18/11/1998	Rat 2 R.Ratus Everton Park 25/5/98 Female
17252	S2R4	6024	20	18/11/1998	Rat 3 R.Ratus Everton Park 25/5/98 Female
17253	S2R4	6024	21	18/11/1998	Rat 4 R.Ratus Everton Park 27/5/98 Female
17254	S2R4	6024	22	18/11/1998	Rat 5 R.Ratus Everton Park 28/5/98 Female
17255	S2R4	6024	23	18/11/1998	Rat 6 R.Ratus Everton Park 3/6/98 Female
17256	S2R4	6024	24	18/11/1998	Rat 7 R.Ratus Everton Park 4/6/98 Female
17257	S2R4	6024	25	18/11/1998	Rat 8 R.Ratus Everton Park 9/6/98 Female
17258	S2R4	6024	26	18/11/1998	Rat 9 R.Ratus Everton Park 9/6/98 Female
17259	S2R4	6024	27	18/11/1998	Rat 10 R.Ratus Everton Park 9/6/98 Female
17260	S2R4	6024	28	18/11/1998	Rat 11 R.Ratus Wacol 23/6/98 Female
21204	S2R4	6024	29	01/03/1999	R.norvegicus F Mature DPI99/107330-1

21205	S2R4	6024	30	01/03/1999	R.norvegicus F Mature DPI99/107330-2
21206	S2R4	6024	31	01/03/1999	R.norvegicus M Pre Breeding DPI99/107330-3
21207	S2R4	6024	32	01/03/1999	R.norvegicus F Mature DPI99/107330-4
21208	S2R4	6024	33	01/03/1999	R.norvegicus F Mature DPI99/107330-5
21209	S2R4	6024	34	01/03/1999	R.norvegicus M Pre-breeding DPI99/107330-6
21210	S2R4	6024	35	01/03/1999	R.norvegicus F Pre-breeding DPI99/107330-7
21211	S2R4	6024	36	01/03/1999	R.norvegicus M Pre-breeding DPI99/107330-8
21212	S2R4	6024	37	01/03/1999	R.norvegicus F Mature DPI99/108511-1
21213	S2R4	6024	38	01/03/1999	R.norvegicus M Mature DPI99/108511-2
21214	S2R4	6024	39	01/03/1999	R.norvegicus M Mature DPI99/108511-3
21215	S2R4	6024	40	01/03/1999	R.norvegicus M Pre-breeding DPI99/108511-4
21216	S2R4	6024	41	01/03/1999	R.norvegicus F Mature DPI99/108511-5
21217	S2R4	6024	42	01/03/1999	R.norvegicus M Pre-breeding DPI99/108511-6
21218	S2R4	6024	43	01/03/1999	R.norvegicus F Mature DPI99/108511-7
21219	S2R4	6024	44	01/03/1999	R.norvegicus F Mature DPI99/108511-8
21220	S2R4	6024	45	01/03/1999	R.norvegicus F Mature DPI99/108511-9
21221	S2R4	6024	46	01/03/1999	R.norvegicus F Mature DPI99/108511-10
21222	S2R4	6024	47	01/03/1999	R.norvegicus F Pre-breeding DPI99/108511-11
21223	S2R4	6024	48	01/03/1999	R.norvegicus M Pre-breeding DPI99/108511-12
21224	S2R4	6024	49	01/03/1999	R.norvegicus M Mature DPI99/108511-13
21225	S2R4	6024	50	01/03/1999	R.norvegicus M Mature DPI99/108511-14
21226	S2R4	6024	51	02/03/1999	Rat DPI 99/103235-1
21227	S2R4	6024	52	02/03/1999	Rat DPI 99/103235-2
21228	S2R4	6024	53	02/03/1999	Rat DPI 99/103235-3
21229	S2R4	6024	54	02/03/1999	Rat DPI 99/103235-4
21230	S2R4	6024	55	02/03/1999	Rat DPI 99/103235-5
21231	S2R4	6024	56	02/03/1999	Rat DPI 99/103235-6
21232	S2R4	6024	57	02/03/1999	Rat DPI 99/103235-7
21233	S2R4	6024	58	02/03/1999	Rat DPI 99/103951-1
21234	S2R4	6024	59	02/03/1999	Rat DPI 99/103951-2
21235	S2R4	6024	60	02/03/1999	Rat DPI 99/103951-3
21236	S2R4	6024	61	02/03/1999	Rat DPI 99/103951-4
21237	S2R4	6024	62	02/03/1999	Rat DPI 99/104304-1
21238	S2R4	6024	63	02/03/1999	Rat DPI 99/104304-2
21239	S2R4	6024	64	02/03/1999	Rat DPI 99/104304-3
21240	S2R4	6024	65	02/03/1999	Rat DPI 99/104304-4
21241	S2R4	6024	66	02/03/1999	Rat DPI 99/104304-5
21242	S2R4	6024	67	02/03/1999	Rat DPI 99/104304-6
21243	S2R4	6024	68	02/03/1999	Rat DPI 99/104304-7
21244	S2R4	6024	69	02/03/1999	Rat DPI 99/104304-8
21245	S2R4	6024	70	02/03/1999	Rat DPI 99/104304-9
21246	S2R4	6024	71	02/03/1999	Rat DPI 99/104304-10
21247	S2R4	6024	72	02/03/1999	Rat DPI 99/104304-11
21248	S2R4	6024	73	02/03/1999	Rat DPI 99/104304-12
21249	S2R4	6024	74	02/03/1999	Rat DPI 99/104304-13
21250	S2R4	6024	75	02/03/1999	Rat DPI 99/104304-14
21251	S2R4	6024	76	02/03/1999	Rat DPI 99/104602
21252	S2R4	6024	77	02/03/1999	Rat DPI 99/104811-1
21253	S2R4	6024	78	02/03/1999	Rat DPI 99/104811-2
21254	S2R4	6024	79	02/03/1999	Rat DPI 99/104811-3
21255	S2R4	6024	80	02/03/1999	Rat DPI 99/104811-4
21256	S2R4	6024	81	02/03/1999	Rat DPI 99/104811-5

11529	S2R4	6025	1	07/08/1998	Serum RAT 97
11530	S2R4	6025	2	07/08/1998	Serum RAT 98
11531	S2R4	6025	3	07/08/1998	Serum RAT 99
11532	S2R4	6025	4	07/08/1998	Serum RAT 100
11533	S2R4	6025	5	07/08/1998	Serum RAT 101
11534	S2R4	6025	6	07/08/1998	Serum RAT 102
11535	S2R4	6025	7	07/08/1998	Serum RAT 103
11536	S2R4	6025	8	07/08/1998	Serum RAT 104
11537	S2R4	6025	9	07/08/1998	Serum RAT 105
11538	S2R4	6025	10	07/08/1998	Serum RAT 106
11539	S2R4	6025	11	07/08/1998	Serum RAT 107
11540	S2R4	6025	12	07/08/1998	Serum RAT 108
11504	S2R4	6026	1	07/08/1998	Serum RAT 72
11505	S2R4	6026	2	07/08/1998	Serum RAT 73
11506	S2R4	6026	3	07/08/1998	Serum RAT 74
11507	S2R4	6026	4	07/08/1998	Serum RAT 75
11508	S2R4	6026	5	07/08/1998	Serum RAT 76
11509	S2R4	6026	6	07/08/1998	Serum RAT 77
11510	S2R4	6026	7	07/08/1998	Serum RAT 78
11511	S2R4	6026	8	07/08/1998	Serum RAT 79
11512	S2R4	6026	9	07/08/1998	Serum RAT 80
11513	S2R4	6026	10	07/08/1998	Serum RAT 81
11514	S2R4	6026	11	07/08/1998	Serum RAT 82
11515	S2R4	6026	12	07/08/1998	Serum RAT 83
11516	S2R4	6026	13	07/08/1998	Serum RAT 84
11517	S2R4	6026	14	07/08/1998	Serum RAT 85
11518	S2R4	6026	15	07/08/1998	Serum RAT 86
11519	S2R4	6026	16	07/08/1998	Serum RAT 87
11520	S2R4	6026	17	07/08/1998	Serum RAT 88
11521	S2R4	6026	18	07/08/1998	Serum RAT 89
11522	S2R4	6026	19	07/08/1998	Serum RAT 90
11523	S2R4	6026	20	07/08/1998	Serum RAT 91
11524	S2R4	6026	21	07/08/1998	Serum RAT 92
11525	S2R4	6026	22	07/08/1998	Serum RAT 93
11526	S2R4	6026	23	07/08/1998	Serum RAT 94
11527	S2R4	6026	24	07/08/1998	Serum RAT 95
11528	S2R4	6026	25	07/08/1998	Serum RAT 96
21257	S2R4	6035	1	02/03/1999	Rat DPI 99/104811-6
21258	S2R4	6035	2	02/03/1999	Rat DPI 99/104811-7
21259	S2R4	6035	3	02/03/1999	Rat DPI 99/104811-8
21260	S2R4	6035	4	02/03/1999	Rat DPI 99/104811-9
21261	S2R4	6035	5	02/03/1999	Rat DPI 99/104811-10
21262	S2R4	6035	6	02/03/1999	Rat DPI 99/104811-11
21263	S2R4	6035	7	02/03/1999	Rat DPI 99/180630-1
21264	S2R4	6035	8	02/03/1999	Rat DPI 99/180982-1
21265	S2R4	6035	9	02/03/1999	Rat DPI 99/189064-2
21266	S2R4	6035	10	02/03/1999	Rat DPI 99/189064-3
21267	S2R4	6035	11	02/03/1999	Rat DPI 99/189926-1
21268	S2R4	6035	12	02/03/1999	Rat DPI 99/191925-1
21269	S2R4	6035	13	02/03/1999	Rat DPI 99/104811-1
22703	S2R4	6035	14	29/04/1999	Jervis Bay - Rattus Rattus, 1 (1999 2946)
22704	S2R4	6035	15	29/04/1999	Jervis Bay - Rattus Rattus, 3 (1999 2947)

22705	S2R4	6035	16	29/04/1999	Jervis Bay - Rattus Rattus, 4 (1999 2948)
22706	S2R4	6035	17	29/04/1999	Jervis Bay - Rattus Rattus, 5 (1999 2949)
22707	S2R4	6035	18	29/04/1999	Jervis Bay - Rattus Rattus, 6 (1999 2950)
22708	S2R4	6035	19	29/04/1999	Jervis Bay - Rattus Rattus, 7 (1999 2951)
22709	S2R4	6035	20	29/04/1999	Jervis Bay - Rattus Rattus, 8 (1999 2952)
22710	S2R4	6035	21	29/04/1999	Jervis Bay - Rattus Rattus, 9 (1999 2953)
22711	S2R4	6035	22	29/04/1999	J B Beecroft - Rattus Rattus, 2(a) (1999 2954)
22712	S2R4	6035	23	29/04/1999	J B Beecroft - Rattus Rattus, 2(b) (1999 2955)
22713	S2R4	6035	24	29/04/1999	J B Beecroft - Rattus Rattus, 3 (1999 2956)
22714	S2R4	6035	25	29/04/1999	J B Beecroft - Rattus Rattus, 4 (1999 2957)
22715	S2R4	6035	26	29/04/1999	J B Beecroft - Rattus Rattus, 5 (1999 2958)
22716	S2R4	6035	27	29/04/1999	J B Beecroft - Rattus Rattus, 6 (1999 2959)
22717	S2R4	6035	28	29/04/1999	J B Beecroft - Rattus Rattus, 7 (1999 2960)
22718	S2R4	6035	29	29/04/1999	J B Beecroft - Rattus Rattus, 8 (1999 2961)
22719	S2R4	6035	30	29/04/1999	J B Beecroft - Rattus Rattus, 9 (1999 2962)
22720	S2R4	6035	31	29/04/1999	J B Beecroft - Rattus Rattus, 10 (1999 2963)
22721	S2R4	6035	32	29/04/1999	J B Beecroft - Rattus Rattus, 11 (1999 2964)
22722	S2R4	6035	33	29/04/1999	J B Beecroft - Rattus Rattus, 12 (1999 2965)