Characterization of the putative wobbly possum disease virus

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Abstract

The objective of this PhD was to characterise a marsupial arterivirus, termed wobbly possum disease virus (WPDV), and to confirm aetiological involvement of the virus in the development of a neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*), termed wobbly possum disease (WPD). An *in vitro* culture system supporting the growth of the virus, comprising primary possum macrophages was developed. Purified virus stock was prepared using iodixanol density gradient ultracentrifugation of infected cell culture lysates and the *in vitro* growth kinetics of WPDV in primary possum macrophages was investigated using a previously described WPDV-specific RT-qPCR. The steepest increase in the levels of intracellular viral RNA was observed between six and 12 hours post infection, followed by a gradual release of cell-free viral RNA between nine and 24 hours post infection. Maximum levels of intracellular and extracellular viral RNA levels occurred at 24 and 48 hours post infection respectively.

Aetiological involvement of the virus in the development of WPD was supported by induction of disease in healthy wild-caught possums following infection with the purified virus. The pathogenesis of viral infection was explored by characterisation of histological lesions and quantification of WPDV RNA in various tissues from experimentally infected possums. Mononuclear inflammatory cell infiltrates of variable size were consistently observed in the liver, kidney, salivary gland and brain. The highest viral RNA levels were found in lymphoid, splenic and liver tissues, suggesting virus tropism for cells of the immune origin, most likely of the monocyte-macrophage system. High levels of viral RNA in tissues and sera from possums euthanased nearly four weeks post-infection indicates that immune response was ineffective in clearing the virus in that time-frame.

To investigate the presence of the virus in wild possum populations in New Zealand, an indirect ELISA using *Escherichia coli*-expressed recombinant viral nucleocapsid (rN) protein as antigen was developed. Pre and post-infection sera from experimentally challenged possums was used for ELISA development. These sera were also characterised using Western-blot against rN antigen. A serological survey of archival possum serum samples that had been collected between the years 2000 and 2016 and from five different regions of New Zealand was also performed using indirect ELISA. Bayesian estimates of parameters for a model of the ELISA data were used to establish ELISA cut-offs for WPDV antibody positive and negative samples. Applying these cut-offs, 50/230 (22%) archival samples were seropositive by indirect ELISA. Altogether, our data suggest that WPDV has been circulating in wild possum populations in New Zealand. Five out of 14 (36%) of pre-infection sera from the challenge study were also seropositive by Western-blot. Development of WPD in these possums following challenge suggests that pre-existing immunity was insufficient for protection against the development of disease. As such, further exploration of virus, host and environmental factors that govern development of disease is required.

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If I have seen further, it is by standing on the shoulders of giants.

- **Isaac Newton, 1675**

Science does not exist in a vacuum, and nowhere is the famous quote by Isaac Newton more apt than in the pursuit of advancing science. Scientists, researchers, academics, teachers, and technicians go to work each day with no more noble a goal than to make the world a better place for those inhabiting it. Whilst individually our contributions may look small, we are all in fact building up a massively interwoven fabric, becoming more and more connected in our understanding, growing larger and greater as every day goes by.

As members of the scientific community we all aspire to be giants for the next generation, and also in our personal lives we must all depend on our own community of giants to guide, support, and nurture us. It is therefore unfortunate that on the front of any one PhD thesis only one name may be present. This is not due to lack of respect or appreciation for the giants upon whose shoulders we stand. Similarly, our families, friends, and colleagues all leave indelible marks on us. We are formed by our scientific, societal, and familial connections, and it would be an impossibility to enumerate even a small fraction of them. To put any other name at the front of this PhD would only severely undermine the fact that so many people deserve so much of the recognition for enabling this research.

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Multiple researchers contributed to the papers presented in this thesis. The contributions that these individuals have made toward the paper have also been stated at the bottom of each chapter, but I would like to thank them here also:

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To everyone - the thesis you are about to read is something I have immense pride in. I have been working towards the results presented here since mid 2012, and as I sit here writing this in mid 2017, I feel a massive sense of accomplishment, pride, and excitement. Here ends a chapter in my life, and onwards I must go! I hope that in doing so, my shoulders become a little bit wider, and my legs a little bit taller, and I too become a giant for someone else.

Julia Giles,

BVSc, MVM, PhD (Candidate),

September 2017

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List of Abbreviations

$3CL^{pro}$	3C-like protease
Ab	Antibody
ACTB	β-actin gene
aPTT	Activated partial thromboplastin time
AUC	Area under the curve
BSA	Bovine serum albumin
C-terminus	Carboxy-terminus
cDNA	Complimentary DNA
cELISA	Competitive ELISA
CI	Control inoculum
CM	Culture medium
CMI	Cell-mediated immune
CNS	Central nervous system
CoV	Coronavirus
CPE	Cytopathic effects
Cq	Quantification cycle
CTL	Cytotoxic lymphocyte
CV	Coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco’s modified eagle medium
DMV	Double-membrane vesicle
DNA	Deoxyribonucleic acid
DOC	Department of Conservation
DUB	Deubiquitinating
E	Envelope

E.Coli	*Escherichia coli*
EAV	Equine arteritis virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
EM	Electron microscopy
ER	Endoplasmic reticulum
EVA	Equine viral arteritis
ExoN	Exonuclease
FA	Fluorescent antibody
FDP	Fibrin degradation product
FP	False positive
GBSS	Gey's balanced salt solution
GM	Growth medium
GP	Glycoprotein
HCT	Haematocrit
HEL	RNA helicase
HRP	Horseradish peroxidase
ICL	Infected cell culture lysate
ICTV	International Committee on Taxonomy of Viruses
IF	Immunostaining
IFA	Immunofluorescent antibody
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IP	Intraperitoneal
ISH	*In situ* hybridisation

LDH	Lactate dehydrogenase
LDV	Lactate dehydrogenase-elevating virus
M	Membrane
mAB	Monoclonal antibody
MHV	Mouse hepatitis virus
MLV	Modified live vaccine
moi	Multiplicity of infection
mRNA	Messenger RNA
N	Nucleocapsid
N-terminus	Amino-terminus
NendoU	Uridylate-specific endoribonuclease
nsp	Non-structural protein
OIE	World Organisation for Animal Health
OMT	Ribose-2'-O-methyltransferase
ORF	Open reading frame
PBS	Phosphate buffered sodium
PBST	Phosphate buffered sodium + Tween 20
PCR	Polymerase chain reaction
pi	Prevalence
PI	Percentage inhibition
PID	Possum infectious doses
PLP	Papain-like protease
pp	Polyprotein
PPM	Primary possum macrophages
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
PT	Prothrombin time
qPCR	Quantitative PCR

RdRp	RNA-dependent RNA polymerase
RFS	Ribosomal frameshift site
rN	Recombinant nucleocapsid protein
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPMI	Roswell park memorial institute medium
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Quantitative reverse transcriptase PCR
RTC	Replication and transcription complex
RVN	Reticulovesicular network
S	Spike
SARS	Severe acute respiratory syndrome
SD	Standard deviation
sg	Subgenomic
sg MRNA	Subgenomic-length mRNA
SHF	Simian haemorrhagic fever
SHFV	Simian haemorrhagic fever virus
SI	Standard inoculum
SPF	Specific pathogen free
TB	Tuberculosis
$TCID_{50}$	Tissue culture infectious dose 50%
TMD	Transmembrane domain
TNF	Tumour necrosis factor
TP	True positive
TRS	Transcription regulating sequence
USG	Urine specific gravity
VNT	Virus neutralization test
WPD	Wobbly possum disease

WPDV	Wobbly possum disease virus
ZBD	Zinc-binding domain

Chapter 1

Literature Review

1. The order *Nidovirales*

1.1. Introduction

The order *Nidovirales* is a monophyletic group of positive-sense, single-stranded, enveloped ribonucleic acid (RNA) viruses that can infect a wide range of host species including a variety of mammals, birds, crustaceans (Cowley and Walker, 2002; Cowley et al., 2000), fish (Schütze et al., 2006) and insects (Zirkel et al., 2011; Zirkel et al., 2013). Infection with nidoviruses can be subclinical or result in overt disease. Classically the latter was considered to be most common in pregnancy and neonates (Holyoak et al., 2008; Nodelijk, 2002; Ziebuhr and Siddell, 2008). Co-morbidities may also contribute to the development and severity of disease (Arabi et al., 2014; Chan et al., 2003). Clinical disease is frequently related to infection of the respiratory or gastrointestinal tracts (*Coronaviridae*) or the mononuclear-phagocyte system (*Arteriviridae*). The order contains some important veterinary pathogens which can result in substantial economic losses and animal welfare concerns. A notable example of this is porcine reproduction and respiratory syndrome virus (PRRSV) which is discussed in further detail in section 2.5.1. The order also contains important emerging pathogens of man, including new human coronaviruses that can cause severe respiratory tract disease such as: human coronavirus HKU1 (HCoV-HKU1) (Woo et al., 2005); human coronavirus NL63 (HCoV-NL63) (Arden et al., 2005; van der Hoek et al., 2004); Middle East respiratory syndrome coronavirus (MERS-CoV)

(Zumla et al., 2015) and the severe acute respiratory syndrome coronavirus (SARS-CoV), a zoonotic pathogen which was responsible for the SARS epidemic in the years 2002 to 2003, affecting at least 8000 people and causing more than 750 deaths (Kuiken et al., 2003; Peiris et al., 2004). Following the SARS-CoV outbreak, interest in the *Coronaviridae* was ignited, resulting in substantial advancements in the understanding of nidovirus replication/transcription and it is now generally accepted that the enzymology involved with nidovirus replication is the most complex of all the plus-strand RNA viruses (Gorbalenya et al., 2006; Schütze et al., 2006).

This literature review is divided into two sections. Section 1 covers common features of nidoviruses, with a focus on taxonomy, genome organization and genome replication. Section 2 provides a more in-depth review of the family *Arteriviridae*.

1.2. Taxonomy

Despite the relatively small size variation of RNA viruses (approximately 3 - 32 kb), five distinct orders of RNA viruses have been created: *Mononegavirales*, *Nidovirales*, *Bunyavirales*, *Picornavirales* and *Tymovirales* (Viruses, 2016). This is in comparison to DNA viruses which comprise only three distinct orders but have far greater variation in genome size.

The order *Nidovirales* (*nidus* in Latin means nest) derives its name from a conserved strategy in which all genes located downstream from the replicase gene are expressed from a 3' co-terminal nested set of subgenomic (sg) mRNAs. The order was first recognized by the International Committee on Taxonomy of Viruses (ICTV) in 1996, and included two families of vertebrate viruses: *Coronaviridae* which contained two genera, *Coronavirus* and *Torovirus*; and *Arteriviridae*, which contained one genus, *Arterivirus*. More recently, the family *Roniviridae*, which has one genus, *Okavirus*, and infects invertebrate hosts (prawns) and the family *Mesoniviridae*, which has one genus, *Alphamesonivirus*, which infects mosquitoes have been included in the order (Adams et al., 2013; Lauber et al., 2012). Although the exact phylogenetic

relationship between members of the order *Nidovirales* requires further investigation, they can be separated into three groups based on the genome size: The 'large nidoviruses' have genome sizes ranging between 26-32 kb and include coronaviruses, toroviruses and roniviruses. Arteriviruses have genome sizes ranging between 13 - 16 kb and are referred to as the 'small nidoviruses'. Finally, mesoniviruses (*in Greek, meso = middle*) with genome size of approximately 20 kb are referred to as ‘intermediate nidoviruses’ (Gorbalenya et al., 2006; Nga et al., 2011).

Despite considerable differences between nidoviruses in genome size and complexity, virion morphology, structural proteins and the conservation of several RNA-processing enzymes, striking similarities both in genome organization and replication strategy exist within the order. However, some of these features are also shared with other RNA viruses and therefore cannot be considered unique and some other common features which were initially considered nidovirus hallmarks are not universally conserved within the order. At present, the only unique feature that can be used to distinguish members of the *Nidovirales* from other RNA viruses is the organization and composition of the multidomain replicase gene (Gorbalenya et al., 2006).

1.3. Genome organisation

The nidoviral genome consists of positive-sense, single-stranded linear RNA that contains a 5' cap structure and a 3' poly (A) tail. Untranslated regions at 5' and 3' termini flank an array of multiple open reading frames (ORFs). The number of ORFs varies both within and between families of the order, with arteriviruses encoding 10 to 15 ORFs, coronaviruses encoding nine to 14 ORFs, toroviruses encoding six ORFs and roniviruses encoding only four ORFs (Gorbalenya et al., 2006; Marra et al., 2003; Snijder et al., 2013).

The nidoviral replicase gene is located at the 5' end of the genome and comprises two large ORFs (ORF1a and ORF1b) that overlap in a small area containing a -1 ribosomal frameshift site (RFS). The RFS directs translation of ORF1b by ribosomes which have initiated protein synthesis at the ORF1a initiator AUG to produce a frameshift polyprotein 1ab (pp 1ab) (Gorbalenya et al., 2006). Together, ORF1a and ORF1b collectively occupy approximately two-thirds to three-quarters of the genome (Ziebuhr et al., 2000). The size of the resulting frameshift protein is variable, ranging from 3,175 amino acids for the arterivirus, equine arteritis virus (EAV) to 7,200 amino acids for the coronavirus, mouse hepatitis virus (MHV) (Ziebuhr et al., 2000). Ribosomal frameshifting is facilitated by the presence of 'slippery' heptanucleotide sequence (UUAAAC), which appears to be conserved among corona-, toro-, mesoni- and arteriviruses but not in roniviruses (Brierley, 1995; Brierley et al., 1987; Cowley et al., 2000; Nga et al., 2011; Zirkel et al., 2013). Located immediately downstream from this sequence is an elaborate RNA pseudoknot which appears to be conserved among the nidoviruses, again with the exception of roniviruses which contain their own putative ‘slippery’ sequence and pseudoknot (Baranov et al., 2005; Brierley et al., 1987; Cowley et al., 2000; Draker et al., 2006). Ribosomal frameshifting occurs in a relatively small proportion of translation events, approximately 20 – 30% for coronaviruses during *in vitro* replication (Ziebuhr, 2005). It has been postulated that ribosomal frameshifting may reflect a need for a specific molecular ratio of ORF1a and ORF1b-encoded protein functions (Ziebuhr, 2005).

The replicase gene encodes a series of conserved domains which are arranged in a unique nidovirus-specific order. From N to C terminus, these are as follows: Papain-like protease domains (PLPs), transmembrane domain 1 (TMD1), transmembrane domain 2 (TMD2), 3C-like protease ($3CL^{pro}$), transmembrane domain 3 (TMD3), a RFS, an RNA-dependent RNA polymerase (RdRp), a (putative) multinuclear zinc-binding domain (ZBD), an RNA helicase (HEL) and a uridylate-specific endoribonuclease (NendoU) (Gorbalenya et al., 2006). Additionally, some viruses with large genomes also encode a 3’-5’ exonuclease (ExoN) (coronaviruses, toroviruses and roniviruses) (Sittidilokratna et al., 2008; Snijder et al., 2003a), a

ribose-2'-O-methyltransferase (OMT) (coronaviruses, toroviruses and roniviruses) (Snijder et al., 2003a; Zeng et al., 2016), and an ADP-ribose 1-phosphatase (coronaviruses and toroviruses) (Saikatendu et al., 2005; Snijder et al., 2003a). Some of these domains are uncommon or have not been reported to occur in other RNA viruses. Domains that are unique to the members of the order *Nidovirales* and therefore qualify as genetic markers include ZBD (Cowley et al., 2000; Seybert et al., 2005; van Dinten et al., 2000) and NendoU domains (Ivanov et al., 2004; Nedialkova et al., 2009; Posthuma et al., 2006), both of which are encoded by ORF1b. The NendoU domain however does not appear to be encoded by mesoniviruses, and therefore cannot be considered to be a nidovirus-wide conserved domain (Lauber et al., 2012; Nga et al., 2011). It is thought that the ZBD is critical for successful nidoviral replication and transcription by modulating enzymatic activities of the helicase domain and through other as of yet unknown mechanisms (Seybert et al., 2005). NendoU domains efficiently cleave double-stranded RNA at specific uridylate-containing sequences in a Mn2+-dependent manner (Ivanov et al., 2004). They can also process single-stranded RNA at sites where uridylate is present, however do so less specifically and effectively than double stranded RNA suggesting a substrate preference (Ivanov et al., 2004). The NendoU domain serves an essential role in coronaviral and arteriviral RNA synthesis and transcription and has additional roles in the viral life-cycle which are yet to be defined (Posthuma et al., 2006). The ExoN encoded by coronaviruses in the N-terminal half of non-structural protein (nsp)14 appears to play an important role in replication fidelity and has been hypothesized to be the first known proofreading enzyme encoded by an RNA virus. (Graepel et al., 2017; Smith et al., 2013). Currently, the organization and structure of the replicase gene is the only conserved feature that can distinguish nidoviruses from other positive strand RNA viruses.

The number and arrangement of ORFs located downstream from the replicase gene varies between the major nidovirus branches. These ORFs are expressed from a 3' coterminal nested set of subgenomic (sg) mRNAs and whilst all but the smallest transcript are polycistronic, they

are functionally monocistronic, with translation of only the 5'-proximal ORF. These ORFs encode nucleocapsid and envelope proteins, family-specific and additional accessory proteins, and NSPs. Accessory genes may be specific for individual viruses, or may be found in several viruses that form a phylogenetic cluster. Products of these genes are often considered to be functionally dispensable in cell culture, however, some of these may have important functions in infectivity or determinance of virulence *in vivo* (Haijema et al., 2004; Kazi et al., 2005; Ortego et al., 2003). The acquisition of these accessory genes may afford their respective nidoviruses rapid adaptability and selection advantages in their natural hosts (Gorbalenya et al., 2006). Compared to the small nidoviruses, the 3'-proximal ORFs or the replicase ORFs are expanded proportionally in the large nidoviruses (Gorbalenya et al., 2006).

1.4. Genome replication, transcription and translation of viral proteins

Nidovirus genomic RNA serves several functions. Firstly, it is a template for synthesis of a full-length antigenome that in turn serves as a template for positive-strand genome replication. Secondly, the genome serves as a template for the translation of the replicase gene. Thirdly, the genome serves as a template for transcription of a nested set of 5'-coterminal sg mRNAs from which viral proteins are translated.

It is important to note that only a limited number of nidoviruses have been extensively studied and sequenced. It is from investigation of these 'model' viruses that current understanding of nidovirus replication and transcription is based. As families within the *Nidovirales* are genetically diverse and only distantly related, information regarding transcription and translation should be extrapolated with caution when discussing members of the *Nidovirales* which have received little attention. Current knowledge of nidovirus replication and transcription is discussed below.

1.4.1. Nidovirus proteinases: processing and function

A feature of many positive strand RNA viruses is the encoding of polyproteins that are processed into multiple protein products by viral proteases during replication (Firth and Brierley, 2012). This strategy enables smaller genome size as multiple protein products can be expressed from a single ORF. For many RNA viruses, either a single polypeptide or separate structural and non-structural polypeptides are produced (Firth and Brierley, 2012). These are subsequently cleaved by proteinases into functional units.

Viral proteinases are required for expression of the nidoviral replicase complex. For nidoviruses, viral replication occurs by the action of the viral replicase complex which is produced by translation of the replicase gene (ORF1a and ORF1b). The resulting replicase polyproteins (pp1a and pp1ab) are co- and post-translationally processed by two to four viral proteases with papain-like and chymotrypsin-like folds into a number of non-structural proteins (nsps) (12 or 13 in arteriviruses, and up to 16 in most coronaviruses) (Snijder et al., 2003a; Ziebuhr et al., 2000). Polyprotein 1ab is produced by programmed ribosomal frameshifting during translation of ORF1a (Namy et al., 2006; Ortego et al., 2003).

Arteriviruses and coronaviruses encode one 'main' proteinase and between one and three 'accessory' proteinases. These proteinases are encoded within ORF1a. The main proteinase is commonly referred to as 3C-like proteinase ($3CL^{pro}$, name simply reflects similarity to the 3C proteinases of the *Picornaviridae*) and resides upstream of the ribosomal frameshift site, RdRp and HEL domains. It is called the 'main' proteinase as it serves a central role in the expression of the major replicative proteins by directing the proteolytic processing of all downstream domains of the replicase polyproteins. The accessory proteinases are commonly referred to as 'papain-like proteases' (PLPs) and are essential for processing the amino-terminal portion of the replicase polyprotein. In contrast to the main proteinase, the accessory proteinases mediate only

a few cleavages. More recently, structural and enzymatic studies have highlighted that the PLPs share similarities with cellular deubiquitinating enzymes (DUBs) and therefore may serve a multifunctional role (Frias-Staheli et al., 2007; Frieman et al., 2009; Sulea et al., 2005). It has been suggested that DUB activities of the PLPs may be involved in blocking the innate immune response to viral infection (Devaraj et al., 2007; van Kasteren et al., 2013) and this is discussed further in regards to the evasion of the immune response by arteriviruses (section 2.6.2).

1.4.3. Entry into cells

Nidoviral infection is reliant on the interaction of the viral glycoproteins with specific surface proteins on target cells. For coronaviruses, the spike (S) protein is integral in mediating viral entry into host cells and determining cell and tissue tropism (Ballesteros et al., 1997; Rottier et al., 2005). It serves a dual purpose, facilitating both receptor binding and fusion of the viral and cellular membranes. Two mechanisms have been described for coronaviral entry into cells: (1) direct entry at the cell surface following receptor binding and fusion and (2) endocytic internalization with fusion occurring in the endosomal compartment (Nash and Buchmeier, 1997). The route of entry may depend on the cell type and/or individual virus and in both entry mechanisms, receptor binding is the trigger for fusion (Belouzard et al., 2012). Additional triggers such as proteolytic activation of the S protein or pH acidification may also be required to activate fusion (Belouzard et al., 2009; Belouzard et al., 2010; Eifart et al., 2007).

1.4.4. Replication of genomic RNA

Upon entry into the host cell the nidovirus particle uncoats and releases its genomic RNA into the cytoplasm, where replication occurs. Translation begins at the replicase ORF1a start codon, yielding two large replicase polyproteins (pp1a and pp1ab). As discussed in section 1.4.1, pp1ab is produced by ribosomal frameshifting during translation of ORF1a and pp1a and pp1ab are co- and translationally processed into a variable number of nsps. Several of these cleavage products

contain hydrophobic domains which may assist assembly of the nsps on modified intracellular membranes, thus creating a membrane-bound complex for RNA synthesis (Van Der Meer et al., 1998). Although the exact mechanism by which these replication complexes are formed requires further investigation, it is currently thought that autophagosome-like double-membrane vesicles (DMVs) are induced by nidoviral infection and that these DMVs anchor the replication complexes (Goldsmith et al., 2004; Pedersen et al., 1999). This is supported by observation of cells infected with SARS-CoV, EAV and MHV in which large DMVs adorned with viral replication complexes are present (Gosert et al., 2002; Snijder et al., 2006; Snijder et al., 2001).

There is some evidence to suggest that DMVs are induced by association of nsps with the endoplasmic reticulum (ER) membrane. In support of this idea, DMVs have been observed in close proximity to or in continuous association with the ER in electron microscopic analysis of infected cells (Pedersen et al., 1999; Stertz et al., 2007). Coronaviral and arteriviral nsps have also been demonstrated to insert into the ER membrane (Kanjanahaluethai et al., 2007; Oostra et al., 2007). Furthermore, coronavirus and arterivirus replicase proteins have been associated with the ER in infected cells (Snijder et al., 2006; Van Der Meer et al., 1998). Recently, the endogenous autophagosome protein LC3/HsAtg8 has been demonstrated in association with DMV's in cells infected with MHV and SARS-CoV. This suggests that these DMVs could in fact be autophagosomes and it has been suggested that nidoviruses may exploit intracellular autophagy machinery for replication purposes (de Haan and Reggiori, 2008). However, there is conflicting evidence as to whether or not a component of the cellular autophagy system (ATG5) is required for replication of MHV in permissive cells (Prentice et al., 2004) and further investigation into the formation of nidoviral replication complexes and their association with DMVs is warranted. Irrespective of the relationship between nidoviral-induced DMVs and cellular autophagy mechanisms, there are further questions which are yet to be answered, specifically, why the nidoviral replication-translation complexes operate on ER-derived DMV's rather than on the ER surface? In a review outlining the evidence for involvement of the

autophagic pathway in nidovirus replication, two potential reasons have been proposed (de Haan and Reggiori, 2008). Firstly, without 'escaping' on autophagosome-like DMV's that bud from the ER, the ER quality control pathways may recognize and eliminate nidoviral protein aggregates, thus destroying viral replication complexes anchored to the ER membrane. Secondly, toxic damage to the host biosynthetic machinery could occur due to accumulation of viral proteins on the ER membrane and this could interfere with viral propagation. Alternatively the formation of autophagosome-like DMVs could be driven by the cell rather than the virus with the autophagic pathways activated as a protective mechanism, triggered by the accumulation of nidoviral nsps on the ER membrane (de Haan and Reggiori, 2008). In support of this notion, recent studies have demonstrated that autophagy is triggered as a protective mechanism in response to accumulation of protein aggregates in the ER (Yorimitsu et al., 2006).

1.4.4. Structural genes

Nidoviruses operate slightly differently to most other RNA viruses; nidoviral structural proteins are primarily produced by the discontinuous synthesis and transcription of 5’ and 3’ coterminal sets of subgenomic-length mRNAs (sg mRNAs) (Sawicki et al., 2001; Sawicki and Sawicki, 1995; Sawicki et al., 2005). This mechanism does not require viral-encoded proteinases for the expression of structural proteins.

A common leader sequence derived from the 5' ends of the viral genomes is located at the 5' end of all sg mRNA's in arteri- and coronaviruses but is not present in all toroviruses or in any roniviruses. In mesoniviruses, two types of conserved 5’ leader sequences result in two major 3’-co-terminal sg mRNAs from the fusion of different pairs of leader and body transcription-regulating sequences (Zirkel et al., 2013). The junction of the leader sequence and each mRNA “body” can be recognized by a short, characteristic AU-rich sequence of approximately 10 nucleotides which has been named as the transcription regulating sequence (TRS) (Sawicki et

al., 2007). It has been proposed that this sequence regulates the discontinuous transcription of the genome template, either by signalling attenuation or discontinuation of RNA synthesis (Sawicki et al., 2001; Sawicki et al., 2007). In arteri- and coronaviruses, only the 5'-most ORF is expressed from each sg mRNA species with few exceptions and accordingly, the number of ORFs located downstream from ORF1b is comparative to the number of sg MRNA species synthesized (Gorbalenya et al., 2006).

The mechanism by which fusion of a common 5' leader sequence to body segments occurs has been a controversial discussion point over the years and several different models have been proposed (Baric et al., 1983; Pasternak et al., 2006; Sawicki and Sawicki, 2005; Sawicki and Sawicki, 1995; Spaan et al., 1983). The controversy between the most popular models (the 'leader-primed transcription model' and the 'discontinuous extension of minus-strand RNA synthesis' model) primarily stems from whether the discontinuous step occurs during minus or positive-strand RNA synthesis (Baric et al., 1983; Sawicki and Sawicki, 1995; Spaan et al., 1983). In both models, the TRS is proposed to have an important role in directing the discontinuous step. In the 'leader-primed transcription model', the discontinuous step is proposed to occur during positive-stranded RNA synthesis. This model arose due to an initial inability to detect minus-strand sg mRNAs in coronavirus-infected cells (Baric et al., 1983; Pasternak et al., 2006; Spaan et al., 1983). Subsequent detection of minus-strand sg mRNAs in both coronavirus and arterivirus-infected cells lead to the development of an alternative model, in which minus-strand RNA synthesis was proposed to be discontinuous (Sawicki and Sawicki, 2005; Sawicki and Sawicki, 1995). At present, the prevailing view is that the discontinuous step occurs during minus-strand synthesis and bears resemblance to homology-assisted copy-choice RNA recombination. In this model, discontinuous synthesis of minus-strand RNA begins with recognition of the 3' end of the plus-strand genome RNA by the replication and transcription complex (RTC). Elongation of the minus-strand RNA proceeds towards the first TRS, and here, elongation of the minus-strand RNA is either discontinued by the RTC or the

TRS is ignored and synthesis is continued towards the next TRS. When the elongation of each minus-strand RNA is discontinued, the RTC relocates to the 5' end of the genome and the translocated minus-strand RNA is then completed by copying the 5' end of the genome (Pasternak et al., 2006; Sawicki and Sawicki, 2005; Sawicki et al., 2007). Additional research is required to provide further support for the pathway in this model. So far, minus-strand sg mRNAs or 'transcription intermediates' that have not yet been coupled with the leader sequence have not been described, but the discovery of such intermediates would provide further support for this pathway (Pasternak et al., 2006).

The discontinuous transcription mechanism used by arteri- and coronaviruses is unique and distinguishes them from other positive-strand RNA viruses, the majority of which produce sg mRNAs by internal initiation from 'promoters' in the antigenome (Miller et al., 1985; Miller and Koev, 2000; Pasternak et al., 2006). In contrast to arteri- and coronaviruses, the sg mRNAs of all but the largest toroviruses and all known roniviruses lack a common leader sequence and are not produced by discontinuous synthesis (Cowley et al., 2002; Van Vliet et al., 2002). Although little is known about the minus-strand RNAs produced by these two families, it has been speculated that similar to arteri-and coronaviruses, minus-strand synthesis is attenuated to produce sg-length mRNA templates for transcription. The discontinuous extension of the nascent minus-strand with a common leader sequence does not occur in roni-and toroviruses, and instead, it is thought that this template is used directly for transcription (Pasternak et al., 2006). This proposed mechanism is similar to that used by a minority of positive-strand RNA viruses, including tomato bushy stunt virus, red clover necrotic mosaic virus, and flock house virus (Pasternak et al., 2006; Sit et al., 1998; White, 2002).

Nidoviral structural proteins are highly variable between families, with only weak similarities reported for corona- and toroviruses (Den Boon et al., 1991; Gorbalenya et al., 2006; Snijder et al., 1990). Nidoviral envelope glycoproteins have principal roles in target cell recognition, entry into host cells and the release of newly synthesized virions from infected host cells. These

proteins stimulate the hosts' immune response and are targets for protective immunity. There is evidence to suggest that in some viruses they may affect virulence (Phillips et al., 1999). The major envelope proteins in nidoviruses are; S and membrane (M) proteins of corona- and toroviruses, M and glycoprotein 5 (GP5) of arteriviruses, and the large spike glycoprotein and small spike glycoprotein (GP116 and GP64, respectively) in roniviruses. The S proteins of corona- and toroviruses have a highly exposed globular domain and a stem-portion containing heptad repeats organized in a coiled-structure (Bosch et al., 2004; Gorbalenya et al., 2006). The M proteins of corona-, toro-, and arteriviruses differ in sequence but are structurally and functionally similar and have a triple- or quadruple-spanning membrane topology with the carboxy-terminus residing inside and the amino-terminus exposed to the virion surface (Escors et al., 2001). In contrast to corona-, toro- and arteriviruses, roniviruses do not appear to possess a discrete M protein gene (Cowley and Walker, 2002). However, GP116 and GP64 appear to be the product of proteolytic cleavage of a precursor protein and the N-terminal fragment of this protein has three membrane-spanning domains characteristic of the M proteins with the exception that the membrane topology is in the reverse topology (i.e. the carboxy-terminus resides external to the membrane) (Jitrapakdee et al., 2003). The nucleocapsid of all nidoviruses includes a single protein species called the nucleocapsid (N) protein, however, the N proteins of viruses from different families differ in size and structure and it is unlikely that they are evolutionary related (Gorbalenya et al., 2006). The function and dispensability of the N protein may also differ between nidovirus families. For example, the coronaviral N protein is thought to be essential for efficient genome replication and sg mRNA synthesis, whereas, the arteriviral equivalent is not (Almazán et al., 2004; Molenkamp et al., 2000; Schelle et al., 2005).

1.4.5. Assembly and release

The coronaviral nucleocapsid is incorporated into the viral particle by budding into the so-called 'budding compartment', which is located between the rough ER and the *cis*-Golgi region

(Narayanan et al., 2000). Molecular interactions between the envelope proteins influence the formation and composition of the viral envelope, however, the exact mechanism by which this occurs is unknown. Studies using MHV as a model have suggested a possible mechanism for the envelopment of the coronaviral nucleocapsid (Narayanan et al., 2000): The nucleocapsid which consists of genome-size mRNA and N protein interacts with M protein, likely at the ER membrane. The nidus for interaction is unknown, but could be due to either the binding of M protein to genomic RNA, mediated by the M protein packaging signal, or direct interaction between N and M proteins. Envelope (E) protein may then interact with M protein either during or after M protein's interaction with N protein. Spike protein is incorporated into the viral envelope via association with M protein. Envelope protein and M protein facilitate the budding of coronaviral particles from the budding compartment. Enveloped virus is then transported out of the cell by the exocytic pathway.

Arterivirus assembly and release show similarities to that of coronaviruses. Arteriviruses are assembled by the budding of preformed nucleocapsids into the lumen of intracellular membranes of the exocytic pathway (including the ER and/or the Golgi complex) (Wada et al., 1995) and like coronaviruses, they are released from host cells by exocytosis (Dea et al., 2000). Glycoprotein 5 and M protein are essential for viral particle formation, whereas the minor surface glycoproteins are dispensable (although they are required for infectivity) (Wieringa et al., 2004; Wissink et al., 2005). As attempts to produce virus-like particles by co-transfection of cells with expression plasmids encoding EAV GP5 and M proteins has been unsuccessful, it has been suggested that additional factors such as coupling to viral genome replication or interaction between viral RNA sequences and major structural proteins are required for arteriviral particle assembly (Wieringa et al., 2004).

2. Arteriviruses

2.1. Taxonomy: recent expansion of the family

About 20 years ago a new family, the *Arteriviridae*, was established within a newly formed order of RNA viruses, the *Nidovirales* (Cavanagh, 1997; Snijder and Meulenberg, 1998). The recognition of the family *Arteriviridae* within the order was important due to recognition of shared replication strategies with the distantly-related family *Coronaviridae* (Snijder and Meulenberg, 1998). Founding members of *Arteriviridae* were EAV, lactate dehydrogenase elevating virus (LDV), PRRSV and simian haemorrhagic fever virus (SHFV). With advances in molecular tools, the family has continued to expand, including the recent discovery of 11 new simian arteriviruses with high genetic diversity (Bailey et al., 2014; Friedrich et al., 2013; Kuhn et al., 2016; Lauck et al., 2013), a virus of the Australian brushtail possum in New Zealand termed wobbly possum disease virus (WPDV) (Dunowska et al., 2012) and a virus of a forest giant pouched rat (Kuhn et al., 2016).

Due to rapid expansion of the family and large evolutionary distance between current members, the official arterivirus taxonomy has recently been revised to be in accordance with the International Code of Virus Classification and Nomenclature. Currently the family includes five genera; *Equartevirus* (containing EAV), *Poratevirus* (containing LDV and PRRSV species), *Simartevirus* (containing SHFV and simian arteriviruses), *Nesartevirus* (containing an arterivirus from forest giant pouched rats), and *Dipartevirus* (containing WPDV) (Viruses, 2016). Revision of taxonomic classification of the family was supported by two independent studies, one that used pairwise sequence comparison (PASC) methodology to evaluate arterivirus relationships (Kuhn et al., 2016) and another that used diversity partitioning by hierarchical clustering (DEmARC) mediated analysis of genomic variation (Brinton et al.,

2016). Taxonomic classification will likely be subject to further changes in the future to ensure more consistent taxonomic nomenclature among the nidovirus families.

2.2. Arterivirus evolution

The replication of RNA viral genomes can be error-prone in the absence of proof-reading /repair and post-replicative error correction mechanisms (Domingo et al., 1996). This may lead to genetic diversification and virus evolution. The most studied example of arteriviral evolution to date is PRRSV, a causative agent of a pig disease which first emerged in North America in the mid 1980's and subsequently in Europe and Asia in the early 1990's (Albina, 1997; Ellis et al., 1999; Keffaber, 1989). In the early 1990's, two isolates of PRRSV, the North American (PRRSV2, NA genotype) and European (PRRSV1, EU genotype) were independently described and their genomic sequences were compared (Collins et al., 1992; Wensvoort et al., 1991). Surprisingly, the amino acid identity between these two isolates was less than 60%, suggesting that either evolution occurred at a remarkable rate compared to other RNA viruses (Murtaugh et al., 1998), or divergence occurred prior to the 1980's with concomitant emergence in different continents at the same time (Nelsen et al., 1999; Plagemann, 2003). More recently, molecular evolutionary analysis suggested that PRRSV was transmitted from another as of yet unknown host species to swine around the 1980's and has since adapted by altering the transmembrane regions (Hanada et al., 2005). By molecular clock analysis, it has been suggested that the most recent common ancestor of PRRSV1 and PRRSV2 existed approximately 100 years ago, and the most common ancestor of PRRSV1 types emerged between 1947 and 1968 (Forsberg, 2005; Stadejek et al., 2013), suggesting that the great genetic diversity of variants originated in ancestral virus population rather than occurring post-emergence. The circumstances that lead to transmission of PRRSV across oceanic boundaries and mysterious transcontinental appearance within a short period of time are unknown, however, a mechanism involving a global triggering factor such as rapid global spread of a 'helper' virus or pathogen has been suggested (Stadejek et al., 2006b). Adding further to the mystery, it appears that Eastern European countries

harbour highly diverse variants of PRRSV1 viruses, with sharp demarcation between variants at the Eastern border of Poland (Stadejek et al., 2006b; Stadejek et al., 2002). This has been suggested to have arisen due to trade restrictions of livestock during the cold war (Stadejek et al., 2006b). Remarkably, the evolutionary rate of PRRSV is among the highest of RNA viruses so far, perhaps due to aerosol route of transmission and ability to induce persistent infection (Hanada et al., 2005), both of which have been reported for other viruses which have high evolutionary rates (Hanada et al., 2004). The housing of swine in large population dense facilities likely also accelerated evolution, particularly as many populations lacked immunity against PRRSV due to its relatively recent emergence (Hanada et al., 2005). The rate of replication errors for PRRSV is approximately similar to other RNA viruses, therefore, the high replication frequency of PRRSV is likely responsible for relatively rapid evolution (Hanada et al., 2005). In the past year, reclassification of taxonomy of the family *Arteriviridae* has resulted in recognition of European and American viruses as separate species, named *Porcine reproductive and respiratory syndrome virus 1* and *Porcine reproductive and respiratory syndrome virus 2* respectively.

High genetic diversity has also been reported for SHFV variants, and currently, the diversity for known SHFV variants is higher than for any other arterivirus (Lauck et al., 2011; Lauck et al., 2013). In fact, the degree of diversity between some newly described SHFV variants and the type strain is approximately equal to the degree of divergence between LDV and PRRSV (Lauck et al., 2011). However, as only a small number of full genome sequences for arterivirus variants are available, the genetic diversity of other arteriviruses may be underestimated (Lauck et al., 2011).

Persistent infection, a feature of arteriviral infection, may also provide important means of genetic evolution for some arteriviruses. For EAV, outbreaks of equine viral arteritis (EVA) are often small and restricted and lead to the generation of minimal virus diversity. Genetic

divergence and evolution is achieved during the course of persistent infection of the reproductive tract of carrier stallions, leading to the emergence of novel variants of EAV (Balasuriya et al., 2013; Balasuriya et al., 1999; Hedges et al., 1999).

2.3. Virion architecture

Arteriviruses are small enveloped spherical or oval shaped viruses approximately 40 – 70 nm in diameter with a buoyant density of 1.13 – 1.17 g/mL in sucrose and 1.17 – 1.20 g/mL in caesium chloride (King et al., 2012). A single N protein that encapsulates the arterivirus genome is surrounded by a lipid envelope containing surface GP and M proteins. Arteriviruses encode two major and five minor envelope proteins. All of these, with the exception of the recently discovered ORF5a protein, are essential for the production of infectious virions (Welch et al., 2004; Wissink et al., 2005).

2.3.1. Nucleocapsid protein

The arterivirus N protein is a relatively small phosphorylated protein with a molecular mass of 12 - 15kDa (De Vries et al., 1992; Magnusson et al., 1970; Snijder and Meulenberg, 1998; Wootton et al., 2002). Phosphorylation of the N protein appears to be a conserved feature among nidoviruses and may confer RNA and protein binding functionality to the arteriviral N protein (Wootton et al., 2002), however, further research is required to understand the biological function. The C-terminal half of the N protein contains the capsid-forming (or 'dimerization') domain (Wootton et al., 2001) and for PRRSV and EAV, the N protein C-terminal domain exists as crystal structures that forms dimers *in vitro* (Deshpande et al., 2007; Doan and Dokland, 2003a, b). The N terminal domain of the N protein contains a positively-charged RNA-binding domain (Yoo et al., 2003). The greatest variation in sequence between and within members of the family *Arteriviridae* are found within this domain (Meng et al., 1995). For PRRSV the N protein is the most immunogenic protein of the virus and comprises

approximately 40% of proteins in the virion. As such, it is the most abundant protein expressed in infected cells (Meulenberg et al., 1995).

2.3.2. Major envelope proteins

Arteriviruses encode two major envelope proteins, the so-called 'major' GP and a non-glycosylated M protein (16-20 kDa) (De Vries et al., 1992). The 'major' GP shows considerable variation in size between different viruses within the family. For PRRSV, EAV and LDV, GP5 is the major GP (De Vries et al., 1992; Faaberg and Plagemann, 1995; Meulenberg, 2000). The major GP and M proteins form a disulphide-linked heterodimer and are essential for virus particle formation and infectivity (Snijder et al., 2003b; Wieringa et al., 2004; Wissink et al., 2005). Arterivirus M protein has similarities to coronavirus M protein, traversing the membrane three times by utilising membrane spanning regions, exposing only a short amino acid sequence at the virion surface (De Vries et al., 1992; Meulenberg, 2000). The major GP is also predicted to span the membrane three times (Dea et al., 2000).

2.3.3. Minor envelope proteins

Most knowledge on the minor structural proteins has been derived from studies on PRRSV and EAV and has been extrapolated to the other arteriviruses. The minor GPs represent approximately 5 – 10% of the arterivirus particle. The minor GPs (GP2, GP3 and GP4) form heterotrimers (GP2, GP3 and GP4) and heterodimers (GP2 and GP4) (Das et al., 2010; De Lima et al., 2009; Wissink et al., 2005). In addition to interacting with other minor GPs, GP4 also interacts with the major GP (GP5) (Das et al., 2010). A small E protein that associates with the minor GP heterotrimer has also been identified (Wieringa et al., 2004). Recently, ORF5, which encodes GP5 (EAV, LDV, PRRSV) has been discovered to contain an additional AUG-initiated ORF, termed ORF5a that is probably expressed from the same sg mRNA as GP5 (Firth et al.,

2011; Johnson et al., 2011a). The importance of ORF5a expression differs between virus species (Firth et al., 2011). Inactivation of ORF5a expression of EAV by reverse genetics yielded a crippled mutant that exhibited significantly lower virus titres, suggesting that expression of ORF5a is important but not essential in the maintenance of virus infectivity for EAV (Firth et al., 2011). Expression of ORF5a is however critical for PRRSV virus viability (Sun et al., 2013).

2.4. Arteriviruses interactions with their hosts

2.4.1. Cell tropism

Arteriviruses often have very restricted cell tropisms and it is thought that this is the result of the presence or absence of specific cell surface receptors and other cellular membrane factors (Kreutz, 1998; Meulenberg et al., 1998). Both *in vivo* and *in vitro*, macrophages and dendritic cells have been shown to be the primary target for arteriviral replication (Duan et al., 1997; Onyekaba et al., 1989; Plagemann and Moennig, 1992).

Due to their cellular specificity, arteriviruses can be difficult to grow in cell culture. An exception to this is EAV which can replicate efficiently both in primary cell culture (horse macrophages and kidney cells) and in a relatively wide variety of commercial cell lines. Simian haemorrhagic fever virus and PRRSV can replicate in primary macrophage cell cultures from their respective hosts as well as in the African green monkey kidney cell line (MA-104) and its derivatives (e.g. MARC-145) (Duan et al., 1998). Lactate dehydrogenase elevating virus has extremely restricted cell tropism in culture and the only cells currently known to support growth of the virus are freshly explanted mouse primary mouse embryo cultures and primary mouse peritoneal macrophage cultures (Rowson and Mahy, 1985; Stueckemann et al., 1982). Further, only a small percentage of primary peritoneal macrophages within culture support viral replication (approximately 15%) and the ability of the cell culture to support replication

progressively declines as the cells age (Brinton-Darnell et al., 1975). Porcine reproductive and respiratory syndrome virus appears to have a strongly restricted tropism for only some sub-populations and specific states of differentiation/activation of porcine monocytes/macrophages in cell culture (Duan et al., 1997). In an *in vitro* study, PRRSV growth was supported by porcine alveolar macrophages and to a far lesser extent by porcine blood monocytes, with permissiveness to infection in both cell types increasing after one day of cultivation (Duan et al., 1997). Treatment of cells in that study with phorbol myristate acetate, a stimulant of cellular differentiation, also significantly lowered virus replication. The low virus growth in blood monocytes in that study contrasts with an earlier study in which much higher virus titres were found in cultured porcine blood monocytes (Voicu et al., 1994). The reasons for variation between studies are unknown, but could be due to differences in susceptibility of the donor animal, differences in cell-isolation methods or differences in PRRSV isolates used.

2.4.2. Entry into cells

Entry into the cell is via clathrin-mediated endocytosis, with release of the viral nucleocapsid into the cytosol from endosomes (Caì et al., 2015; Nauwynck et al., 1999). The endocytic pathway is acid-dependant, with low pH proposed to induce conformation changes that trigger or facilitate membrane fusion (Nauwynck et al., 1999). Whilst the exact mechanisms that determine cell tropism have not yet been fully elucidated, two receptors on macrophages have been identified which facilitate the entry of PRRSV into macrophages; heparan sulfate for binding (Delputte and Nauwynck, 2006) and sialoadhesin for internalization (Van Gorp et al., 2008; Vanderheijden et al., 2003). Recently, the cell surface receptor CD163 which is mainly expressed in cells of the monocyte/macrophage lineage (Van den Heuvel et al., 1999) has been shown to be involved in PRRSV entry into permissive macrophages, possibly through uncoating/viral release (Van Gorp et al., 2008). A model of PRRSV entry into macrophages has been recently suggested (Van Breedam et al., 2010). In this model, initial contact with the virus

occurs via heparan sulfate glycoaminoglycans on the macrophage cell surface. Following this sialoadhesin is engaged by the viral M/GP5 complex and uptake of the virus-receptor complex occurs via clathrin-mediated endocytosis. Interaction of the multimeric glycoprotein complex formed by the minor surface glycoproteins GP4 and GP2 with the scavenger receptor CD163 and acidification of the endosome are likely required for genome release.

The importance of the minor glycoproteins in determination of cellular tropism *in vitro* has recently been shown (Tian et al., 2012). In one study, an infectious PRRSV cDNA clone that encoded EAV minor GP and E proteins acquired the unusually broad *in vitro* cellular tropism characteristic for EAV (Tian et al., 2012). Similar studies swapping the EAV and PRRSV major glycoproteins did not dramatically change cellular tropism of the resulting infectious EAV or PRRSV clone (Dobbe et al., 2001; Verheije et al., 2002).

2.4.3. Cellular structures associated with arterivirus replication

Similarly to other nidoviruses (section 1.4.4), arterivirus infection triggers the formation of modified intracellular membranes, termed DMVs within the peri-nuclear region of the infected cell. The ORF1a encoded putative membrane-spanning proteins nsp2, nsp3 and nsp5 have been implicated in the formation of the DMVs, however, only the expression of the self-cleaving nsp2-3 polyprotein induced the formation of DMVs in *in vitro* studies (Snijder et al., 2001). How these nsps interact with host cells is currently unknown. Similar to coronaviruses, DMVs can interconnect with each other and cellular ER via their outer membrane, thereby forming a reticulovesicular network (RVN) (Knoops et al., 2012). It is thought that these DMVs provide a scaffold upon which the RTC can associate. Nucleocapsid protein tubules have also been associated with the RVN, raising the possibility that RNA synthesis and assembly are coordinated in the intracellular space (Knoops et al., 2012). There is conflicting evidence that the autophagy pathway plays an important role in the arterivirus replicative cycle. For EAV, one study demonstrated no replicative differences between autophagy-competent and autophagy-

deficient cells *in vitro* (Monastyrska et al., 2013). However, for PRRSV, induction of autophagy significantly enhanced viral titres of PRRSV whilst inhibition of autophagy significantly reduced virus yield (Liu et al., 2012). Further work is required to understand the role of autophagy in arterivirus replication.

2.5. Diseases associated with arterivirus infections

2.5.1. Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome (PRRS) is a serious swine disease caused by the arterivirus, PRRSV, and can present as reproductive failure in pregnant sows or respiratory tract disease and growth restriction in piglets (Goyal, 1993; Pejsak et al., 1997; Terpstra et al., 1991). In pregnant sows, abortions, stillbirth of fresh or mummified foetuses, and cyanosis of the ear and vulva may occur. In piglets, respiratory distress and increased susceptibility to other respiratory infections such as Glasser's disease may occur. The syndrome was first reported in the mid 1980's in North America and subsequently in Europe and Asia in the early 1990's (Albina, 1997; Ellis et al., 1999; Keffaber, 1989). The virus has spread globally since then and is considered endemic in most parts of the world, although some countries remain PRRS free, including New Zealand, Australia and Sweden. In countries where PRRSV is endemic, subclinical disease is common however outbreaks of the more severe forms of PRRS occur occasionally. Despite the availability of vaccines, control of PRRSV remains difficult (see section 2.7.2).

In an experimental live animal infection study using a PRRSV cell culture isolate delivered intranasally to eight pregnant seronegative sows, typical signs of PRRS were observed after four to seven days (Terpstra et al., 1991). In another study, specific pathogen free (SPF) pigs that were exposed by contact to clinically-affected sows showed clinical signs of illness within two

days of exposure (Wensvoort et al., 1991). Variable incubation times have been reported in field outbreaks, ranging from two to 37 days (Dee, 1992; Robertson, 1992; Varewyck, 1991; Wensvoort et al., 1991). There are inherent differences with comparing data from field outbreaks as the circumstances under which infection occurred and how it was monitored differed between the outbreaks. However, differences in observed incubation period may have been due to differences in virulence between viruses involved, dose, route of exposure, pre-existing immunity, or observer acuity. Transmission can occur by direct contact or by aerosols, and airborne spread or movement of stock may facilitate infection of new herds (Terpstra et al., 1991; Wensvoort et al., 1991).

As described in section 2.2, PRRSV has recently been divided into two species; *Porcine reproductive and respiratory syndrome virus 1* (European) and *Porcine reproductive and respiratory syndrome virus 2* (North American) based on phylogenetic analysis. Despite their genetic diversity, the clinical syndromes they cause remain similar. Substantial genetic diversity also exists between PRRSV variants west and east of the eastern Polish border, which was postulated to have developed as a result of restricted movement of stock during the cold war (Stadejek et al., 2006b).

2.5.2. Equine arteritis virus

Equine arteritis virus is the causative agent of a respiratory and reproductive syndrome of equids, termed equine viral arteritis (EVA), and is considered the prototype virus of the family *Arteriviridae*. EVA was first isolated in 1953 following an outbreak of abortion and respiratory disease in a Standardbred breeding farm in the U.S (Bryans et al., 1957a; Bryans et al., 1957b). Since then, serological surveys have demonstrated evidence of infection in most continents (Eichhorn et al., 1995; Huntington et al., 1990; Timoney and McCollum, 1993); however, some countries remain virus-free including Iceland and Japan. On the basis of temporal serological

data, EAV is currently considered to be eradicated from the horse population in New Zealand following an EVA control program (McFadden et al., 2013).

Consistent with other members of the family *Arteriviridae*, EAV infection is highly species specific and infection has only been reported in members of the family *Equidae* (horses, donkeys, zebras, mules) (Balasuriya et al., 2013; Stadejek et al., 2006a; Timoney and McCollum, 1993). Only one serotype of EAV has been identified so far and is termed the 'Bucyrus strain'. Differences in phenotype and virulence exist among field viruses with some variants possessing the ability to cause moderate to severe disease whilst infection with others may only result in fever (Balasuriya et al., 2007; McCollum and Timoney, 1999; Patton et al., 1999; Vairo et al., 2012).

Transmission of the virus generally occurs either venerally from carrier stallions to naïve mares, or via respiratory infection from acutely infected animals during outbreaks (McCollum et al., 1971). Following infection, the virus may be excreted in respiratory secretions for a short period of time, usually less than three weeks (McCollum et al., 1971). Approximately 10 – 70% of stallions develop persistent infection following acute infection with EAV (Timoney and McCollum, 1993). The virus is able to persist in the reproductive tract (primarily in the ampulla of the vas deferens) and testosterone plays an essential role in the establishment and maintenance of the carrier state, with no carriers among geldings (McCollum et al., 1994)

Clinical signs of EAV infection may differ depending on individual animal-related factors such as the age and physical condition (including gravidity) of the animal, virus dose and route of infection (Balasuriya et al., 2013). The majority of infections are subclinical, but primary infection can result in a range of clinical signs including any combination of the following: fever, depression, anorexia, rhinitis, nasal discharge, ocular signs (conjunctivitis, lacrimation,

photophobia, periorbital edema), gait stiffness, urticaria, respiratory distress, petechiation of mucous membranes and edema which may involve the limbs, scrotum, prepuce and ventral body wall (Timoney and McCollum, 1993). Abortion may occur in pregnant mares infected between third and tenth month of gestation and infection of neonatal or young foals may result in a fulminating pneumonia or pneumoenteritis (Piero et al., 1997). Whilst death can occur due to infection with the Bucyrus strain of EAV, it occurs only rarely following infection with EAV field viruses (Pronost et al., 2010; Timoney and McCollum, 1993).

2.5.3. Lactate dehydrogenase-elevating virus

Lactate dehydrogenase-elevating virus was first discovered in 1960 during investigation of plasma enzyme levels in tumour-bearing mice (Riley et al., 1960). It was found that within three days of transplantation of many different types of tumours, there was a five to ten fold increase in plasma lactate dehydrogenase (LDH). Subsequent investigation found that inoculation of clinically normal mice with cell-free plasma from affected mice also reproduced the condition, supporting the notion that an infectious agent rather than effects of the transplanted tumour was responsible for the elevation in LDH. Whilst infection in experimental laboratory colonies is less prevalent nowadays due to awareness of the virus and availability of RT-PCR for monitoring purposes (Chen and Plagemann, 1997), the main importance of the virus from a research perspective is that it may affect results of *in vivo* studies, particularly those involving the immune system.

Infection with LDV in mice results in persistent viraemia. Lactate dehydrogenase elevating virus is able to persist so efficiently because the primary target cell is a renewable, continuously regenerated and apparently ‘non-vital’ subpopulation of macrophages (Plagemann et al., 1995). The only known function of this subpopulation is clearance of a few enzymes, including the muscle type of LDH (Rowson and Mahy, 1985). There also appears to be no severe consequences caused by the effects of persistent LDV on the immune system. Because of the

persistent viraemia, the virus can be spread by blood-sucking ectoparasites, and this is thought to be an important mechanism for transmission in feral mice. Mice persistently infected with LDV have been detected in Europe, America and Australia (Brinton, 2012; Li et al., 2000). Transplacental infection can occur following acute infection during pregnancy, but is less likely in chronically-infected females (Broen et al., 1992). Fighting and cannibalism also facilitate spread of the virus (Rowson and Mahy, 1985). In laboratory colonies, virus transmission via virus-contaminated tissues or tumours is an important source of infection (Chen and Plagemann, 1997).

Two neuropathic LDV laboratory mutants (LDV-C and LDV-v) have been described and infection of C58 and AKR strains of mice with these mutants leads to a generally fatal age-dependant poliomyelitis caused by infection of anterior horn neurons (Anderson et al., 1995). Elderly mice of these strains can spontaneously develop poliomyelitis following infection, whereas younger mice are generally resistant unless the anti-LDV immune response is suppressed by medication or X-irradiation prior to infection (Anderson et al., 1995). Interestingly, neuropathogenic isolates of LDV gradually lose their ability to induce poliomyelitis in susceptible mice during the course of persistent infection of either poliomyelitis-susceptible or non-susceptible mice. It is currently thought that impaired ability to establish persistent infection and out-competition by non-neuropathogenic variants that coexist within the neuropathogenic LDV stocks occurs following *in vivo* infection (Chen et al., 1997; Plagemann et al., 2001).

2.5.4. Simian haemorrhagic fever virus

Simian haemorrhagic fever virus causes a severe and often fatal disease termed simian haemorrhagic fever (SHF) in macaques (*Macaca sp.*), although, asymptomatic infection in macaques has also been described (Palmer et al., 1968). SHFV was first isolated in 1964

following outbreaks of disease in Soviet and American captive primate centres (Gravell et al., 1986; Palmer et al., 1968), likely as the result of contact with, or iatrogenic transmission (shared medical and tattoo needles) from, captive African non-human primates (e.g. patas monkeys). In contrast to Asian macaques, Patas moneys are capable of maintaining asymptomatic infection for periods exceeding 10 years (London, 1977; Vatter et al., 2015).

Clinically, SHF is characterized by acute fever with generalized impairment of hemostasis and coagulation resulting in bleeding diatheses (Abildgaard et al., 1975). Clinical signs may first become apparent as early as 48 – 72 hours post infection (Abildgaard et al., 1975; Gravell et al., 1986; London, 1977; Palmer et al., 1968). In the terminal stage of the disease, anorexia, photophobia, ataxia, facial edema, abortions and gastrointestinal disturbances may also be seen (Zack, 1993). Disseminated intravascular coagulation (DIC) results from release of pro-inflammatory cytokines from infected macrophages and dendritic cells (Bray, 2005). In a recent study, bacterial sepsis was detected in 12 of 16 SHFV-infected animals that did not survive, suggesting that secondary bacterial infection may play a role in the pathogenesis in some animals (Johnson et al., 2011b). The mortality rate in sporadic outbreaks has ranged from 11% to 100% (Gravell et al., 1986; London, 1977; Palmer et al., 1968).

There appears to be differences in pathogenicity in Patas monkeys between different virus isolates, with some isolates in one study possessing the ability to induce acute but rarely fatal infection (LVR, P-180) whereas others establish asymptomatic persistent infections (P-248, P-741) (Gravell et al., 1986). The immune response to these isolates also differed in this study. Isolates that induced acute disease also induced high titres of SHFV-specific IgG antibody. Interestingly, of the four isolates included in this study, none induced cross-neutralizing antibodies in infected patas monkeys.

The results of haematological and coagulation analysis in clinically-affected macaques is reflective of consumptive coagulopathy, with varying degrees of thrombocytopaenia, decreased

haematocrit (HCT), increased activated partial thromboplastin time (aPTT), prothromin time (PT), and elevated fibrin degradation products (FDPs) (Johnson et al., 2011b; Palmer et al., 1968). Lymphopaenia, neutropaenia and monocytopaenia may also be seen on haematology. On biochemical analysis, increased ALT, AST, ALP and decreased albumin may occur and proteinuria may be seen on urinalysis (Johnson et al., 2011b).

Although zoonotic transmission of SHFV has not been demonstrated, there has been recent interest in SHFV as the clinical outcome of infection with these viruses bears similarities to other important zoonotic primate-associated viruses such as Ebola, Marburg and Lassa viruses. Furthermore, SHFV displays biological properties (e.g. high viral load and high genetic diversity within their hosts) that are associated with the potential for rapid evolution, a shared feature of many emergent RNA viruses (Bailey et al., 2014; Lauck et al., 2013).

2.6. Immune responses to arterivirus infection

2.6.1. Innate immune responses

Broadly speaking, the innate immune response is a non-antigen specific response and is important in limiting initial virus replication (Janeway Jr and Medzhitov, 2002). It does so via the release of cytokines and interleukins from infected cells, macrophages and natural killer cells. The innate immune response to arteriviruses can be variable, but is often poor (Lopez-Fuertes et al., 2000; Snijder et al., 2013; Sun et al., 2012). *In vitro* studies of EAV-infected macrophage cells have demonstrated increased transcription of genes encoding cytokine mediators, with virulent and avirulent strains differing in the magnitude of the cytokine response stimulated (Moore et al., 2003). In contrast, similar *in vitro studies* with PRRSV have failed to demonstrate considerable cytokine response and even a down-regulation in the proinflammatory cytokine tumour necrosis factor- α (TNF-α) (Lopez-Fuertes et al., 2000). In

another PRRSV study, field isolates differed in their ability to induce interferon-α (IFN-α) expression in alveolar macrophages *in vitro* and it is possible that variable effect on the innate immune system between genetically diverse arterivirus variants could account for differences in virulence also observed *in vivo* (Lee et al., 2004).

2.6.2. Arterivirus evasion of innate immune responses

Arteriviruses appear to have developed an ability to manipulate the innate immune response to facilitate survival and potentially long-term or life-long persistence within infected hosts (Kimman et al., 2009; Sun et al., 2012). It is likely that arteriviruses employ multiple mechanisms to disarm the innate immune response, but only a few potential mechanisms have been described so far.

Several viral proteins that can dampen innate immune signalling have recently been identified (Beura et al., 2010; Go et al., 2014; Han and Yoo, 2014). These include nsp1, which has shown to be a potent interferon (IFN) antagonist (Han and Yoo, 2014). Interferon is an important antiviral cytokine that has roles in both the innate and adaptive immune responses and PRRSV nsp1 and nsp1 subunits have been demonstrated to suppress the stimulation of IFN (Beura et al., 2010; Han and Yoo, 2014; Sun et al., 2012). Similar to PRRSV, nsp1 of EAV, LDV and SHFV also appear to contain IFN suppressive activities (Go et al., 2014; Han and Yoo, 2014).

Arteriviruses encode a dual-purpose protease (papain-like protease 2, PLP2) that is essential for processing the N-terminal portion of the replicase polyprotein, but also has DUB properties (section 1.4.1). Recently, it has been suggested that DUB activities of the PLPs may be involved in blocking the innate immune response to viral infection (Devaraj et al., 2007; van Kasteren et al., 2013). Ubiquitin is a small (approximately 8-kda) regulatory protein moiety that when found on target proteins may signal these as targets for proteomal degradation (Enesa et al., 2008; Kayagaki et al., 2007; Wertz et al., 2004). This activity can be negatively regulated

through the action of DUB enzymes. Arteriviruses (as well as other RNA viruses), may take advantage of this effect, resulting in down-regulation of IFN and other pro-inflammatory cytokines, and thus ultimate evasion of the hosts innate immune response (van Kasteren et al., 2013). This is supported by a study by van Kasteren and colleagues in which infection with EAV mutants containing a PLP2 engineered to have decreased DUB activity resulted in enhanced immune signalling in infected equine cells (van Kasteren et al., 2013).

Arterivirus N protein may also may have a modulatory effect on the innate immune response by induction of interleukin 10 (IL-10) (Liu et al., 2015; Wongyanin et al., 2012), an immunoregulatory cytokine that can inhibit transcription and translation of a variety of other inflammatory cytokines (Donnelly et al., 2004). In a recent study of PRRSV strain NT0801, it was demonstrated that amino acids 33-37 (N-N non-covalent domain) of the N protein were important for induction of IL-10 expression (Liu et al., 2015). Further research is required to understand whether other members of the family *Arteriviridae* possess N proteins with similar characteristics.

2.6.3. Cell-mediated immune responses

The cell-mediated immune (CMI) responses to arterivirus infections have not been investigated in depth and potential mechanisms by which arteriviruses may evade the CMI response require further investigation.

Interferon-γ is integral in the CMI response to PRRSV by blocking infection of porcine macrophages and other cells *in vitro* (Bautista and Molitor, 1999; Rowland et al., 2001) and it therefore follows that the degree of this response *in vivo* may influence infection outcome. A small amount of information pertaining to PRRSV has been published and this has shown a delayed development of PRRSV-specific IFN-γ- secreting cells following infection or

vaccination with European-origin PRRSV (Diaz et al., 2006; Díaz et al., 2005). Similar findings have been demonstrated for an American modified live vaccine strain of PRRSV (Meier et al., 2003). In this study, virus-specific IFN-γ cells, mainly comprised of double-positive CD4+CD8+ T cells, first started to appear within three weeks following vaccination. The cell count was variable, fluctuating between 50 – 100 per million peripheral blood mononuclear cells (PBMCs) for the following 10 weeks before gradually increasing to 400 – 500 per million PBMCs at 48 weeks (Meier et al., 2003). In a field study involving four herds, the magnitude of IFN-γ production was determined using enzyme linked immunospot (ELISPOT) assay and was compared between sows which developed reproductive signs of PRRS and those that did not. Close to 100% of pigs sampled in this study had pre-existing PRRSV-specific antibodies suggesting that most, if not all pigs had already been exposed to PRRSV. In three of the four herds, a strong CMI response was correlated with protection of sows against PRRS however there was large intra-herd variability with the degree of immune responsiveness and clinical protection. However, the presence of multiple environmental and animal-related (e.g. genetics) factors that were not controlled or consistent between herds precluded identification of reasons for this variability. Identification of such factors however could have the potential to decrease the incidence of reproductive signs within endemically infected herds through implementation of management strategies. However, virus-related factors, such as the existence or emergence of multiple viral genotypes within a farm would be difficult to control, and these variants could potentially differ in their ability to induce CMI.

It has been demonstrated that PRRSV M, N, GP4 and GP5 proteins induce CMI (Kwang et al., 1999). Of the structural proteins, N and M appear to be the strongest inducers of CMI (Díaz et al., 2009; Jeong et al., 2010). T cell epitopes have been identified for N, GP4, GP5 and nsp9 of PRRSV (Díaz et al., 2009; Vashisht et al., 2008). There is little published information on the CMI response to arteriviruses other than PRRSV. For EAV, one study demonstrated that cytotoxicity induced by EAV was virus-specific, mediated by CD8+ T cells and that EAV-cytotoxic lymphocyte (CTL) precursors persisted for at least one year following infection

(Castillo-Olivares et al., 2003). For LDV, specific CD4+ and CD8+ T-cell immune responses were insufficient to clear the virus and CTL precursors disappeared close to four weeks post-inoculation, likely due to high dose-clonal exhaustion in acute infection (Even et al., 1995).

2.6.4. Humoral immune responses

Glycoprotein 5 appears to be the main target for development of neutralizing antibodies following arterivirus infections (Balasuriya et al., 1997; Plagemann et al., 2002) and GP5/M interactions may be essential for the induction of this response in some arteriviruses (Balasuriya et al., 2004; Lopez and Osorio, 2004). The induction of neutralizing antibodies following arterivirus infection is often poor and this has been postulated to be due to glycosylation of GP5, resulting in less efficient binding of neutralizing antibodies (Li et al., 1998; Vu et al., 2011). For the development of complement fixing antibodies, the inciting immunodominant proteins differ somewhat between members of the *Arteriviridae*. For EAV, sera from horses other than carrier stallions most consistently recognize the M protein whereas, the response to the membrane-associated glycoproteins and the N protein are more variable (Hedges et al., 1998; MacLachlan et al., 1998; Wagner et al., 2003). In contrast, sera from EAV carrier stallions consistently reacted with the N protein (Hedges et al., 1998). The N protein of PRRSV is the most abundant and antigenic of the structural proteins and pigs mount a rapid antibody response to the N protein following infection with PRRSV (Dea et al., 2000; Meulenberg, 2000). These early antibodies however are not protective and have been hypothesized (but not confirmed) to contribute to antibody dependant enhancement of disease by increasing virus internalization by macrophages (Cancel-Tirado et al., 2004; Rascón-Castelo et al., 2015; Tirado and Yoon, 2003). Antibody responses against all GPs, protein M, nsp1, nsp2 nsp4 and nsp7 of PRRSV have also been described (Brown et al., 2009; Rascón-Castelo et al., 2015).

2.7. Diagnosis and prevention of arterivirus diseases

2.7.1. Diagnosis

Laboratory diagnosis of EAV infection can be achieved by RT-PCR of nasopharyngeal or conjunctival swabs, blood or semen samples, monoclonal antibody (mAB)-based IHC on fixed tissue samples or by serological assays (Holyoak et al., 2008). The most common serological assay used by many diagnostic laboratories is the virus neutralization test (VNT) and at present, this is the recommended gold standard assay (Holyoak et al., 2008; Timoney and McCollum, 1993) and is also the World Organisation for Animal Health (OIE) prescribed test for international trade. Problems with the VNT assay have included the cost, time, variability of results between laboratories and unreadable tests at lower dilutions caused by serum cytotoxicity (Newton et al., 2004). Competitive ELISA (cELISA) and protein-specific ELISA have been developed, however, differences in protein and positive-sera sources result in variable results (Balasuriya and MacLachlan, 2004) and these assays are not approved for international trade due to historically lower sensitivities and specificities than the VNT. More recently, commercially available cELISA (VMRD EAV antibody cELISA test kit) with high sensitivity and specificity relative to the VNT has been evaluated and proposed to be considered as an alternative approved assay for the detection of antibodies to EAV (Pfahl et al., 2016).

Detection of PRRSV by IHC, fluorescent antibody (IFA or FA), virus isolation, or RT-PCR assays for detection of viral RNA in semen, tissue and serum samples have been described (Jorsal et al., 2014; Morgan et al., 2014; Snijder et al., 2013). RT-PCR can also be used to distinguish between highly pathogenic and classical PRRSVs (Hao et al., 2007). Numerous serological assays have also been developed, however, the most common commercial ELISA used for diagnosing the serostatus of herds is an IDEXX ELISA based on the N-protein antigen (Brown et al., 2009). More recently, the use of oral fluid for diagnosis of PRRSV using PCR,

ELISA and fluorescent microsphere assay has been described, offering a less invasive means to conventional blood testing (Biernacka et al., 2016; Langenhorst et al., 2012).

2.7.2. Prevention

Both attenuated modified live vaccine (MLV) (ARVAC, Zoetis Animal Health Inc., licenced for use in US and Canada) and inactivated adjuvanted vaccine (Artervac, Zoetis Animal Health, licenced for use in Europe) are commercially available (Balasuriya, 2014). Guidelines for vaccination and prevention of EAV-associated disease have been described in detail (Balasuriya, 2014). The MLV provides sustained protection against clinical EVA, with neutralizing antibodies appearing five – eight days post vaccination and persisting for at least two years (Timoney et al., 1988). Modified live vaccine provides good protection against development of clinical EVA and the development of carrier state in naïve colts and stallions, however limited replication of field viruses may still occur (Balasuriya, 2014; Timoney et al., 1988). Modified live vaccine has been credited with limiting large-scale outbreaks of EVA in the U.S (Balasuriya and MacLachlan, 2004). Implementation of infection-control programs on breeding farms, horse events and veterinary clinics may help prevent outbreaks of EVA and should an outbreak occur, proper isolation, disinfection and cessation of breeding may help to prevent further spread of the virus (Balasuriya, 2014). As carrier stallions maintain and transmit the virus, identification and appropriate management of carrier stallions should be implemented in countries where EAV is endemic. Carrier stallions may continue to be used for breeding, but must be housed at separate premises from immune-naïve animals and bred only to seropositive mares (Balasuriya, 2014).

A variety of commercial MLV and inactivated vaccines are available across the U.S and Europe for prevention of PRRS outbreaks and management and husbandry procedures for disease control have been devised (Mateu and Diaz, 2008; Mengeling, 2005). In spite of this, PRRSV is

widespread and reinfection on farms following eradication is relatively common (Mateu and Diaz, 2008). The protection afforded by vaccination with MLV is considered to be greater than that offered by inactivated vaccines (Kimman et al., 2009). However, there have been concerns regarding the use of MLV including the efficacy against all variants of field viruses, reversion of attenuated vaccine strains to virulence, or recombination between MLV strains and field viruses (Kimman et al., 2009; Li et al., 2009; Nielsen et al., 2001).

3. Wobbly possum disease

3.1. Introduction

The Australian brushtail possum (*Trichosurus vulpecula*) is an omnivorous marsupial that was first introduced to New Zealand from Australia in the 19th century. Due to the lack of predators and lush environment, possums have thrived in New Zealand and have since become a significant pest, causing substantial destruction of New Zealand's unique ecosystem and wildlife (Cowan and Waddington, 1990; King, 1990). Further, possums are considered to be the primary wildlife reservoir of bovine tuberculosis (TB) in New Zealand (Coleman, 1988; Coleman and Caley, 2000).

The severity of the situation in New Zealand and the protected status of the species in some states of Australia lead to several investigations into infectious pathogens of possums (Perrott, 1998; Rice and Wilks, 1996; Thomson et al., 2002a). Despite these efforts, only a limited number of viruses have been identified from possums. These include possum enteroviruses (Zheng, 2007), possum adenovirus (Thomson et al., 2002b), and possum papillomavirus (Perrott et al., 2000c). In addition, herpesvirus- and coronavirus-like particles have been observed in faecal samples from possums by electron microscopy (Rice and Wilks, 1996).

Unexpectedly in 1995, an outbreak of a severe and previously undescribed neurological disease, termed wobbly possum disease (WPD), occurred in a captive possum colony (Ag Research, Invermay) in New Zealand (Mackintosh et al., 1995). Several years thereafter, the disease was also reported in free-living possums in New Zealand in the Rotorua district (Perrott, 1998; Perrott et al., 2000a). Since then, there have been anecdotal reports of the disease and possums with consistent histological features of the disease have been described in a report by the New Zealand Department of Conservation (DOC) (McLeod, 2007). Whilst it is suspected that this disease continues to exist in wild possum populations in New Zealand, there is no active monitoring scheme in place and the true prevalence of WPD is unknown.

3.2. Biology and ecology of the Australian brushtail possum

The Australian brushtail possum (also known as the common brushtail possum) is a nocturnal and semi-arboreal marsupial belonging to the family *Phalangeridae* that is native to Australia. Whilst the predominant diet consists of foliage, they may also consume birds' eggs and chicks, insects, other small vertebrates such as rats and mice and carrion (Brown et al., 1993; Ragg et al., 2000). They are able to rapidly adapt to different kinds of vegetation and different environments, and can inhabit forests, farmlands and urban environments (Nowak, 1999). This adaptability and lack of predators has allowed the species flourish in New Zealand. On the other hand, possum populations have been in decline in several regions of Australia (How and Hillcox, 2000; Woinarski et al., 2010). In addition to predators native to Australia, such as snakes, tiger quolls and powerful owls, possums are threatened by introduced animals such as cane toads, cats and foxes and also by adverse events such as bush fires (Woinarski et al., 2010).

Adult brushtail possums weigh between 1.3 and 5.0 kg, have a head and body length of 32 – 58 cm and a tail length of 24 – 35 cm (Nowak, 1999). The body and all of the tail except for a

hairless ventral patch at the tip are covered in a thick and woolly coat that can vary in colour from silver-grey, brown, red or black (Nowak, 1999). A scent gland that is used to mark territory is located on the chest, the secretions of which cause a reddish discolouration to the surrounding fur (Nowak, 1999). The tail is prehensile and adapted to grasping branches and the first digit of each hind-paw lacks a claw but is strong and opposable.

The gestation period of the brushtail possums is short, on average 17.5 days (Nowak, 1999; Pilton and Sharman, 1962). Following this, a tiny 1.5 cm long and approximately 2 g neonate crawls to the mother's forward opening pouch and attaches to one of her 2-4 teats. They remain in the pouch for a further four – five months before becoming back-riders for a further two – five months (Nowak, 1999). Milk constituents undergo marked quantitative and qualitative changes during lactation (Cowan, 1989). As such, establishing a pathogen-free colony of research possums by hand-rearing poses considerable challenges. Females reach sexual maturity at one year of age and males by two years of age (Nowak, 1999). In the wild, they may live for over 13 years (Nowak, 1999).

Although possums are generally solitary animals, they sometimes share dens, however, the incidence of den-sharing varies markedly between different geographical areas and appears to be related to population density (Caley et al., 1998). Dens are usually made in sheltered places like tree hollows, crevices and caves but in urban spaces may be found in roof spaces. During den sharing, there is close physical contact and affiliative behaviours such as grooming and food sharing may occur (Day et al., 2000). It has been suggested that den sharing may provide an excellent mechanism for transmission of infectious diseases (Day et al., 2000). Most contacts between possums occur in the peak mating season (March-May) and are likely related to mating behaviours, including sexual contact and agonistic behaviours such as threats/vocalisation and fighting (Ji et al., 2005).

3.3. Early WPD transmission studies

Following the original outbreak of WPD in the Invermay research facility, a transmission study was performed using filtered tissue homogenates derived from tissues from affected possums and administered to healthy wild-caught possums via intraperitoneal (IP) injection (O'Keefe et al., 1997). In this study, WPD was reproduced in 15/16 inoculated possums and not in un-inoculated control possums.

Further transmission studies were undertaken to investigate the routes of transmission of WPD. A standard inoculum (SI) comprised of homogenized and filtered tissues from seven WPD-affected possums (Invermay-origin) was titrated *in vivo* and determined to have $>10^5$ possum infectious doses $(PID)_{50}$/mL. The inoculum was then administered to six healthy wild caught possums via intragastric route (4 mL per possum, $n = 2$), intratracheal route (2 mL per possum, $n = 2$) and intradermal route (0.1 – 0.2 mL per possum, $n = 2$). All possums that received the standard inoculum developed consistent clinical and histological signs of WPD irrespective of the route (Perrott et al., 2000b). To further investigate the routes of disease transmission, blood, urine and homogenized mites derived from WPD-affected possums were then administered to healthy wild-caught adult possums by the following routes: blood (intradermal, $n = 1$ and intraperitoneal, $n = 1$), urine from an acutely ill possum (intraperitoneal, $n = 2$), urine from a convalescent possum (intraperitoneal, $n = 1$) and homogenised mites (intradermal, $n = 1$). One juvenile possum received a transfer of live mites derived from two affected adults (Perrott et al., 2000b). All possums in the study developed disease with the exception of one possum that received urine from a convalescent possum and the juvenile possum that received a transfer of live mites. This demonstrated that WPD can be transmitted by multiple routes and from multiple infectious sources. In the same study, WPD also occurred in healthy possums sharing a group enclosure with infected possums, but not in individually-housed possums sharing the

same air space, suggesting that the virus is spread by direct contact and not by aerosol. However, the natural routes of transmission in the wild and the infectious dose are unknown.

Wobbly possum disease was also reproduced experimentally using tissues from possums from the Rotorua outbreak, however, clinical signs and histological changes in infected possums were relatively mild compared to possums inoculated with Invermay-origin tissues (Perrott, 1998; Perrott et al., 2000a). The disease was presumed to be viral in origin due to the successful reproduction of disease using filtered homogenates (O'Keefe et al., 1997; Perrott, 1998), however, the aetiological agent remained a mystery. Also, as all transmission studies to date ended in euthanasia, the proportions of animals which may have recovered from infection were unknown.

3.4. Clinical and pathological features

The disease was characterised clinically by the progressive development of neurological signs including; fine head tremor, ataxia, reluctance to move (possibly as the result of ataxia) and apparent blindness. Prior to the onset of overt neurological signs, a decreased appetite and altered behavioural responses (timidity or extreme aggression) were seen in some possums. Clinical signs of colitis, including frank blood and/or mucus in the faeces was observed in some possums (Mackintosh et al., 1995; Perrott, 1998; Perrott et al., 2000a). The majority of experimentally infected possums developed severe clinical signs within two weeks of inoculation (Perrott, 1998; Perrott et al., 2000b). On post-mortem investigation, cachexia was the only consistent abnormality noted (Mackintosh et al., 1995; Perrott, 1998). Histologically the disease was characterised by perivascular mononuclear infiltrates comprising predominantly plasma cells and lymphocytes in multiple tissues, but most prominently in the liver (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott, 1998). Interstitial nephritis and mild non-suppurative mengingoencephalitis were also reported. No neuronal-cell damage was observed. In the absence of molecular or serological diagnostics, a weighted scoring system based on clinical

and histological features has been developed to facilitate an objective case definition of WPD (Perrott, 1998; Perrott et al., 2000b).

3.5. Identification of a nidovirus in tissues of WPD-affected possums

In 2012, Dunowska and colleagues identified the partial (9525 nt) genomic sequence of a nidovirus in archival tissue samples from WPD-affected possums using next generation sequencing (Dunowska et al., 2012). The nidovirus clustered with other arteriviruses, however, relatively large genetic distances existed between the virus and other current members of the *Arteriviridae*. More recently, this virus has been officially recognized as a member of the family Arteriviridae (Viruses, 2016), however, it appears to be highly divergent compared to other current arteriviruses (Dunowska et al., 2012; Gulyaeva et al., 2017; Kuhn et al., 2016). Results of recent phylogenetic analysis have placed WPDV in a basal position in the arteriviral phylogenetic tree, and as such, WPDV is currently the closest known member to ancestral arterivirus (Gulyaeva et al., 2017; Kuhn et al., 2016).

3.6. Aetiological links to WPD

The aetiological link of the novel virus to WPD was first explored in the same study discussed in section 3.3. Dunowska and colleagues tested archival tissue from WPD-affected and WPD-unaffected possums using WPD-specific PCR. In that study, the proportion of possums positive for the novel virus by PCR was significantly higher ($p < 0.0001$) among possums with WPD than clinically healthy possums and the virus was suggested to be involved aetiologically in the development of WPD. *In-situ* hybridisation (ISH) was also performed on nine tissues from two WPD-affected possums that demonstrated compatible histological changes and seven tissues from two healthy possums (Dunowska et al., 2012). Virus-specific signal of variable intensity

was detected in 8/9 tissues from WPD-affected possums and not in healthy controls, thus providing further support for aetiological involvement.

More recently, a quantitative reverse transcription PCR (RT-qPCR) assay to detect the novel nidovirus has been developed (Dunowska et al., 2013). Using this assay, viral RNA was detected in all 22 individual tissues tested from 11 WPD-affected possums but not in any of the four tissue samples from two healthy possums. Whilst both of these studies strongly support the aetiological involvement of the novel nidovirus in development of WPD, an alternative and less likely explanation for the presence of virus in the tissue could be as a coincidental bystander or accidental finding due to the favourable conditions created during disease. Thus, further research, such as fulfilment of original or modified Koch's postulates for disease causation is required to build upon current PCR-based data.

3.7. Organisation and structure of the genome

The predicted genomic organisation of WPDV is typical for that of arteriviruses (Dunowska et al., 2012; Gulyaeva et al., 2017). Two ORFs (*ORF1a* and *ORF1b*) that were predicted to encode a large replicase polyprotein (pp1ab) were found at the 5'end of the genome. A slippery sequence overlapping *ORF1a* and *ORF1b* has been identified, as has a downstream sequence that was predicted to fold into a pseudoknot. The position of the slippery sequence and pseudoknot in the genome suggests that the two 5'most ORFs are expressed via a -1 RFS, a common feature of current members of the family *Arteriviridae* (Brierley et al., 1992). Analysis of the predicted pp1ab protein has also identified the presence of a conserved motif that is characteristic of chymotrypsin-like proteases and has been designated as the putative $3CL^{pro}$. As described above in section 1.4.1, the $3CL^{pro}$ is the "main" proteinase encoded by nidoviruses and is responsible for the cleavage of central and C-terminal portions of pp1ab into mature polypeptides. From N to C terminus, the order of domains in WPDV replicase gene are as follows: PLPs (PLP1a, PLP 1b, PLP1c and PLP2), transmembrane domains, $3CL^{pro}$, a RFS,

RdRp, ZBD, HEL1 and NendoU (Dunowska et al., 2012; Gulyaeva et al., 2017). The PLPs of WPDV are highly divergent compared to other arteriviruses, and zinc finger (ZNF) and nuclease domains which are conserved in other arteriviruses are not encoded by WPDV PLP1a.

Downstream from *ORF1a* and *ORF1b* are a further eight ORFS (designated *ORF 2 – 9*), a 3' untranslated region and a polyA tail. *ORFs 2-9* (comparable to ORFs 2a, 2b, 3, 4, 5a, 5, 6 and 7 of arteriviruses) were predicted to encode structural proteins (Dunowska et al., 2012). *ORF9* (analogous to ORF 7 of arteriviruses) and *ORF8* (analogous to ORF 6 of arteriviruses) were predicted to encode the N and M proteins, respectively. *ORF2* and *ORF4* were predicted to encode proteins that bear similarities to the major GPs of other arteriviruses. Viral or cellular homologues of the structural genes were not identified using BLAST searches, and all structural genes were annotated based on size, charge and structure of their predicted protein products, thus highlighting the diversity of the structural genes of different arteriviruses.

3.8. Scope of thesis

Discovery of this novel marsupial nidovirus raised questions about its possible use as a biocide. However, further research to understand the molecular and biological properties of the virus would be required in order to assess its suitability for this purpose. The work described in this thesis comprises basic research that was aimed at elucidating some of the biological properties of the putative WPDV. The first aim was to develop an *in vitro* culture system for the virus in order to acquire a source of the purified virus for further experimentation (Chapter 2). The second aim was to satisfy Koch's postulates of causation by reproduction of WPD in healthy possums with purified WPDV isolate (Chapter 3). The third aim was to evaluate the pathological consequences of WPDV infection including analysis of the virus load and histological changes in tissues collected post-mortem from experimentally infected possums (Chapter 4). The fourth and final aim was to develop a serological test for detection of WPDV

infection to allow for serological surveys of possum populations both within New Zealand (Chapter 5) and overseas.

The layout of this thesis is paper-based. Each chapter covers research data that were intended to be published in one peer-reviewed paper.

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Chapter 2

Primary possum macrophage cultures support the growth of a nidovirus associated with wobbly possum disease

Summary

The objective of the study was to establish a system for isolation of a thus far uncultured, marsupial nidovirus associated with a neurological disease of possums, termed wobbly possum disease (WPD). Primary cultures of possum macrophages were established from livers of adult Australian brushtail possums (*Trichosurus vulpecula*). High viral copy numbers (up to 6.9 x 10^8/mL of cell lysate) were detected in infected cell culture lysates from up to the fifth passage of the virus, indicating that the putative WPD virus (WPDV) was replicating in cultured cells. A purified virus stock with a density of 1.09 g/mL was prepared using iodixanol density gradient ultracentrifugation. Virus-like particles approximately 60 nm in diameter were observed using electron microscopy in negatively stained preparations of the purified virus. The one-step growth curve of WPDV in macrophage cultures showed the highest increase in intracellular viral RNA between six and 12 hours post infection. Maximum levels of cell-associated viral RNA were detected at 24 hours post infection, followed by a decline. Levels of extracellular RNA increased starting at nine hours post infection, with maximum levels detected at 48 hours post infection. The establishment of the *in vitro* system to culture WPDV will facilitate further characterisation of this novel nidovirus.

Introduction

Wobbly possum disease virus (WPDV) is a possum nidovirus that was discovered by next generation sequencing of archival tissue samples from possums affected by a severe neurological disease, termed wobbly possum disease (WPD) (Dunowska et al., 2012). Aetiological involvement of the virus in the development of WPD was first suggested based on the results of WPDV-specific reverse transcriptase PCR (RT-PCR) using liver DNA as template (Dunowska et al., 2012) and was further supported by the demonstration of viral RNA in various archival tissues from WPD-affected possums using *in situ* hybridisation (ISH) and quantitative reverse transcriptase PCR (RT-qPCR) (Dunowska et al., 2012; Dunowska et al., 2013). This was consistent with the multi-organ distribution of histological lesions observed in WPD (O'Keefe et al., 1997; Perrott et al., 2000a; Perrott et al., 2000b) and further supported aetiological involvement of the virus in WPD. Unfortunately, Koch's postulates for disease causation could not be tested as no source of purified WPDV existed. As such, to facilitate further investigation of this marsupial nidovirus, there was a need to generate purified virus stock for further experiments. Development of an *in vitro* culture system that supports growth of WPDV was thus required.

The work presented in this chapter describes establishment of *in vitro* cultures of primary possum macrophages (PPM), which support WPDV replication. We also describe kinetics of the viral growth in those cultures, and the initial electron microscopy (EM) images of the purified virus isolate.

Materials and methods

Preparation of PPM cultures

A modified protocol based on those published by Kitani et al (Kitani et al., 2010; Kitani et al., 2011) was used to establish PPM cultures. The liver of an adult brushtail possum was dissected aseptically from the body cavity immediately after euthanasia using an inhalational halothane chamber followed by exsanguination (all manipulations were approved by Massey University Animal Ethics Committee). The entire liver was macerated in petri dishes using crossed scalpel blades under sterile conditions, and washed three times in sterile Gey's balanced salt solution (GBSS: 0.166 g/L $CaCl_2$, 0.97 g/L KCl, 0.03 g/L KH_2PO_4, 0.21 g/L $MgCL_2$-$6H_2O$, 0.07 g/L $MgS0_4$-$7H_2O$, 8.00 g/L NaCl, 0.227 g/L $NaHCO_3$, 0.12 g/L Na_2HPO_4, 1.0 g/L glucose, pH 7.2). For each wash, the liver pieces were agitated gently for two minutes at room temperature in 250 mL of GBSS and allowed to settle prior to aspiration of GBSS. Following the final wash, the tissue was digested in 300 mL of GBSS containing 0.05 w/v % collagenase (Invitrogen) and 0.14 w/v % dispase (Invitrogen) for 30 minutes at 37 °C, pipetted up and down to break the remaining large tissue fragments and passaged through a sterile sieve. The cells were pelleted at 300 x g for 10 minutes, washed three times in GBSS, and seeded at a density of 4 x 10^4 cells/cm in growth medium (GM) consisting of Advanced DMEM (Invitrogen) supplemented with 2% Penicillin-Streptomycin (final concentration of 200 U/mL penicillin and 200 µg/mL streptomycin, Invitrogen), 1% Glutamax (Invitrogen), 5% heat inactivated fetal calf serum (Thermo Scientific), 100 µM 2-mercaptoethanol (Sigma-Aldrich) and 10 µg/mL insulin (ACTRAPID Penfill, Novo Nordisk Pharmaceuticals Ltd). The cell suspensions (15 mL/flask) were added to 75 cm tissue culture flasks (Nunc Thermo Scientific) and maintained at 37 °C in a humidified atmosphere with 5% CO_2 for up to 21 days. Growth medium was completely replaced a day after seeding, and every two to four days thereafter. Round macrophage-like cells that started to develop on the monolayer of liver cells from around day five were collected every two to three days by gently shaking the flasks using a temperature-controlled shaker (Gyromax 737, Amarex Instruments Inc.) at 60 rpm for 45 min at 37 °C. Media containing macrophage-like cells were transferred into 250 mm non-tissue culture grade plastic petri dishes (Grenier Bio-One, 2.5 mL per dish) and fresh GM added to the flasks. Non-adherent cells were

removed from petri dishes by replacement of the media six hours later, and the dishes were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Phagocytic assay

One-day-old macrophages were scraped from the surface of the petri dishes using a cell scraper (Corning) and seeded into an 8-well chamber slide (Nunc) at a density of 10^5 cells/well in 100 µL of GM. Following an overnight incubation at 37 °C in a humidified 5% CO_2 atmosphere, the medium in six wells was replaced with 100 µL of GM containing 1.25 x 10^7 carboxylate-modified red fluorescent latex beads (2.0 µm diameter, Sigma-Aldrich, L303). The cells were incubated as above for either one and a half or four hours, and 4',6-diamidino-2-phenylindole (DAPI, NucBlue Fixed Cell ReadyProbes Reagent, Invitrogen) was then added to the culture medium according to the manufacturer's instructions. The cells were rinsed four times with PBS to remove non-phagocytised beads, fixed briefly with 100% ethanol at room temperature and examined using a fluorescent microscope (Ziess Axiophot) and image capture system (Olympus DP70: Cellsens dimensions).

In vitro growth of WPDV

Macrophages grown on monolayers of mixed possum liver cells (typically in the final stages of culture, when monolayers were judged to be too fragile to shake macrophages off them) or in petri dishes (one day after seeding) were inoculated with 100 µL of a 1:10 dilution of a standard inoculum (SI) that had been used during previous WPD transmission studies [4]. The SI comprised a filtered (0.2 µm) 10% suspension of liver, spleen and brain from seven WPD affected possums, and had been previously shown to contain at least 10^5 possum infective doses (PID) 50% of the WPD agent. Up to five blind passages of the virus were then performed. On each passage the macrophage culture was freeze-thawed three to five days following infection, and an aliquot of cell lysate was transferred into a petri dish (100 µL) with a fresh culture of

PPM or into a flask (600 µL) containing macrophages growing on a monolayer of mixed liver cells. For the fifth passage of the virus, petri dishes and flasks were inoculated with a 1:1000 dilution of cell lysate from the fourth passage. Cultures were observed daily for cytopathic effects (CPE) using inverted light microscopy.

The growth of the virus was detected using RT-qPCR specific for WPDV (Dunowska et al., 2013). Briefly, total RNA was extracted from cell culture lysates using Total RNA mini kit (Geneaid Biotech Ltd). RNA was eluted in 50 µL of water and 8 µL was used for cDNA synthesis using qScript™ cDNA Supermix (Quanta Biosciences) according to the manufacturer's instructions, in a total volume of 10 µL. The qPCR assays were run using an Eco real-time instrument (Illumina Inc., San Diego, CA, USA). Each qPCR reaction was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences Inc., Gaithersburg, MD, USA), a final concentration of 0.4 µM of each primer (WPD.A1.F: 5'-TCCCAGCGTTGTGGCTCCGA-3') and WPD.A5.R: 5'-TGCAGGCTGGTGTGGTACGGT-3') and 2 µL of template cDNA in a total volume of 10 µL using previously published cycling conditions (Dunowska et al., 2013). The viral copies per µL of template cDNA were calculated from quantification cycle (Cq) values using the Eco or EcoStudy software (Illumina Inc.) based on the standard curve generated using serial dilutions (10^7 to 10^1 copies/µL) of linearized plasmid containing the expected PCR product comprising 165 bp from *ORF1b* (GenBank accession JN116253). Samples were considered positive if the amplification curve crossed the threshold, and there was a corresponding melting peak between 82.9 °C and 83.9 °C. All standards and samples were run in duplicate.

In addition to PPM, the following primary cells and continuous cell lines were tested for their ability to support the growth of WPDV: primary possum kidney cells, potoroo kidney cell line (PtK2, ATCC-CCL-56), opossum kidney cell line (OK, ATCC-CRL-1840), African green monkey kidney (MA-104) cells, and mouse macrophage cell lines RAW264.7, J774 and P388D1 (all three kindly provided by Dr Patrick Reading). The cells were maintained either in

Advanced RPMI (Invitrogen, RAW264.7, J774 and P388D1 cells) or Advanced DMEM (PK, PtK2, OK, and MA104 cells) media supplemented with 1% Penicillin-Streptomycin (final concentration of 100 U/mL penicillin and 100 µg/mL streptomycin), 1% Glutamax, and 2-5% heat inactivated fetal calf serum. Either SI or WPDV isolate from the original culture in the PPM (denoted as passage 0) were used as infectious inocula. One or two blind passages, up to six days each, were performed. Cells were observed daily for viral CPE and the cell lysates from the final passage were tested for WPDV RNA by RT-PCR (Dunowska et al., 2012) or RT-qPCR (Dunowska et al., 2013).

Purification of the WPDV isolate

The infected cell culture lysates (approximately 80-85 mL) were combined and clarified by centrifugation at 3,000 x g for 20 min at 4 °C. The clarified lysates were layered on top of 1 mL 50% iodixanol (Optiprep, Sigma-Aldrich) cushion in each of six 15 mL ultraclear ultracentrifuge tubes (Sorvall Instruments) and centrifuged (Sorvall Combi-OTD ultracentrifuge) at 75,000 x g for 90 min at 4 °C using SW41 swinging bucket rotor. The supernatants were aspirated and discarded except for a volume equivalent to that of the cushion (1 mL). The remaining contents were combined and divided into two crimp-seal TV865 vertical rotor tubes (Sorvall Instruments). Iodixanol (25% w/v in PBS pH 7.0) was added as required to appropriately fill and balance the tubes, which were then centrifuged at 200,000 x g for six and a half hours at 4 °C. The gradients formed were unloaded dense end first in 0.75 mL fractions by bottom-tube puncture using an 18 gauge needle. The load of viral RNA in each fraction was estimated by WPDV-specific RT-qPCR as described above, and results were presented as viral copy numbers per µL of template cDNA. The density of different fractions was calculated based on the weight of 100 µL aliquots of each fraction. Fractions that contained the highest number of viral copies from 16 separate gradients were combined to form a purified virus stock (12 mL). The purified virus stock was filtered through 0.45-µm pore-size filters and frozen at -80 °C until use.

EM of purified WPDV

Transmission EM was performed on negatively stained preparations of the purified virus. Briefly, a drop of the purified virus was placed on a strip of parafilm, and formvar-coated square 200 mesh Cu grid (Agar Scientific) was placed on top of the drop for four minutes. Extra liquid was absorbed using filter paper and then a drop of 2% saturated uranyl acetate (BDH) in 50% ethanol was placed on the grid for four minutes. Extra liquid was absorbed using filter paper and the grid was allowed to dry before viewing. Images were taken using a Philips CM10 TEM (Eindhoven, The Netherlands) with Olympus SIS Morada camera and iTEM software (Olympus Soft Imaging Solutions, Germany).

Titration of WPDV

The WPDV isolate was titrated using a modification of a standard end-point titration method. Briefly, each well of a 96-well cell culture plate (Corning) was seeded with 100 µL PPM containing $1x10^5$ cells. An aliquot (100 µL) of each of serial 10-fold dilutions (10^{-1} to 10^{-10}) in GM of the virus (either purified virus stock or cell lysate from the fifth passage) was then added in triplicate to separate wells on the plate. The plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 72 hours and then frozen at -80 °C. Each well was scored as positive or negative for WPDV based on results of WPD specific RT-qPCR test, as described above. Wells were considered positive for replication of WPDV if more than 100 viral RNA copies/µL of cDNA were detected. The viral titre was calculated according to the method of Reed and Muench and expressed as tissue culture infectious dose 50% ($TCID_{50}$) per mL.

Kinetics of WPDV replication *in vitro*

Replication kinetics of WPDV was assessed based on the one-step growth curve. Four wells in each of eight 96-well tissue culture plates were seeded with 1×10^5 PPM in 100 µL of GM per well, and three of these wells per plate were inoculated with 100 µL of the purified WPDV

each, at a multiplicity of infection (moi) of 0.5. Negative control on each plate comprised one well with the uninfected macrophages, and non-replication control consisted of one well with viral inoculum incubated in a cell-free media. Viral replication was assessed at eight time-points (0.5, 3, 6, 9, 12, 18, 24 and 48 hours post infection, each in triplicate). At each time-point, cell culture media were transferred from appropriate wells on one 96-well plate to 1.5 mL microtubes (Axygen) and centrifuged at 3,000 x g for 10 minutes to pellet any non-adherent macrophages. The supernatants were removed and used for determination of the levels of extracellular viral RNA by qPCR as described above. The cell pellets were resuspended in 350 µL of the lysis buffer each (RB buffer from the Total RNA extraction mini kit) and transferred back to their respective wells on the 96 well plate in order to lyse macrophages that remained adherent at the bottom of the well. This preparation was used for determination of the levels of cell-associated viral RNA.

Results

Preparation of PPM cultures

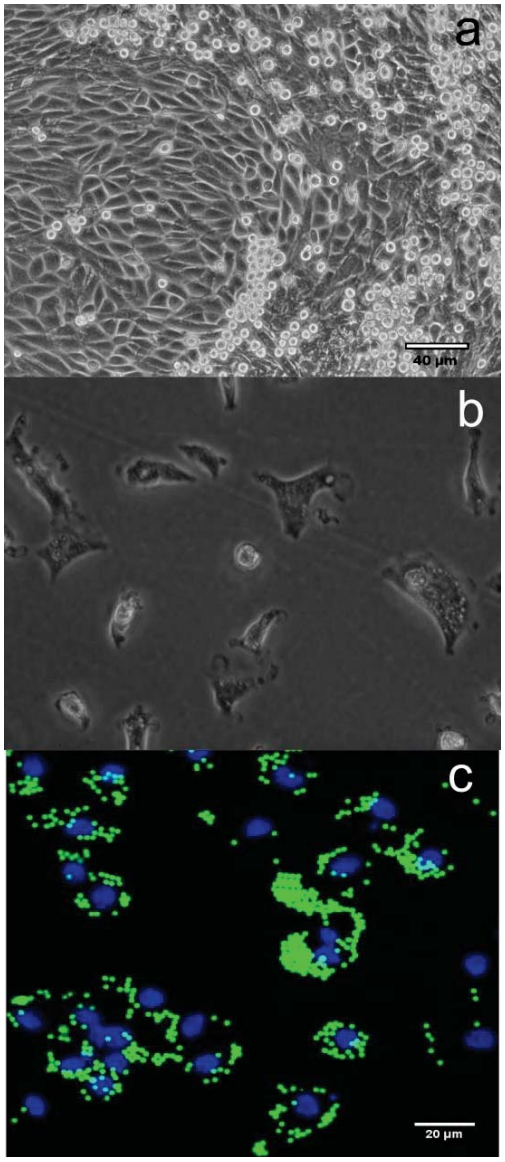
Within one day of seeding mixed hepatic cells attached to the surface of cell culture flasks and appeared round to polygonal. Contaminating red blood cells and non-adherent mixed liver cells were removed with subsequent media changes. Within one to two days, areas containing sheets of polygonal, cobblestone-like cells were visible. With further time in culture, the cells assumed a more elongated, fibroblast-like appearance. Around the same time (day five to six), the monolayer was almost confluent, and round macrophage-like cells were beginning to appear on the surface of this monolayer. By day nine, large numbers of round macrophage-like cells were observed (figure 2.1A). After 21 days, the number of macrophage-like cells appeared to decline and the fibroblast-like monolayer began to degenerate.

Macrophage-like round cells were collected off the mixed liver cell monolayer every two to three days from as early as six days after seeding. The collected cells adhered to the surface of non-culture grade plastic petri dishes, and displayed typical macrophage morphology with a ruffled appearance to the cytoplasmic margins and pseudopodia (figure 2.1B).

Phagocytic assay

After incubation for one and a half hours with fluorescent-labelled latex beads, the majority of the macrophage-like cells contained numerous latex beads within their cytoplasm (figure 2.1C).

Figure 2.1: Primary possum macrophages



Macrophage-like round cells growing on the top of primary mixed liver cells originally imaged at 200x magnification (a). The same cells collected off the monolayers adhered to the surface of non-culture grade plastic petri dishes, displayed typical macrophage morphology (b) and phagocytosed fluorescent latex beads (c). Both pictures were originally taken at 400x magnification. The phagocytic activity was recorded one and a half hours following addition of the beads to the culture.

In vitro growth of WPDV

High viral copy numbers (up to 1.1 x 10^6/µL of cDNA, corresponding to 6.9 x 10^8/mL of cell lysate) were detected in cell culture lysates from the fifth passage of the virus. Growth of WPDV was not accompanied by consistent clearly recognisable CPE. In some, but not all, of the infected cultures clumps of rounded cells attached to the base of the petri dish and an apparent increase in the numbers of floating cells was observed.

None of the other cell types tested showed any CPE following inoculation with WPDV. Viral RNA was not detected in any of the cell lysates tested from MA-104, PtK2, PK, or OK cells that had been inoculated with the virus. However, low levels of WPDV RNA were detected in some of the cell lysates of RAW264.7, J774 and P388D1 from the second passage of WPDV. The levels of viral RNA were very low (<100 copies/µL) and the virus could not be consistently passaged.

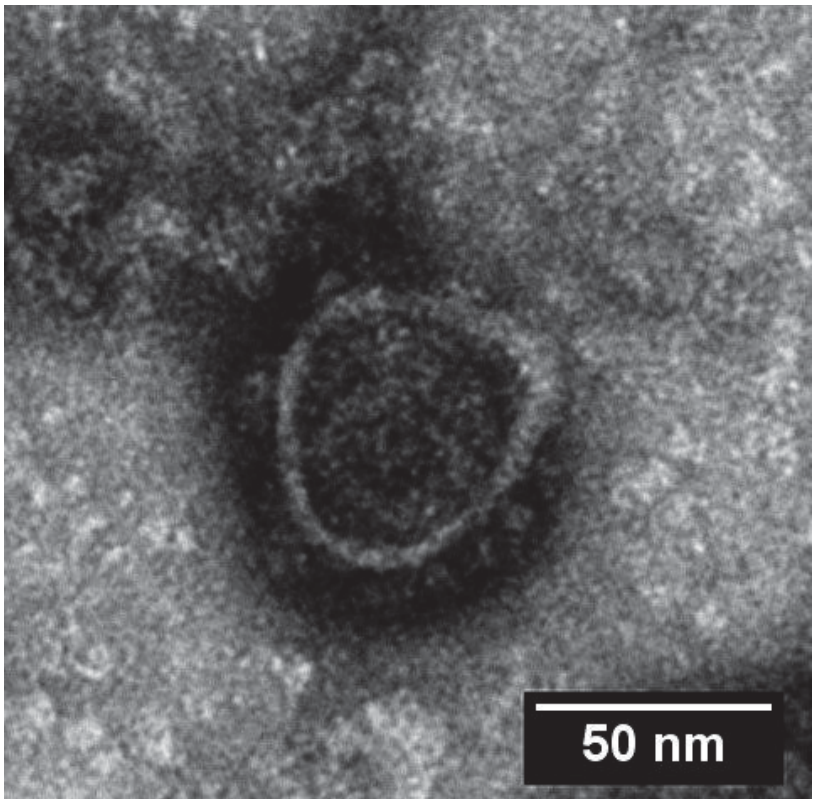
Purification of the WPDV

A faint band was visible in each vertical rotor tube after density gradient ultracentrifugation. Fractions collected from each gradient contained 0 to 2.3 x 10^7 viral RNA copies/µL, with fractions collected from the area of the band showing the highest viral copy numbers. Fractions containing the highest viral copy numbers from various runs (range 3.0 x 10^5 to 2.3 x 10^7 viral RNA copies/µL) were combined to create a purified WPDV stock with a density of 1.09 g/mL.

Electron microscopy

Uniform, spherical to icosahedral virus-like particles approximately 60 nm in diameter were observed in the purified WPDV preparation under EM (figure 2.2). The particles appeared to be enveloped and with indistinct surface projections.

Figure 2.2: Electron micrograph of a negatively stained preparation of putative wobbly possum disease virus (WPDV)

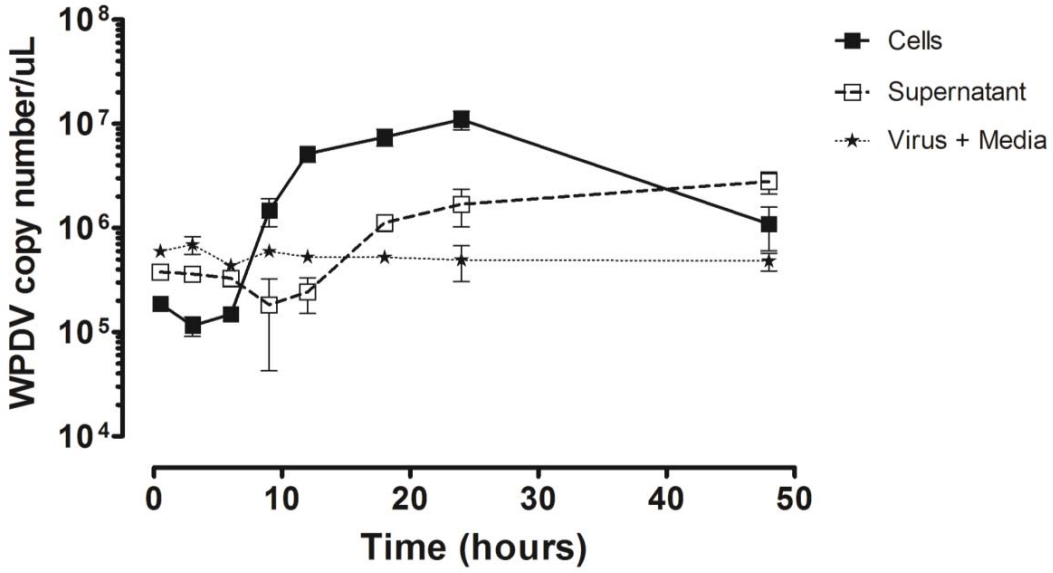


Kinetics of WPDV replication *in vitro*

The titre of the purified virus was calculated to be 1.0 x 10^7 $TCID_{50}$/mL. There was a clear demarcation between wells that were scored as positive or negative for WPDV replication on a titration plate, with similarly high average viral RNA copy numbers in wells inoculated with the first five dilutions of the stock virus ranging from 4.3 x 10^5 to 1.29 x 10^6 per µL of cDNA.

The one-step growth curve of WPDV in PPM is shown in Figure 2.3. The steepest increase in the intracellular viral RNA levels was observed between six and 12 hours post infection, with maximum levels reached at 24 hours post infection. The subsequent decline in cell-associated viral RNA was accompanied by an increase in the levels of extracellular viral RNA. This increase was steepest between nine and 18 hours post infection, with cell-free viral RNA reaching peak levels at 48 hours post infection. The average maximum level of extracellular viral RNA (2.8 x 10^6 copies/µL) was approximately one log lower than the average maximum level of cell-associated viral RNA (1.1 x 10^7 copies/µL). Negative control wells (uninfected possum primary macrophages) were negative for WPDV RNA.

Figure 2.3: One-step growth curve of wobbly possum disease virus (WPDV) in primary possum macrophages



Primary possum macrophages (1 x 10^5/well) were infected with WPDV at moi of 0.5. Cell associated (Cells) and cell-free (Supernatant) viral RNA was extracted and quantified using a previously described RT-qPCR assay at eight time points, each in triplicate. The results were expressed as viral copy numbers per µL of template cDNA. Negative controls at each time-point (uninfected macrophage cultures) were negative for WPDV RNA. Non-replication control (Virus + Media) consisted of WPDV incubated in a cell-free media. Mean values for each time point are shown. Error bars represent standard deviations (SD).

Discussion

To our knowledge, this is the first description of primary culture of possum liver macrophages. The cells were characterised as macrophages based on their ability to adhere to non-treated plastic, morphological features and strong phagocytic activity, which is a functional characteristic of macrophages (Aderem and Underhill, 1999). Further phenotypic analysis of the cells by immunostaining was hindered by the lack of possum-specific reagents.

Of the primary cells and continuous cell lines tested, only PPM consistently supported the growth of WPDV *in vitro*. Some replication of WPDV also occurred in all three mouse macrophage cell lines tested. However, the levels of WPDV detected in those cultures were low and the virus could not be consistently passaged. This is consistent with characteristics of other arteriviruses, which tend to show restricted host and cell specificity both *in vivo* and *in vitro* (Onyekaba et al., 1989; Plagemann and Moennig, 1992; Snijder and Meulenberg, 1998).

We targeted liver macrophages, as the highest levels of WPDV RNA had been previously detected in livers of experimentally infected possums (Dunowska et al., 2013), and macrophages have been shown to be the primary target cells for arteriviral replication both *in vivo* and *in vitro* (Duan et al., 1997; Onyekaba et al., 1989; Plagemann and Moennig, 1992). Some isolates of porcine reproductive and respiratory syndrome virus (PRRSV) and simian haemorrhagic fever virus (SHFV) can also replicate efficiently in MA-104 African green monkey kidney cells or derivatives of this cell-line, MARC-145 and CL2621 cells (Kim et al., 1993; Plagemann and Moennig, 1992). In contrast, equine arteritis virus (EAV) replicates efficiently in a variety of primary and established cell lines including African green monkey kidney (VERO), rabbit kidney (RK-13) and baby hamster kidney (BHK-21) cells (Moore et al., 2002; Snijder and Meulenberg, 1998). Based on our data, it appears that WPDV shows a restricted host cell type *in vitro*, similarly to arteriviruses other than EAV. It remains to be established whether macrophages from different sources e.g. different tissue types, or donor

possums of different ages, differ in their ability to support WPDV replication *in vitro*. The ranges of cells that can be infected with WPDV *in vivo* also remain to be determined.

Growth of WPDV could not be reliably assessed by the presence of viral CPE in infected macrophage cultures. Therefore, virus titration was performed using results of WPDV RT-qPCR to assess the presence of replicating WPDV in wells inoculated with serial dilutions of the virus preparation. While visual observation for viral CPE or immunostaining (IF) with virus-specific antibody is commonly used for this purpose, a similar qPCR based approach has been recently described for titration of human herpesvirus 6 (Gustafsson et al., 2012). Although we have not confirmed our results using a second detection method such as IF due to unavailability of anti-WPDV antibody, detection of similarly high levels of viral RNA in wells inoculated with the serial dilutions of the virus supports validity of our approach. Without replication of WPDV in the wells, the amount of viral RNA detected would be expected to follow a standard curve corresponding to 10-fold dilutions of the inocula.

Although CPE characterised by rounding of cells and detachment from the cell culture plate are common for some arteriviruses such as EAV, SHFV and PRRSV (Benfield et al., 1992; Hyllseth, 1969), viral CPE is not observed in cultures of mouse macrophages inoculated with lactate dehydrogenase elevating virus (LDV). This likely reflects the fact that the proportion of LDV-permissive cells *in vitro* is not high enough to observe cell death (Snijder and Meulenberg, 1998; Stueckemann et al., 1982). By extrapolation, it is possible that only a proportion of cultured macrophages were permissive for WPDV replication *in vitro*, particularly considering heterogeneity of Kupffer cells (Armbrust and Ramadori, 1996). We have not determined the viability of WPDV-infected macrophages at any of the time points. However, similar studies with LDV failed to demonstrate presence of any dead cells in infected cultures, despite the fact that LDV infection of permissive macrophages is cytocidal (Onyekaba et al., 1989; Plagemann and Moennig, 1992). The authors suggested that the phagocytic activity of remaining macrophages, not permissive to LDV infection, was a likely explanation for the rapid

removal of any dead cells from the LDV infected cultures. By extrapolation, lack of clearly recognisable CPE in WPDV-infected PPM cultures suggests that similar dynamics may be occurring during WPDV infection. Determination of the proportion of infected cells *in situ*, coupled with assessment of cell death would be necessary to test this hypothesis.

One-step growth experiments with other arteriviruses showed maximal rates of RNA synthesis between six and 10 hours post infection, with maximal rates of viral release occurring between 10 and 24 hours post infection (Snijder and Meulenberg, 1998). This is comparable with the results obtained in the current study, with the steepest increase in the levels of intracellular viral RNA observed between six and 12 hours post infection, followed by a gradual release of cell-free viral RNA between nine and 24 hours post infection.

A relatively large decline in levels of intracellular viral RNA between 24 and 48 hours post infection was accompanied by only a moderate increase in the levels of extracellular viral RNA. This likely related the fact that intracellular viral RNA would be expected to include not only genomic viral RNA but also a pool of viral RNAs that serve a role during replication of the virus, but are not incorporated into the virion (Snijder et al., 2013). It is also possible that a proportion of the intracellular viral RNA was synthesised during abortive (non-productive) infections, without the release of virions from the cells. It has been suggested that most LDV virions become degraded following entry into *in vitro* cultured macrophages and a high moi is necessary for a few virions to escape this process and establish productive infection (Plagemann and Moennig, 1992). Even with high moi, only a maximum of five to 15% of cultured macrophages from adult mice were susceptible to LDV infection (Onyekaba et al., 1989). Our data suggest that a similar process may be occurring during *in vitro* WPDV infection of PPM, but *in situ* detection of infected cells would be necessary to further support this conclusion.

The density of WPDV in iodixanol (1.09 g/mL) was lower than the reported densities of other arteriviruses in sucrose (1.13-1.17 g/mL) and caesium chloride (1.17-1.20 g/mL) gradients (King et al., 2012). However, the buoyant densities of several viruses in iodoxanol were

reported to be lower than their densities in caesium chloride, sucrose, or glycerol gradients (Gias et al., 2008; Lawrence and Steward, 2010). Thus, the buoyant density of WPDV in iodoxanol may be comparable with the reported densities of other arteriviruses in traditional density gradient media.

The appearance of purified WPDV under electron microscopy is consistent with the current members of the family *Arteriviridae*, which are described as 40 – 70 nm diameter spherical particles with indistinct surface projections (King et al., 2012). The appearance of arteriviruses is distinct from other members of the *Nidovirales* and the morphological appearance of WPDV therefore further supports classification of this virus within the family *Arteriviridae*.

Conclusions

We have established primary macrophage cultures from fresh liver tissues of the Australian brushtail possum, and have shown that these cultures support the growth of WPDV, thereby providing a suitable *in vitro* culture system for further research. Several lines of evidence suggest that productive infection of PPM with WPDV is not very efficient. Despite this limitation, up to 10^6 WPDV copy numbers per µL of cDNA were detected in cell culture lysates after five *in vitro* passages. The restriction of cell types permissive for *in vitro* WPDV infection to macrophages, rapid kinetics of WPDV growth *in vitro*, and the EM appearance of viral particles all provide further support for the proposed classification of the virus within the family *Arteriviridae*. Establishment of an *in vitro* system for growth of WPDV will facilitate further characterisation of this marsupial nidovirus, development of the serological assays for investigation of WPDV epidemiology, and provision of the final proof of its aetiological involvement in WPD via fulfilment of Koch's postulates for disease causation.

Disclaimer

I have performed all experiments and analyses described in this chapter, with the exception of growth of WPDV in commercial cell lines and primary kidney cells, which were done by my co-author and PhD supervisor, Dr Magda Dunowska. Manuscript describing work presented in this chapter has been published in Journal of Virological Methods, Volume 222, 15th September 2015, pages 66-71.

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Chapter 3

The aetiology of wobbly possum disease: reproduction of the disease with purified nidovirus

Summary

The objective of this study was to investigate the role of a recently discovered marsupial nidovirus in the development of a neurological disease, termed wobbly possum disease (WPD), in the Australian brushtail possum (*Trichosurus vulpecula*). Four possums received 1 mL of a standard inoculum that had been prepared from tissues of WPD-affected possums, four possums received 1.8 mL (1 x 10^6 $TCID_{50}$) of a cell lysate from inoculated cultures, and four possums received 1 mL (1 x 10^7 $TCID_{50}$) of a purified WPD isolate. All but one possum that received infectious inocula developed neurological disease and histological lesions compatible with WPD. High levels of viral RNA were detected in livers from all possums that received infectious inocula, but not from control possums. Altogether, the data presented provides strong experimental evidence for the causative involvement of WPD virus in development of neurological disease in infected animals.

Introduction

Wobbly possum disease is a severe neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*) that has only been described on several occasions in captive (Mackintosh et al., 1995) and wild (Perrott, 1998; Perrott et al., 2000a) possums in New Zealand. On the basis of clinical signs and transmission potential, the aetiological agent,

currently unknown, has been suggested as a potential biocide agent in New Zealand for this marsupial pest (Perrott, 1998).

There are several lines of evidence to suggest that a virus is aetiologically involved in the development of wobbly possum disease (WPD). Firstly, filtered tissue lysate derived from WPD-affected possums and administered to healthy possums reproduced the disease (O'Keefe et al., 1997; Perrott, 1998; Perrott et al., 2000b). Secondly, virus-like particles were observed in preparations of livers from two experimentally-infected possums under electron microscopy (O'Keefe et al., 1997). More recently, the results of PCR-based studies supported aetiological involvement of a recently discovered marsupial nidovirus, wobbly possum disease virus (WPDV), in the development of wobbly possum disease. Further investigation into the outcome of the virus *in vivo* has been hindered by lack of purified virus stock for experimentation. In the previous chapter, growth of WPDV in primary possum macrophage and mixed liver cell cultures, and purification of cell culture-derived virus were described. Following on from this, the objective of the current study was to extend previous PCR-based data by providing experimental support for the hypothesis that infection of adult Australian brushtail possums with this nidovirus is aetiologically linked to development of WPD.

Materials and Methods

Possums

Sixteen Australian brushtail possums were caught from the wild in the Manawatu region of New Zealand using live animal traps over a period of 18 days commencing in June 2013. Possums were individually housed in purpose-made cages in a Massey University research facility as described previously (Perrott, 1998) and fed a diet consisting of *ad-libitum* commercial rabbit pellets and a daily selection of fresh fruits and vegetables. Water was provided *ad-libitum* via rabbit sipper bottles. All female possums (n = 6) had pouch-bound

joeys. Possums were assigned consecutive ID numbers, with joeys identified by the ID of their dams followed by a "j" suffix. Cages were cleaned daily while possums were confined in detachable nesting boxes. The amount of consumed food and water were assessed at the time of cage cleaning. The weights of possums were monitored by weighing the nesting boxes with possums inside. Live animal traps and housing cages were kindly provided by Landcare Research, Palmerston North, New Zealand.

Inocula

Three types of infectious inocula were used. The first inoculum comprised a standard inoculum that had been used previously in animal transmission studies and was shown to contain at least 10^5 possum infectious doses $(PID)_{50}$ / mL of the WPD agent (Perrott, 1998; Perrott et al., 2000b). It consisted of 10% tissue suspension (brain, liver, and spleen from seven WPD-affected possums) that had been filtered through a 0.45 μm syringe filter. The second inoculum comprised lysates of primary possum macrophage cells infected with the standard inoculum. The primary possum macrophage cell cultures were prepared as described previously in chapter 2 (Giles et al., 2015). Lysates from the fifth passage of the virus were clarified by low-speed centrifugation (3,000 x g for 20 minutes at 4 °C) and filtered through 0.45 μm filter. The third inoculum comprised purified WPDV isolate. The virus was grown in possum primary macrophages and purified using iodixanol density gradient ultracentrifugation as described previously (chapter 2). Fractions containing the highest levels of WPD RNA as determined by WPDV-specific RT-qPCR (Dunowska et al., 2013) were combined and filtered through 0.45 μm filter. Both the purified cell culture isolate and the infected cell culture lysate had been titrated in primary possum macrophages as described previously (chapter 2). Control inoculum comprised a gradient material prepared from uninfected cell culture lysate.

Experimental design

Possums were allocated to one of the experimental groups at the time of catchment, starting from group 1 and finishing on group 4 (table 3.1). Possums in group 1 were retained as uninfected controls, and each possum in groups 2-4 received one of the three different types of infectious inocula. All inoculations were performed after an acclimatisation period of between two and four weeks. Possums were sedated with 40 mg/kg ketamine (Phoenix Pharm Distributer Ltd) and 2 mg/kg diazepam (*Pamlin*; Parnell Laboratories *NZ* Ltd) administered intramuscularly. Each possum was examined for external signs of disease by a veterinarian (Julia Giles) and 2 to 5 mL of whole blood was collected into red-top serum tubes (BD Vacutainer®) by jugular venepuncture. Following this, each possum in group 2 (possums E5-E8) received 1 mL (2×10^7 $TCID_{50}$) of purified WPDV, each possum in group 3 (possums E9-E12) received 1.8 mL (1×10^6 $TCID_{50}$) of infected cell culture lysate, and each possum in group 4 (possums E13-E16) received 1 mL of standard inoculum. Group 1 possums received either a control inoculum (possums E3 and E4) or nothing (possums E1 and E2). All inoculations were performed via intraperitoneal injection. Possums were observed daily for changes in behaviour and the presence of neurological deficits. Changes in food and water intake were also recorded. To minimize stress, the initial observations were made with possums confined to their cages. When changes in behaviour or neurological deficits were first suspected, each possum was removed from its cage and individually assessed in a closed room for alterations in gait, vision, response to the environment and ability to climb a wire-mesh wall. Assessment out of the cage was performed every one to three days thereafter. Weight was recorded prior to inoculation, weekly thereafter, and upon euthanasia. The possum weights were estimated indirectly based on weights of nested boxes with and without possums.

All animal manipulations were approved by the Massey University Animal Ethics Committee (MUAEC protocol 12/15).

Euthanasia, post-mortem examination and sample collection

Possums were euthanized as soon as they showed clear signs of neurological disease or sooner if deemed necessary based on ethical grounds. Consistent neurological signs comprised one or more of the following; behavioural changes, tremors, head tilt, crouched hindlimb gate, ataxia and decreased ability to climb. Individual possums were sedated with 40 mg/kg ketamine (Phoenix Pharm Distributer Ltd) and 2 mg/kg diazepam (*Pamlin*; Parnell Laboratories NZ Ltd) administered intramuscularly and euthanized with an intracardiac injection of 5 mL of sodium pentobarbital (Provet). Control possums (group 1) were euthanized 14 – 15 days following euthanasia of all possums from groups 2 – 4. Blood was collected immediately post-euthanasia by cardiocentesis or venipuncture of the great vessels. Post-mortem examination was conducted immediately and two sets of tissue samples from all major internal organs, including brain, were collected. One piece of each tissue was stored frozen at -80°C and another was fixed in paraformaldehyde buffered formalin at room temperature.

Histopathology

Histological examination was performed on the following tissues: liver, spleen, cerebral cortex, heart and kidney from all adult possums and liver, spleen, brain, kidney and lung from two joeys (10j and 13j) whose mothers received infectious inocula. Briefly, tissues were collected into 2% paraformaldehyde in phosphate buffer (pH 7.4) with added lysine (0.15M) and sodium-m-periodate (0.01M) following the method of McClean and Nakane (1974). Periodate was added just prior to tissue immersion. Fixed tissues were held in 60% ethanol prior to dehydration, clearing and paraffin embedding. Sections (4 μm) were stained using a variation of Gill's haematoxylin and eosin method and permanently mounted with Entellan (Merck). Slides were imaged using a light microscope (Ziess Axiophot) and image capture system (Olympus DP70: Cellsens dimensions).

Determination of WPDV RNA load in tissues

The presence of viral RNA in livers and spleens from all adult possums was detected using WPDV specific RT-qPCR as described previously (Dunowska et al., 2013), with the exception that the results were presented as viral copy numbers per µL of template cDNA. Total RNA was extracted from ~20 mg of tissue (liver or spleen) using Total RNA Mini Kit for tissue (Geneaid Biotech Ltd) according to the manufacturer's instructions. RNA was eluted in 50 µL of water and 8 µL was used for cDNA synthesis using qScript™ cDNA Supermix (Quanta Biosciences) according to the manufacturer's instructions, with the modification that reactions were scaled down to a total volume of 10 µL. Positive (serial dilutions of a plasmid containing WPDV PCR fragment) and negative (water) controls were included in each RT-qPCR run. Spleen and liver WPDV RNA levels were determined on separate runs. The load of WPDV RNA was also evaluated in livers from two clinically normal joeys (10j and 13j), and two joeys (12j and 16j) that died during the study. The mothers of all joeys tested had received infectious inocula. Tissues were not available for testing from the remaining joeys.

To evaluate the quality of extracted RNA, all samples that were negative for WPDV were also evaluated for the possum β-actin gene (ACTB) using qPCR with the same conditions as described above. Primer sequences used for amplification were as follows: ACTB.F 5'-TGGTACCACCAGACAATACGGT-3' and ACTB.R 5' – TCCGATGCCCAGAAGCCCTCT-3'. Positive (serial dilutions of a plasmid containing ACTB PCR fragment) and negative (water) controls were included in each qPCR run.

Application of a numerical grading system for WPD

A previously described weighted scoring system (Perrott, 1998; Perrott et al., 2000b) was used to objectively classify possums as affected or not affected by WPD. Briefly, five criteria in each of two equal categories (clinical signs and histological indices) were graded to give a maximum score of six per category. The six points for clinical signs of WPD were weighted for ataxia and immobility (three points), observable behavioural changes (one point), decreased appetite (one

point) and weight loss of greater than 20% (one point). In the original description of the system, the six points for histological indices were weighted for the presence of meningitis or encephalitis evidenced by perivascular mononuclear infiltrates in the cerebral cortex (two points), with further points allocated for presence of perivascular cuffing or an accumulation of lymphocytes or plasma cells in liver (one point), myocardium (one point), kidney (one point) and other tissues (one point). The only "other tissue" examined in the current study was spleen. A cumulative score from both categories of five or more satisfied a numerical diagnosis of WPD.

Results

Possums during acclimatization period

All possums acclimatized well to the captive environment as judged by their appetite and behaviour. Although six out of 12 possums lost some weight during the acclimatization period, the remaining six possums gained weight in the same period (table 3.1). The initial weight losses were less than 5% for four possums, with the highest weight loss (24%) recorded for one of the control possums (possum E3). This possum, however, regained its appetite during the course of the study, which was evidenced by a 12% weight gain recorded at the time of euthanasia.

None of the possums displayed any overt signs of external disease on clinical examination prior to inoculations. Group 1 comprised three males and one female with a dependent joey. Group 2 comprised three males and one female with a dependent joey. Group 3 comprised two males and two females, each with a dependent joey. Possum E12 was lacking a tail. However, the tail stump was covered in fur, without any signs of recent trauma. Group 4 comprised two males and two females, each with a dependent joey.

Clinical Signs

All but one infected possum developed neurological signs consistent with WPD between eight and 27 days post inoculations and were subsequently euthanized (table 3.1). Due to difficulties assessing neurological signs in wild possums, where signs were mild, possums were not euthanised until neurological signs became clearer. Decreased appetite was observed in 12/12, weight loss in 11/12, and neurological signs in 11/12 of infected possums. Possum E15 did not develop neurological signs prior to death at six days post inoculation. Three (6j, 12j, and 16j) of the five dependant joeys of WPDV-inoculated possums died. The two remaining joeys (10j and 13j) appeared clinically normal at the time of euthanasia. As joeys were still pouch-bound, this assessment was based on vigorousness of movement when removed from the pouch and external body condition.

A decreased appetite was first observed in all possums that received infectious inocula between days one and six post-infection, with an average onset of three days post inoculation. Neurological signs (table 3.2) observed in infected possums were indistinguishable between groups 2-4 and consisted of: decreased ability to climb (11/12), behavioural changes (11/12), tremors (9/12), ataxia (8/12), a crouched hindlimb gait (8/12), head tilt (4/12), and circling (2/12). Neurological signs were first observed between days eight and 19, with an average onset of nine days post inoculation. Ataxia was characterized by a stumbling, rolling, swaying gait with or without hypermetria. Tremors were fine, generalized and often worsened with movement. Behavioural changes included loss of pre-existing fear response towards humans. Control possums (group 1) including one dependent joey did not develop any clinical signs of WPD.

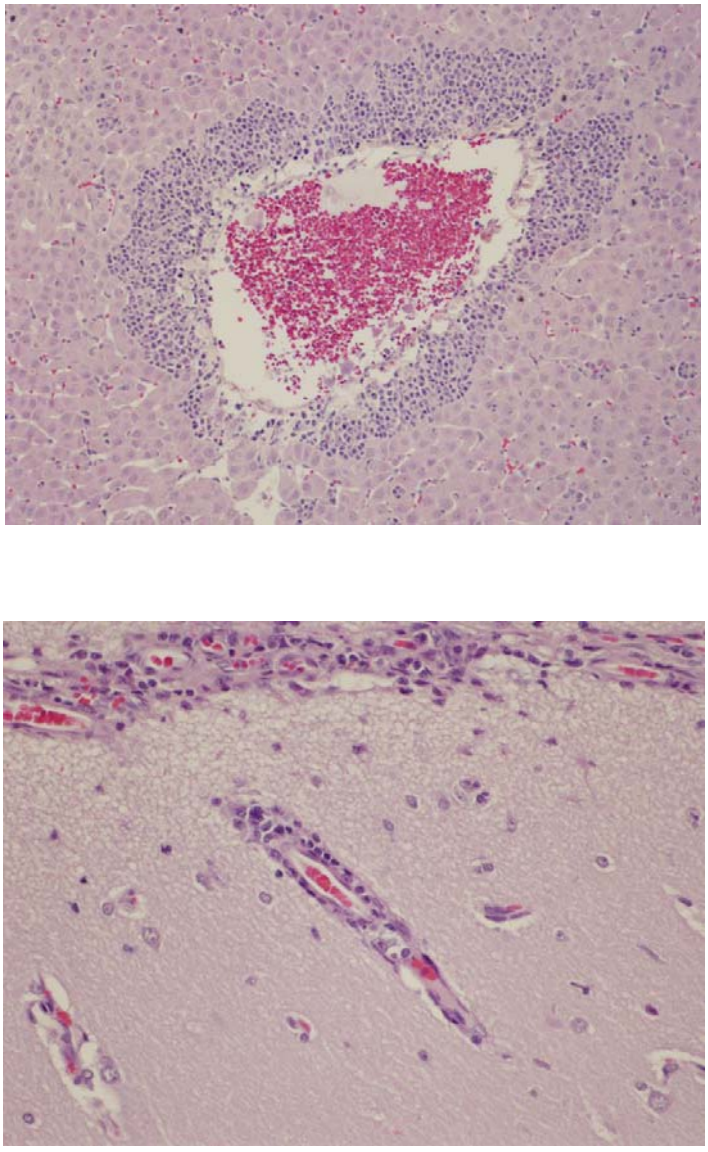
Post-mortem examination

Gross pathological changes observed in infected adult possums included: poor body condition (all possums), cloudy cerebrospinal fluid (possum E7), and petechiation of the gastrointestinal serosa, meninges and renal capsule (possum E9). Pitted kidneys and a large volume gastrointestinal haemorrhage were observed in possum E15, who died prior to the development of neurological signs. Bright yellow pigmentation of the kidneys was observed in one control possum (possum E2). No other gross pathological changes were observed in control possums. No gross pathological changes were observed in two joeys (10j and 13j) that underwent post-mortem examination.

Histopathology

Histological changes that have been previously described as characteristic for WPD (O'Keefe et al., 1997; Perrott, 1998; Perrott et al., 2000a) were observed in multiple tissues from all but one of the diseased possums in the current study (table 3.2). Briefly, foci of mononuclear inflammation, often centred around the vasculature were found in multiple tissues including the heart, brain parenchyma, meninges, liver and kidney. Inflammatory aggregates comprised predominantly plasma cells and lymphocytes with fewer macrophages. Possum E15 that died prior to the onset of neurological signs lacked histological lesions characteristic for WPD. Small foci of perivascular mononuclear inflammation were also observed surrounding the central veins in livers from two control possums (possums E1 & E3). Histological changes consistent with WPDV infection were observed in all tissues of joey 13j, with the most severe lesions observed in the liver. Mild, focal perivascular mononuclear infiltration was observed in the brain of joey 10j, but not in other tissues. Tissues from three joeys (joey's 6j, 12j and 16j) were too autolysed for histological examination.

Figure 3.1: Histology of liver (top) and brain (bottom) tissue from WPDV-infected possums



Mononculear inflammatory cells surrounding blood vessels in; liver (top image), viewed under 10 x objective and brain (bottom image), viewed under 20 x objective. Slides were created from tissues from possum E5. Slides were imaged using a light microscope (Ziess Axiophot) and image capture system (Olympus DP70: Cellsens dimensions).

Table 3.1: Details of clinical signs observed in adult possums and % weight change over the duration of the study

Possum ID (F/M)[a]	Group	Inoculum type[b]	Onset of clinical signs (days post-inoculation)							% weight change		Euthanasia (days post inoculation)
			Decreased appetite	Behavioural change	Tremors	Head Tilt	Crouched hindlimb gait	Ataxia	Decreased ability to climb	During acclimatization	Post-inoculation	
E1 (M)	1	None	-	-	-	-	-	-	-	+29	-5	42
E2 (M)	1	None	-	-	-	-	-	-	-	-0.3	+7	42
E3 (M)	1	CI	-	-	-	-	-	-	-	-24	+14	41
E4 (F)	1	CI	-	-	-	-	-	-	-	-3	+6	41
E5 (M)	2	WPDV	1	19	19	19	-	19	19	0	-0.5	20
E6 (F)	2	WPDV	1	8	-	-	8	-	-	-14	-2	8
E7 (M)	2	WPDV	2	19	20	-	8	20	20	+13	-34	20
E8 (M)	2	WPDV	5	19	-	-	8	19	19	+18	-26	27
E9 (M)	3	ICL	5	8	8	-	19	8	8	-5	-27	19
E10 (F)	3	ICL	5	8	8	-	8	-	9	+11	-17	9
E11 (M)	3	ICL	6	22	22	19	-	19	22	+4	-30	22
E12 (F)	3	ICL	6	27	27	19	-	27	27	-4	-49	27
E13 (F)	4	SI	5	19	23	19	19	19	23	+12	-22	23
E14 (M)	4	SI	5	8	8	-	8	-	8	+56	-28	9
E15 (M)	4	SI	5	-	-	-	-	-	-	-9	-	6 (Died)
E16 (F)	4	SI	1	19	8	-	19	9	19	+20	-45	20

[a] F=female, M=male

[b] CI=control inoculum, WPDV= purified WPD virus isolate, ICL=infected cell culture lysates, SI=standard inoculum

All females had dependent joeys at the beginning of the study. Joeys of possums E6, E12 and E16 died during the period of the study, while joeys of possums E4, E10 and E13 appeared normal. Possums were inoculated with one of the following inocula: control inoculum (CI) comprising gradient material from uninfected cell culture lysates, standard inoculum (SI) prepared from tissues of WPD-affected possums, infected cell culture lysates (ICL), or WPDV (purified WPD virus isolate). Days post inoculation for possums E1 and E2 were considered the same as for possums E3 and E4.

Table 3.2: Application of a numerical grading system for diagnosis of wobbly possum disease (WPD)

Possum ID (F/M)[a]	Group	Inoculum Type[b]	Clinical Signs					Histological Indices					Score	WPD
			Ataxia	Immobility	Behavioural change	Decreased appetite	Weight loss ≥ 20%	Cerebral cortex	Liver	Kidney	Heart	Spleen		
E1 (M)	1	None	-	-	-	-	-	-	1	-	-	-	1	-
E2 (M)	1	None	-	-	-	-	-	-	-	-	-	-	0	-
E3 (M)	1	CI	-	-	-	-	-	-	1	-	-	-	1	-
E4 (F)	1	CI	-	-	-	-	-	-	-	-	-	-	0	-
E5 (M)	2	WPDV	3	-	1	1	-	2	1	1	1	1	11	+
E6 (F)	2	WPDV	-	1	1	1	-	2	1	1	1	1	9	+
E7 (M)	2	WPDV	2	1	1	1	1	2	1	1	1	1	12	+
E8 (M)	2	WPDV	2	1	1	1	-	2	1	1	1	1	11	+
E9 (M)	3	ICL	2	1	1	1	1	2	1	1	1	1	12	+
E10 (F)	3	ICL	2	1	1	1	-	2	1	1	1	1	11	+
E11 (M)	3	ICL	2	1	-	1	1	2	1	1	1	1	11	+
E12 (F)	3	ICL	3	-	-	1	1	2	1	1	1	1	11	+
E13 (F)	4	SI	2	1	1	1	-	2	1	1	1	1	11	+
E14 (M)	4	SI	-	1	1	1	-	2	1	1	1	1	9	+
E15 (M)	4	SI	-	-	-	1	-	-	-	-	-	-	1	-
E16 (F)	4	SI	2	1	1	1	1	2	1	1	1	1	12	+

[a] F = female, M = male

[b] CI = control inoculum, WPDV = purified WPD virus isolate, ICL = infected cell culture lysates, SI = standard inoculum

Possums were inoculated with one of the following inocula: control inoculum (CI) comprising gradient material from uninfected cell culture lysates, standard inoculum (SI) prepared from tissues of WPD-affected possums, infected cell culture lysates (ICL), or purified WPD virus isolate (WPDV), and observed daily for the presence of neurological deficits. Following development of neurological disease, the possums were euthanised and tissues subjected to histological examination. Criteria from two categories (clinical signs and histological indices) were graded with a combined maximum score of 12. For histopathological indices, demonstration of perivascular mononuclear infiltrates in the cerebral cortex or meninges incurred two points, whilst demonstration of perivascular mononuclear infiltration in the liver, spleen, kidney or heart each incurred one point. For clinical signs, the observation of ataxia or immobility incurred up to three points, whilst the observation of behavioural changes, decreased appetite and a weight loss of greater than 20% of initial body weight each incurred one point. A cumulative WPD score greater than or equal to five satisfied a numerical diagnosis of WPD (+).

Determination of WPDV RNA levels in tissues

High levels of viral RNA were detected in livers ($6.64 \times 10^4 – 8.84 \times 10^5$ copies/μL) and spleens ($171.5 - 3.42 \times 10^5$ copies/μL) from all possums that received infectious inocula (groups 2 – 4). Control possums (group 1) were negative for WPDV RNA. The viral RNA was also detected in liver samples from three out of four dependant joeys tested, with high levels of viral RNA detected in livers of joeys 13j (3.39×10^5 copies/μL) and 16j (3.62×10^5 copies/μL), and low levels of viral RNA detected in the liver from joey 10j (87.34 copies/μL). The liver of joey 12j was negative for WPDV RNA. High levels of ACTB RNA were detected using the β-actin primers in all samples that were negative for WPDV RNA.

Application of a numerical grading system for WPD

All but one adult possum in groups 2 - 4 had a score ≥ 9 and were hence classified as affected by WPD (table 3.2). Possum E15 was the only possum that did not satisfy a numerical diagnosis of WPD. None of the control possums (group 1) was classified as affected by WPD, with the highest score of one. The WPD score was not assigned to joeys, as detailed neurological assessment of joeys was not possible.

Discussion

Although WPD was first described and experimentally reproduced more than 20 years ago (Mackintosh et al., 1995; Perrott, 1998), the aetiology of this disease remained a mystery until recently, when a partial sequence of a novel nidovirus was identified in archival tissues from WPD-affected possums (Dunowska et al., 2012). While detection of nidoviral RNA in possum livers was strongly associated with disease, the possibility that the real cause of WPD was infection with another, as yet undiscovered pathogen, could not be excluded. In this scenario, the presence of the nidovirus in diseased animals would represent an accidental finding due to

favourable environment created by infection with the real aetiological agent for WPD. However, lack of a suitable culture system for the novel nidovirus hindered any further studies to support the PCR-based association between the virus and WPD. The recent development of such system (chapter 2) made it possible to generate a stock of highly purified virus, which was used in the current experiments.

There are many factors that affect the outcome of a viral infection including virulence of the virus, specific immunity of the host as well as presence of concurrent diseases. The difficulties associated with establishment of causative links between viral infections and specific diseases have been already noted by Koch himself, as well as other authors (Frobisher, 1949; Huebner, 1957; Rivers, 1937; Robinson, 1958). Despite the fact that the general guidelines developed by these authors for investigation of disease causation many years ago may be difficult to apply to some of the viral diseases (e.g. slow viruses, multifactorial diseases etc.), they provide a useful frame of reference for investigations of acute viral diseases such as WPD.

The canonical Koch's postulates for disease causation have been extended and modified to accommodate the unique characteristics of viruses by River (1937) and summarised by Frobisher (1949) under four main points. Firstly, "the virus must be present in the host cells showing the specific lesions, at the time of the disease, or in the blood or other body fluids." We have clearly demonstrated that WPDV is present in tissues from diseased animals. In the current study, this was achieved by WPD-specific RT-qPCR testing of livers and spleens from experimentally infected possums. In addition, the cell culture isolate (inocula 2 and 3) was derived from a mix of tissues from WPD-affected possums. Moreover, the presence of viral RNA has been demonstrated in various tissues with histological lesions of WPD by RT-qPCR (Dunowska et al., 2013) and by *in situ* hybridisation (Dunowska et al., 2012).

Secondly, "filtrates of the infectious material (blood, et cetera, or tissue triturates), shown not to contain bacteria or other visible or cultivable organisms, must produce the disease or its

counterpart in appropriate animals or plants" and thirdly, "similar filtrates from such animals or plants must transmit the disease". The first two of these requirements have been fulfilled in the current study by inclusion of the standard inoculum comprising filtered tissue homogenates from WPD-affected possums, and the virus isolated from the same standard inoculum, in the experimental protocol. Thus, three types of "infectious filtrates" were used: standard inoculum, purified WPDV isolate and clarified lysates of WPDV-infected cultures. All but one possum inoculated with the infectious material developed WPD based on clinical and histological indices, providing strong evidence that the agent responsible for development of WPD was in fact possum nidovirus, and not another pathogen that might have been present in infected tissues or in lysates from the infected cell cultures.

Finally, "immunological and histological studies should confirm the specificity and identity of the diseases caused, but this is not always possible." We have demonstrated presence of histological lesions typical for WPD (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott, 1998) in all but one of the possums that had been inoculated with the infectious material. In agreement with previous studies (O'Keefe et al., 1997), the most severe histological lesions were observed in livers of experimentally infected possums. This corresponded well with detection of high levels of viral RNA in the same tissues, and was consistent with results of the previous study (Dunowska et al., 2013), in which the highest levels of viral RNA were detected in spleens and livers of WPD-affected possums. Histological changes and tissue RNA levels from experimentally-infected possums will be explored further in chapter 4.

The cause of the few small foci of perivascular mononuclear infiltrates observed histologically in the liver of two out of four control possums is unknown. These findings are unlikely to represent current WPD as control possums did not develop compatible clinical signs, lesions were not observed in other tissues examined, and WPDV RNA was not detected in the same tissues. Infiltration of a single tissue with mononuclear cells is simply a sign of inflammation, which is not specific for WPD. However, the widespread pattern of inflammation in several

internal organs is considered consistent with WPD, and has formed the basis for the histological component of the scoring system utilised in the current study (Perrott, 1998; Perrott et al., 2000b). According to this system, none of the control possums were affected by WPD.

The clinical assessment of possums was not straightforward due to inherent difficulties associated with neurological assessment of wild animals. However, the clinical signs observed in WPD-affected possums were similar to those described previously (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott, 1998). Ataxia was characterized by stumbling, rolling or leaning gait with or without hypermetria. This is suggestive of disease involving the cerebellum and/or vestibular system (Lorenz M.D, 2004). A crouched hindlimb gait could have represented paresis from disease affecting the lumbar spinal cord, peripheral nerves and/or muscles. In contrast to previous reports (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott, 1998), we did not observe signs of blindness or aggression in any of the WPD-affected possums. Instead, these possums showed a decreased fear response towards their human carers. As this apparent "friendliness" was not observed in control possums, it was unlikely to be due to taming and desensitization in captivity, and was thus likely to represent central neurological disease.

It is unknown what proportion of possums naturally infected with WPDV can survive in the wild. The neurological impairments associated with WPD would make the affected possums vulnerable to predation in Australia, where possums form a part of native protected flora. In New Zealand, they are considered pests due to uncontrollable expansion associated with lack of natural predators (Nugent et al., 2001). However, the difficulties in climbing would make it challenging for the diseased possums to forage for food and to find shelter. The experimentally infected possums in the current study were euthanised soon after development of neurological disease. Thus, it remains to be determined whether some of the WPD-affected possums could recover from disease if provided with appropriate supportive care. This would be of interest from the wildlife conservation point of view should WPD be shown to be present in Australia (this is currently undetermined).

Despite difficulties assessing the neurological status of pouch-bound joeys, two out of five joeys of the infected possums appeared clinically normal, suggesting the existence of some age-related factors in susceptibility to WPD. Similar apparent resistance to WPD in some of the joeys experimentally exposed to the infectious material has been reported previously (Perrott, 1998; Perrott et al., 2000b). Two clinically normal joeys from WPD-affected mothers tested in the current study were positive for WPD RNA, thus confirming infection in the absence of overt disease, within the limitations of accurate clinical assessment of pouch-bound joeys. However, joey 13j displayed multi-organ histological lesions consistent with WPD and high levels of WPDV RNA were detected in its spleen. We cannot exclude the possibility that joey 13j would have progressed to clinical WPD if euthanasia had not intervened. The second clinically normal joey (10j) lacked multi-organ histological changes consistent with WPD and only low levels of WPDV RNA were detected in its spleen. This joey was euthanized on day nine, two weeks before euthanasia of joey 13j. Thus, it is possible that the differences in the degree of histological changes and the levels of WPDV RNA observed in the tissues of these two joeys simply reflected the timing of euthanasia. The cause of death in the remaining three joeys in the study is unknown. Unfortunately, tissues from these three joeys were autolysed and not suitable for histological examination. Altogether, the role of age on the susceptibility to WPD and the potential for survival requires further exploration.

Conclusions

We have successfully reproduced WPD in possums following inoculation with a purified cell culture isolate of a recently described marsupial nidovirus. The experimentally infected possums developed disease which was indistinguishable from disease developed by possums inoculated with the standard inoculum prepared from tissue homogenates of WPD-affected possums based on previously described clinical and histological criteria. Demonstration of consistent clinical signs and high levels of viral RNA in the livers and spleens of all but one infected possums and

in none of the controls provides strong evidence that the virus is aetiologically involved in development of WPD.

Disclaimer

I have performed all experiments and analyses described in this chapter, with the exception of creation of buffers for tissue fixation, and preparation of histological slides, both of which were done by my PhD supervisor, Dr Matthew Perrott. Manuscript describing work presented in this chapter is published in Published in Virology, Volume 491, April 2016, pages 20-26

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Chapter 4

Viral RNA load and histological changes in tissues following experimental infection with wobbly possum disease virus

Summary

The objective of the study was to describe histological lesions and viral RNA levels in tissues from Australian brushtail possums (*Trichosurus vulpecula*) that had been experimentally infected with wobbly possum disease virus (WPDV). Mononuclear inflammatory cell infiltrates of variable size were consistently observed in the liver, kidney, salivary gland and brain. Two out of 12 (17%) WPDV-inoculated possums had marked azotaemia and 11/12 (91.6%) WPDV-infected possums had histological renal lesions that included varying degrees of interstitial nephritis. The highest viral RNA levels were found in lymphoid, splenic and liver tissues consistent with presumed tropism of WPDV for cells of the immune origin, most likely of the monocyte-macrophage system. Viral RNA levels in the hindbrain and spinal cord were significantly lower than in the forebrain, suggesting site-dependant preference for viral replication within nervous tissue. Moderately high levels of viral RNA were found in the salivary gland and serum suggesting that the virus may be transmitted via fighting in the wild. Presence of the viral RNA in the kidney, bladder and urine suggests that urinary excretion may also play a role in virus transmission via environmental contamination and direct contact with the virus during den sharing.

Introduction

Recently the full genomic sequence of wobbly possum disease virus (WPDV) has been described (Gulyaeva et al., 2017). Based on the analysis of this sequence, WPDV has now been classified within the family *Arteriviridae* as a sole member of the newly established genus *Dipartevirus* (Kuhn et al., 2016). While WPDV shares some features common to arteriviruses, it is only distantly related to other members of the family. Phylogenetically, WPDV comprises a single-virus lineage separate to another lineage that includes all other arterivirus species from different placental hosts (Gulyaeva et al., 2017; Kuhn et al., 2016). Its basal position in the phylogenetic tree suggests that WPDV is the closest to the ancestral arterivirus. Hence, deciphering pathogenesis and biological properties of WPDV is interesting not only from the point of view of possum health, but also from the viral evolutionary perspective.

In the previous chapter, aetiological involvement of the virus in the development of WPD was confirmed by induction of disease by experimental infection of wild-caught possums with purified WPDV isolate. The aim of the current study was to describe histological lesions and viral RNA levels in tissues from possum experimentally infected with WPDV. The data presented adds to results of previously reported studies (Dunowska et al., 2013; O'Keefe et al., 1997; Perrott et al., 2000b) by increasing the number of diseased and healthy possums examined and by inclusion of additional tissue types. The results of the present study add to our understanding of the pathogenesis of WPD and virus-host interactions.

Materials and methods

Source of tissues

Material for the current study comprised archival tissue and body fluid samples from 16 experimental possums used in a previous live-animal transmission study (Giles et al., 2016).

Briefly, wild-caught possums were individually housed and assigned consecutive ID numbers, before being inoculated via intraperitoneal (IP) injection with purified WPDV isolate (possums E5-E8), infected cell culture lysate (possums E9-E12), or standard inoculum that comprised a filtered 10% tissue suspension of brain, liver and spleen from seven WPD-affected possums (possums E13-E16). Possums E1 and E2 received nothing and possums E3 and E4 received control inoculum. Following the development of neurological signs consistent with WPD, possums were euthanized and two sets of tissue samples from all major internal organs, including multiple regions of the brain, were collected from each possum on post-mortem examination. One piece of each tissue was stored at -80 ˚C and another was fixed in 2% paraformaldehyde in phosphate buffer (PLP) pH 7.4 with added lysine (0.15M) and sodium-m-periodate (0.01M) following the method of McClean and Nakane (1974). Urine, where possible, was collected by cystocentesis and stored at -80 ˚C. Blood was collected into red-top vacutainer tubes (BD Vacutainer®) by cardiocentesis or venipuncture of the great vessels. Serum was separated from the clot by centrifugation at 1,500 x g for 10 minutes and stored at -80 ˚C.

All animal manipulations were approved by the Massey University Animal Ethics Committee (MUAEC protocol 12/15).

Determination of WPDV RNA load in tissues

Total RNA was extracted from ~20 mg of tissue (liver, spleen, kidney, forebrain, hindbrain, lumbar spinal cord, salivary gland, retropharyngeal lymph node and bladder) using Total RNA Mini Kit for tissue (Geneaid Biotech Ltd) and from 100 µL samples of body fluids (urine and serum) using Total RNA Mini Kit for blood and cultured cells (Geneaid Biotech Ltd) according to the manufacturer's instructions, with the exception that body fluids were used instead of cell culture media or lysate. Full sets of tissues were available for seven WPD-affected possums. Urine was the only missing sample for two possums (possums E10 and E16) and lymph node was missing for one possum (possum E14). For the remaining two possums (E6 and E11) only

liver, spleen and sera were available for testing. RNA from tissues and body fluids were eluted with 50 µL of water and NanoDrop™ (Thermo Fisher Scientific) was used to determine the concentration and quality of RNA. Up to 1 µg RNA was used for cDNA synthesis using qScript™ Supermix (Quanta Biosciences) according the manufacturer's instructions, with the exception that reactions were scaled down to a total volume of 10 µL.

The viral RNA load was determined using WPDV-specific reverse transcriptase quantitative PCR (RT-qPCR) as previously described (Dunowska et al., 2013) with the modification that the assay was performed using MIC instrument (Bio Molecular Systems) and Luminaris HiGreen qPCR mastermix (Thermo Fisher Scientific) with the following conditions: 50 ˚C for 2 minutes (uracil DNA glycosylase activation), 95 ˚C for 10 minutes (initial denaturation and activation of DNA polymerase), 40 cycles of 95 ˚C for 5s, 60 ˚C for 5s and 72 ˚C for 15s, followed by melt from 72 ˚C to 95 ˚C at 0.3 ˚C/s. Several dilutions of a plasmid containing WPDV PCR fragment and water were used in each RT-qPCR run as positive and negative controls, respectively. All available tissues from WPD-affected possums were tested. In addition, tissues from possums E1 and E3 were included as negative controls.

Results were expressed as viral copy numbers per µg of RNA (solid tissues) or per µL of template cDNA (body fluids). Differences in viral load between tissue types in infected possums were determined by least mean squares using SAS software. Two possums (possums E6 and E11) were removed from the analysis due to multiple missing data points. Two negative data points from WPD-affected possums (spinal cord from possum E7 and hind brain from possum E8) were also removed from the analysis. Values were log transformed to be normally distributed and a mixed model with tissue as fix effect and possum as random effect was fitted to the data (Proc MIXED, SAS). The significance level was set at $p < 0.05$.

Histology

Histological examination was performed on the following tissues: liver, spleen, brain, kidney, bladder and salivary gland from all possums, and a lymph node from the head region from all possums except possums E9, E10, E14 and E15. All tissue samples were fixed in paraformaldehyde in phosphate buffer (PLP) as described above, then transferred and held in 60% ethanol prior to processing and embedding into paraffin blocks. Sections (4 μm) were stained using Gill's haematoxylin and eosin method (Bancroft and Stevens, 2001) and permanently mounted with Entellan (Merck). Kidney slides were also stained for collagen using Masson's trichrome and permanently mounted with Entellan (Merck). Slides were observed using a light microscope (Olympus BX 51) and images captured using an Olympus XC 50 digital camera. Slides were examined by four researchers (Julia Giles, Matthew Perrott, Wendi Roe, Danielle Aberdeen) blinded. The severity of lesions within the renal interstitium, glomeruli and tubules were individually graded on a scale of 0 to 3, where 0 represented no discernible lesions and 3 represented severe lesions. A total score was given by summation of individual lesion scores. Statistical significance for individual parameters and total grade between inoculated and control possums were determined by Kruskal-Wallis Test (SAS). The significance level was set at $p < 0.05$.

Serum Biochemistry

Testing for urea and creatinine levels in stored serum samples was performed by IDEXX laboratories (Palmerston North, New Zealand). Sera from possums E1 – E4 (control possums) and 11 out of 12 WPDV-inoculated possums were tested. Insufficient serum from possum E15 was available for testing. Possums were considered to have elevated urea if it exceeded 15.8 mmol/L and elevated creatinine if it exceeded 100 μmol/L (Viggers and Lindenmayer, 1996). Mild azotaemia was considered as a serum creatinine of 100-150 μmol/L and urea of 15.8-45 mmol/L. Severe azotaemia was considered as a serum creatinine > 400 μmol/L and urea of >45 mmol/L.

Leptospira qPCR

Leptospirosis is endemic in New Zealand possums and infection with pathogenic serovars can cause of kidney pathology. Therefore, to rule out infection with *Leptospira* spp. as a cause of kidney pathology, *Leptospira* qPCR targeting the DNA gyrase subunit B gene was performed using DNA extracted from frozen kidney tissues according to the method of Slack *et al* (Slack et al., 2006). Samples from 14/16 experimental possums were tested. Frozen kidneys from possums E6 and E11 were not available.

Immunohistochemistry

Immunostaining for WPDV antigen was performed on liver, brain and kidney tissues from all 16 possums. Briefly, sections (5 μm) were cut and mounted onto positively charged slides (HDD Thermo Scientific) and oven-dried at 65 ˚C for approximately one hour. After deparaffinization with xylene, sections were rehydrated through graded ethanol to water. Formalin pigment was removed by incubation with saturated picric acid in alcohol for five minutes. Slides were rinsed in water for five minutes, quenched with 3% H_2O_2 in methanol for 10 minutes and rinsed in water for two minutes. Heat-based antigen retrieval (Tris EDTA pH 8.5 for 30 minutes at 95 ˚C) was followed by permeabilization (0.3% Triton X in PBS pH 7.0 for five minutes). Slides were then washed three times in PBS pH 7.0 containing 0.2% Tween 20 (PBS/Tween20), blocked with SuperBlock blocking buffer (Thermo Scientific) containing 0.1% Tween 20, and incubated overnight (4 ˚C) with anti-WPDV antibody diluted 1:500 (0.7 μg/mL) in SuperBlock blocking buffer. Affinity purified polyclonal rabbit IgG raised against *E.coli*-expressed recombinant nucleocapsid (rN) protein (anti-WPDV) was commercially produced (Lifeome). The rN protein had been shown to be recognised by sera from WPDV infected possums by enzyme-linked immunosorbent assay and Western blot (Giles JC, manuscript in preparation). The following morning, the slides were washed three times in PBS/Tween20. The presence of bound primary antibody was detected using the ABC universal

detection system (Vector Laboratories) according to the manufacturer's instructions and visualised with ImmPACT™ DAB substrate (Vector Laboratories). After approximately five minutes of incubation with the chromogen, the slides were washed in running tap water for five minutes and counterstained with haematoxylin. To assess non-specific binding, rabbit polyclonal anti-epidermal growth factor receptor antibody (Ab-3, Calbiochem®, Merck Millipore) was used as irrelevant antibody.

Results

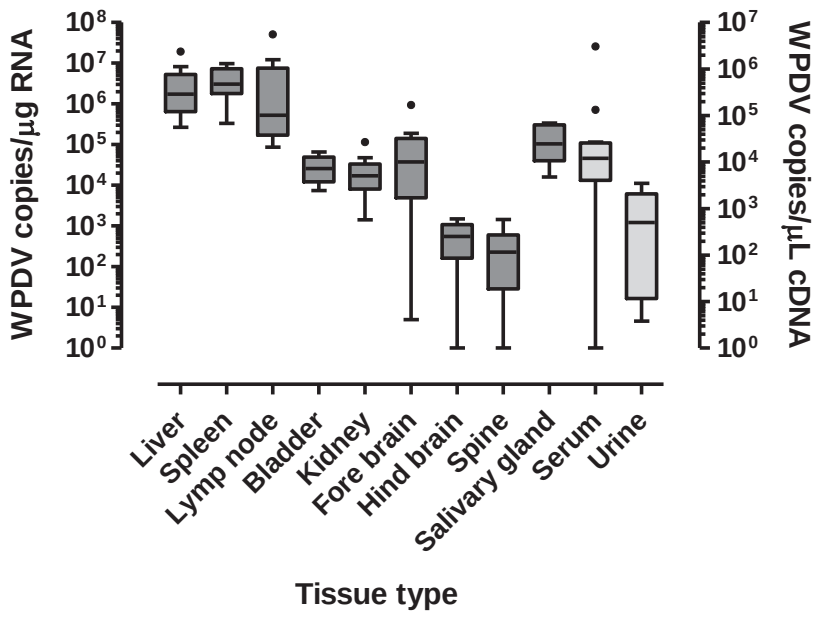
Determination of WPDV RNA levels in tissues

Viral RNA levels in different tissue types and body fluids from individual possums are shown in table 4.1 and viral RNA levels by tissue and body fluid type are shown in figure 4.1.

On average, the highest levels (> 10^6 copies/ µg RNA) of viral RNA were detected in the retropharyngeal lymph node, spleen and liver of WPDV-infected possums (figure 4.1). Moderately high levels of viral RNA (approximately 10^4 – 10^5 copies/ µg RNA) were found in the salivary gland, kidney, bladder and forebrain. Comparatively low levels of viral RNA (< 10^3 copies/ µg RNA) were found in the spinal cord and hindbrain. The mean viral load in the liver was significantly higher than the mean viral load in the kidney, fore brain, hind brain, salivary gland and bladder ($p < 0.05$) but was not significantly different from the mean viral RNA levels in the spleen and lymph node ($p > 0.05$). The mean viral load in the fore brain was significantly higher than the mean viral load in the hind brain and spinal cord ($p < 0.05$).

Moderately high levels of viral RNA were detected in sera from all but one infected possums (average 2.76 x 10^5 copies/µL), however, there were marked differences between individual possums (table 4.1). Lower levels of viral RNA were detected in the urine than the serum (1.02 x 10^3 copies/µL), with similar variability between individual animals.

Figure 4.1: Quantity of viral RNA in tissues and body fluids from WPDV-infected possums



Viral RNA was extracted and quantified using a previously described WPDV-specific RT-qPCR. The results were expressed as WPDV copies/µg RNA for tissues (liver, spleen, lymph node, bladder, kidney, fore brain, hindbrain, spine and salivary gland) and as WPDV copies/µL cDNA for body fluids (serum and urine). Data are presented as box-and-whisker Tukey plots, with outliers shown as dots.

Table 4.1: Viral load in tissues and body fluids from WPDV-infected possums

Possum ID # (M/F)[a]	Inoculum type[b]	Euthanasia (days post inculation)	Quantitative RNA levels (copies/µg RNA)									Quantitative RNA levels (copies/µL template cDNA)	
			Liver	Spleen	Forebrain	Hindbrain	Spinal Cord	Salivary gland	Lymph node	Kidney	Bladder	Urine	Serum
E1 (M)	None	42	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
E3 (M)	CI	41	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
E5 (M)	WPDV	20	$5.07x10^6$	$3.01x10^6$	$5.76x10^3$	$3.99\ x10^2$	$1.05x10^2$	$2.67x10^5$	$1.05x10^6$	$9.03x10^3$	$1.24x10^4$	3.8	$9.68x10^3$
E6 (F)	WPDV	8	$6.12x10^5$	$4.96x10^6$	-	-	-	-	-	-	-	-	0
E7 (M)	WPDV	20	$8.17x10^6$	$2.71x10^6$	$6.83x10^4$	$5.32x10^2$	Neg	$3.00x10^5$	$2.87x10^6$	$1.93x10^4$	$4.87x10^4$	$4.87x10^1$	$2.26x10^4$
E8 (M)	WPDV	27	$5.32x10^6$	$8.02x10^6$	$9.38x10^5$	Neg	$9.41x10^2$	$3.38x10^5$	$8.61x10^4$	$4.74x10^4$	$6.55x10^4$	$2.88x10^1$	$1.33x10^5$
E9 (M)	ICL	19	$1.98x10^6$	$6.85x10^6$	$3.76x10^4$	$1.49x10^3$	$4.86x10^2$	$1.60x10^4$	$5.12x10^7$	$1.85x10^4$	$3.28x10^4$	$2.32x10^3$	$9.81x10^3$
E10 (F)	ICL	9	$3.00x10^5$	$4.79x10^5$	5	$9.71x10^2$	$3.52x10^2$	$1.16x10^5$	$2.91x10^5$	$8.54x10^3$	$4.22x10^4$	-	$5.45x10^2$
E11 (M)	ICL	22	$2.45x10^6$	$1.65x10^6$	-	-	-	-	-	-	-	-	$1.44x10^4$
E12 (F)	ICL	27	$1.92x10^7$	$9.76x10^6$	$3.74x10^4$	$2.06x10^2$	$4.23x10^2$	$3.29x10^5$	$2.00x10^5$	$1.16x10^5$	$4.95x10^4$	$9.51x10^2$	$3.07x10^6$
E13 (F)	SI	23	$1.49x10^6$	$2.25x10^6$	$1.27x10^5$	$1.44x10^3$	$2.93x10^1$	$4.12x10^4$	$5.26x10^5$	$6.49x10^3$	$1.14x10^4$	$3.49x10^3$	$3.03x10^3$
E14 (M)	SI	9	$2.68x10^5$	$3.30x10^5$	$2.52x10^3$	$7.55x10^2$	$2.58x10^1$	$9.20x10^4$	-	$1.42x10^3$	$1.54x10^4$	$1.31x10^3$	$7.11x10^3$
E15 (M)	SI	6 (died)	$7.38x10^5$	$3.06x10^6$	$2.14x10^4$	$3.1x10^1$	$8.86x10^1$	$7.49x10^4$	$1.41x10^5$	$1.59x10^4$	$1.86x10^4$	5.8	$2.69x10^4$
E16 (F)	SI	20	$1.16x10^6$	$7.51x10^6$	$1.89x10^5$	$5.79x10^2$	$1.43x10^3$	$3.73x10^4$	$1.22x10^7$	$2.83x10^4$	$7.38x10^3$	-	$1.61x10^4$
Average RNA levels (possums E5-E16)			$3.90x10^6$	$4.22x10^6$	$1.43x10^5$	$6.40x10^2$	$3.88x10^2$	$1.61x10^5$	$7.62x10^6$	$2.71x10^4$	$3.04x10^4$	$1.02x10^3$	$2.76x10^5$

RNA was extracted from archival tissue samples from 12 WPDV-infected possums (possums E5-E16) and two uninfected controls (possums E1 and E3). Viral load was quantified using a previously described WPDV-specific RT-qPCR. Results were expressed as viral copy numbers per µg of RNA (solid tissues) or per µL of template cDNA (body fluids). [a] F (female); M (male). [b] Possums were inoculated with one of the following inocula: control inoculum (CI) comprising gradient material from uninfected cell culture lysates, standard inoculum prepared from tissues of WPD-affected possums (SI), infected cell culture lysates (ICL), or WPDV (purified WPDV isolate).

Histology

Histological examination was performed on possums E1-E4 (controls) and all WPDV-infected possums (possums E5-E16).

Kidney: Kidney lesions are summarised in table 4.2 and image presented in figure 4.2. Mild – severe interstitial nephritis was present in 2/4 (50%) control possums and 11/12 (91.6%) WPDV-infected possums. Additionally, mild – severe tubular lesions were present in 10/12 (83.3%), mild – moderate glomerular lesions were present in 5/12 (41.7%) and mild-severe interstitial fibrosis were present in 4/12 (33.3%) WPDV-infected possums. Scores for tubular lesions ($p = 0.009$), interstitial nephritis ($p = 0.032$), total scores ($p = 0.014$) and immunopositivity for WPDV antigen ($p = 0.004$) were significantly higher for WPDV-infected possums than control possums. The extent of interstitial fibrosis and glomerular lesions did not differ significantly between infected and control possums ($p>0.05$). Interstitial nephritis was present in 11 WPDV-infected possums, but mild interstitial nephritis was also present in two control possums (possums E3 and E4). In 10/11 WPDV-infected possums and 2/4 control possums (50%), the interstitial infiltrate was lymphoplasmacytic, while a mixed infiltrate (predominantly lymphocytes and plasma cells with fewer neutrophils) was present in one (8.3%) WPDV infected animal (possum E5). In possums with moderate to severe interstitial nephritis (grade 2-3; $n = 7$), renal tubules were often lined by degenerating or proliferating epithelial cells and contained eosinophilic material (proteinaceous fluid). Glomerular changes were variable and mild in 3/12 (25%) WPDV-infected possums and moderate in 2/12 (16.7%) of WPDV-infected possums. Where present, glomerular changes comprised one or more of the following; periglomerular fibrosis, hyperplasia or hypertrophy of parietal epithelium, synechiae, and increased glomerular cellularity. In 1/12 cases (8%) there were perivascular aggregates of moderate numbers of mononuclear cells in the peri-renal adipose tissue (possum E5), but these were also present in one of the four (25%) control animals (possum E3). Masson's trichrome was strongly positive for collagen in the interstitium of both of these possums.

Brain: Lesions are summarised in table 4.3 and images provided in figure 4.2. Mild to severe non-suppurative encephalitis (*n = 1*; control possum) or meningoencephalitis (*n = 10*; WPDV-infected possums) was present in 11 possums. Meningoencephalitis in these cases was characterised by infiltration of the subarachnoid space by few to moderate numbers of lymphocytes, plasma cells and macrophages, with perivascular cuffs of mononuclear cells 1-4 layers thick surrounding vessels in the superficial cortex and periventricular grey matter. The inflammatory infiltrate was partially (possum E9) or predominantly (possum E7) neutrophilic in two possums. In 2/12 (16.7%) infected possums (possums E5 and E7) the perivascular inflammation extended into the surrounding neuropil. Inflammatory changes were often more apparent in subependymal regions. Lymphoplasmacytic perivascular inflammation was present within the choroid plexus of 6/12 (50%) infected possums (possums E5, E7, E12, E13, E14 and E16). Multifocal dense aggregates of glial cells (glial nodules) involving both grey or white matter were present in 5/12 (41.7%) infected possums (possums E5, E7, E8, E12, E16) and 1/4 (25%) control possum. Occasional foci of malacia were present in the forebrain of possum E9, while multiple suppurative foci and necrotising vasculitis were present within the forebrain of possum E7.

Liver: Small, multifocal inflammatory aggregates were disseminated throughout the hepatic parenchyma in all (12/12) WPD-infected possums. Inflammatory aggregates comprised predominantly plasma cells with fewer macrophages, and frequently appeared to be clustered around macrophages with increased amounts of vacuolated cytoplasm. Moderate-sized aggregates of the same cell population were also observed surrounding vessels in periportal (figure 4.2) and centrilobular regions. Small foci of perivascular mononuclear inflammation around the central veins were also observed in sections of liver from two control possums (possums E1 and E3), however, control possums lacked mononuclear cell infiltration in the hepatic parenchyma.

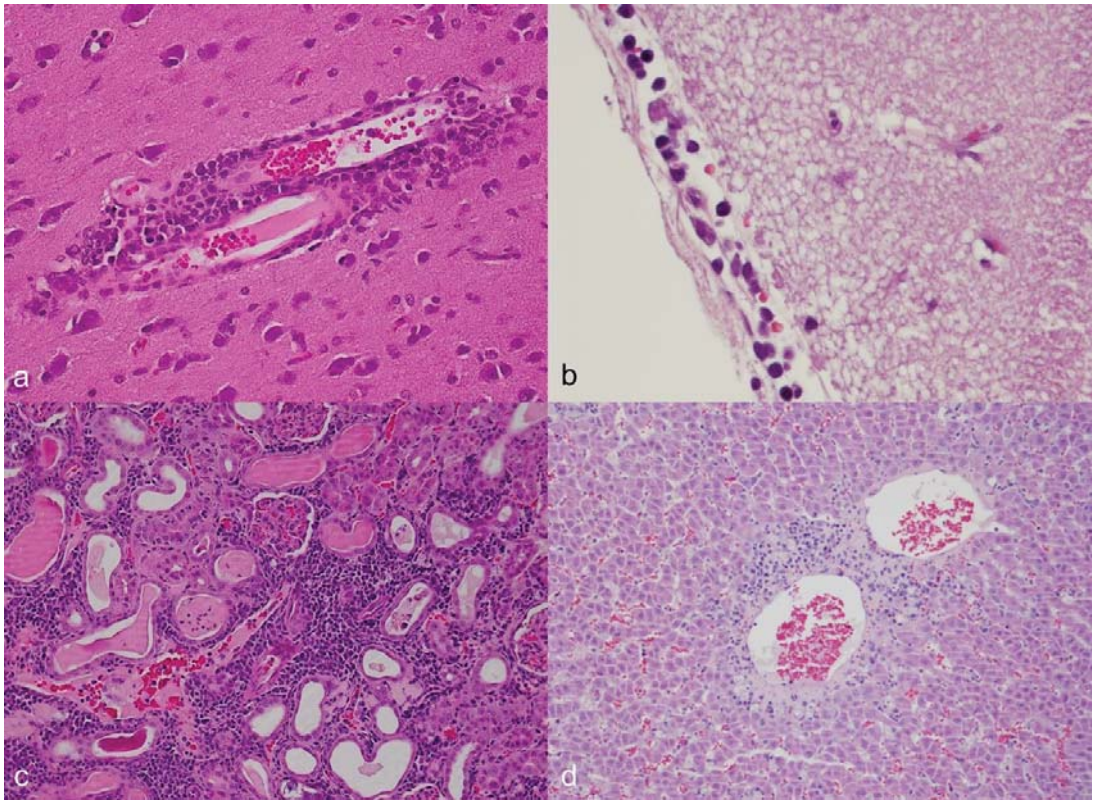
Spleen: In all WPD possums, lymphoid follicles were poorly organised, with few obvious germinal centres. Variable numbers of foamy macrophages were also present in the red pulp of the spleens of all WPD possums. Changes were not present within spleens of control possums.

Lymph node: Variable degrees of lymphoid necrosis and an increased number of foamy macrophages were present in the lymph nodes of 7/8 (88%) of WPD possums examined. The lymph nodes of control possums did not contain lesions.

Salivary gland: Moderate numbers of mononuclear cells were present in the peri-acinar and perivascular regions in salivary glands from all 12 WPD possums but not from control possums.

Bladder: Occasional mild perivascular infiltrates comprising several plasma cells and lymphocytes were observed in the submucosa of the bladder of 6/12 (50%) WPD possums and in none of the control possums.

Figure 4.2: Histology of brain, kidney and liver tissue from WPDV-infected possums



a) Possum E13 brain, viewed under 20 x objective. Mononuclear perivascular cuff in the forebrain b) Possum E9 brain, viewed under 40 x objective. Infiltration of the subarachnoid space with lymphocytes, plasma cells and macrophages c) Possum E12 kidney, viewed under 20 x objective. Severe interstitial nephritis and tubular damage. The interstitial infiltrate is lymphoplasmacytic. Renal tubules are dilated, and proteinacious fluid and cellular debris are present in the tubules d) Possum E16 liver viewed under 10 x objective. Inflammatory aggregates surrounding central veins. Aggregates comprise predominantly plasma cells with some macrophages. Slides were observed using a light microscope (Olympus BX 51) and images captured using an Olympus XC 50 digital camera.

Table 4.2: Histopathological, biochemical and qPCR renal parameters

Possum ID # (M/F)[a]	Inoculum Type[b]	Euthanasia (days post inoculation)	Urea (mmol/L)[c]	Creatinine (µmol/L)[d]	Histologic features of kidney disease[e]					IHC[f]	qPCR *Leptospira* (-/+)[g]
					Interstitial nephritis	Tubular lesions	Glomerular lesions	Interstitial Fibrosis	Total score		
E1 (M)	Control	42	10.3	47	0	0	0	0	0	-	-
E2 (M)	Control	42	5.6	48	0	0	0	0	0	-	+
E3 (M)	Control	41	6.2	48	1	0	0	0	1	-	-
E4 (F)	Control	41	6.8	82	1	0	0	0	1	-	-
E5 (M)	WPDV	20	6.4	34	1	1	0	0	2	+	-
E6 (F)	WPDV	8	17.1	136	0	0	0	0	0	-	N/A
E7 (M)	WPDV	20	17.8	53	1	1	0	0	2	+	-
E8 (M)	WPDV	27	43.9	145	2	1	1	0	4	+	+
E9 (M)	ICL	19	10.2	42	2	1	1	0	4	+	-
E10 (F)	ICL	9	109.9	1108	3	3	2	3	11	+	-
E11 (M)	ICL	22	6.4	43	1	1	0	0	2	+	N/A
E12 (F)	ICL	27	3.1	23	3	3	0	1	7	+	-
E13 (F)	SI	23	5.6	35	2	1	1	1	5	+	-
E14 (M)	SI	9	107.8	951	3	3	2	3	11	+	-
E15 (M)	SI	6 (died)	N/A	N/A	1	0	0	0	1	+	-
E16 (F)	SI	20	8.8	26	2	1	0	0	3	-	-

[a] F (female); M (male). [b] Possums were inoculated with one of the following inocula: control inoculum (CI) comprising gradient material from uninfected cell culture lysates, standard inoculum prepared from tissues of WPD-affected possums (SI), infected cell culture lysates (ICL), or WPDV (purified WPDV isolate). [c] Above 15.8 mmol/L = elevated. [d] Above 100 µmol/L = elevated. [e] Severity of histological changes are graded as follows: 0 = no significant lesions, 1 = mild lesions, 2 = moderate lesions, 3 = severe lesions. A total grade was calculated by summation of grades from individual categories. [f] Immunohistochemistry using antibodies directed against expressed WPDV nucleocapsid (N) protein, - = no signal, + = signal. [g] = + = PCR positive, - = PCR negative, N/A = tissues not available for testing

Table 4.3: Description of histological brain lesions in WPDV-infected possums

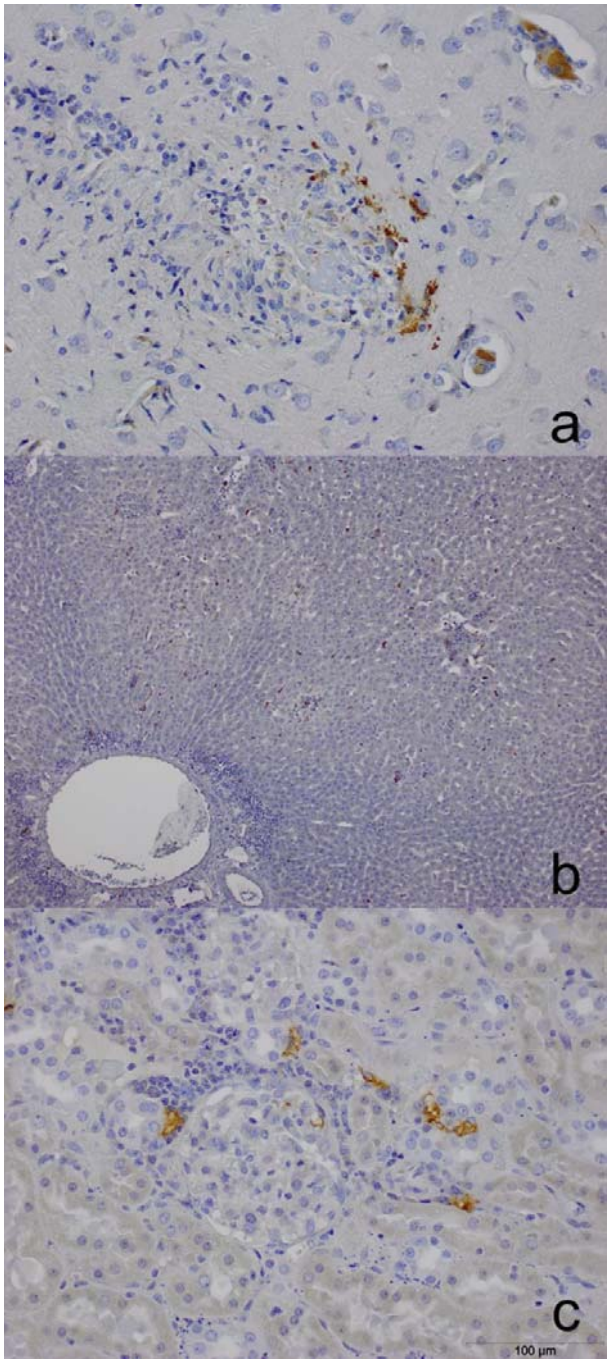
Possum ID # (M/F)[a]	Inoculum Type[b]	Euthanasia (days post inoculation)	Histologic features of brain lesions (+/-)[c]								IHC (+/-)[d]
			Encephalitis	Meningitis	Inflammatory infilitrate	Cuffing	Gliosis	Glial nodules	Endothelial Hypertrophy	Choroiditis	
E1 (M)	Control	42	-	-	-	-	-	-	-	-	-
E2 (M)	Control	42	-	-	-	-	-	-	-	-	-
E3 (M)	Control	41	-	-	-	-	-	-	-	-	-
E4 (F)	Control	41	+	-	Nonsuppurative	-	-	+	-	-	-
E5 (M)	WPDV	20	+	+	Nonsuppurative	+	-	+	+	+	+
E6 (F)	WPDV	8	-	-	-	-	-	-	-	-	-
E7 (M)	WPDV	20	+	+	Neutrophilic	+	+	+	-	+	+
E8 (M)	WPDV	27	+	+	Nonsuppurative	-	-	+	-	-	+
E9 (M)	ICL	19	+	+	Nonsuppurative	+	+	-	+	-	+
E10 (F)	ICL	9	+	+	Nonsuppurative	+	-	-	+	-	+
E11 (M)	ICL	22	+	+	Nonsuppurative	-	-	-	-	-	-
E12 (F)	ICL	27	+	+	Nonsuppurative	+	+	+	-	+	+
E13 (F)	SI	23	+	+	Nonsuppurative	+	+	-	-	+	+
E14 (M)	SI	9	+	+	Nonsuppurative	+	+	-	+	+	-
E15 (M)	SI	6 (died)	-	-	-	-	-	-	-	-	-
E16 (F)	SI	20	+	+	Nonsuppurative	+	+	+	-	+	+

Possums E5-E16 were infected with WPDV and possums 1 – 4 were uninfected controls. [a] F (female); M (male). [b] Possums were inoculated with one of the following inocula: control inoculum (CI) comprising gradient material from uninfected cell culture lysates, standard inoculum prepared from tissues of WPD-affected. possums (SI), infected cell culture lysates (ICL), or WPDV (purified WPDV isolate). [c] + = lesions present, - = lesions absent. [d] Immunohistochemistry using antibodies directed against expressed WPDV nucleocapsid (N) protein, + = immunopositive, - = immunonegative, N/A = not available

Immunohistochemistry

Immunopositivity for WPDV antigen, where present, was identified as the appearance of granular staining within the cytoplasm of cells. Immunopositivity was present in macrophages in the livers of all experimentally infected possums (figure 4.3), with no immunopositivity in the livers of control possums. Inflammatory aggregates often appeared to be clustered around immunopositive macrophages. Immunostaining was also detected in the kidneys of 10/12 (83.3%) WPDV-infected possums and none of the control possums (table 4.2, figure 4.3). Here, immunostaining was detected in macrophages within inflammatory aggregates in the renal interstitium. Immunostaining was also detected in the brains of 8/12 (66.6%) WPDV-infected possums and none of the control possums. Where present, immunostaining was present in cells within the subarachnoid space (n = 3) and glial nodules (n = 1).

Figure 4.3: WPDV-specific immunohistochemistry of liver, brain and kidney tissues from WPDV-infected possums



Specific immunostaining of WPDV-antigen in the cytoplasm of macrophages in; a) a glial nodule in the forebrain of possum E7, b) in macrophages in the hepatic parenchyma of possum E13. A smaller number of immunopositive macrophages also surround the central vein. Inflammatory aggregates appear to cluster around immunopositive cells, and c) renal interstitium of possum E16. Images courtesy of Dr Matthew Perrott.

Biochemistry

Mild azotaemia was present in two WPD-affected possums (possums E6 and E8) and severe azotaemia in another two possums (possums E10 and E14). Urea was mildly elevated in one additional possum (possum E7), and both values were within references ranges in the remaining experimental possums including control possums (table 4.2).

Leptospira qPCR

Two of the 14 possum kidneys tested were positive for *Leptospira* DNA by qPCR (table 4.2) with Ct values of 19.89 (possum E1, uninoculated control) and 31.56 (possum E8, WPD).

Discussion

We have reported viral RNA levels in tissues from WPD-affected possums. Whilst RNA levels in selected tissues (liver, spleen, brain and kidney) from WPD-affected possums have been previously reported (Dunowska et al., 2013), we have expanded both the number of possums and the tissue types tested to provide a greater understanding of the tissues targeted by the virus *in vivo*. We have also demonstrated for the first time the presence and localisation of the viral antigens within lesions in selected tissue types.

To allow for comparison of RNA levels between tissues, qPCR results were normalised to μg of total RNA based on amount of RNA used for the cDNA synthesis step, while RNA levels in body fluids (urine and serum) were expressed as copy numbers/μL cDNA per RT reaction. Because total RNA levels extracted from body fluids were extremely low, normalisation to total RNA would have resulted in significant inaccuracies. As such, a direct comparison between viral load in solid tissues and body fluids was not possible. Consistent with results of the previous study (Dunowska et al., 2013), high levels of viral RNA were present in livers and

spleens of all WPD-affected possums. In addition, high levels of viral RNA were also detected in lymph nodes examined. These results suggest that WPDV, similarly to other arteriviruses (Teifke et al., 2001), has *in vivo* tropism for macrophage-rich tissues, which may result in generalized infection of the mononuclear-phagocyte system. This is further supported by our IHC data, with WPDV antigen detected within macrophages in the liver and kidney of infected possums. These results also correspond well with the *in vitro* growth requirements for WPDV, as the virus has been cultivated so far only in primary possum macrophages (chapter 2).

Based on our results, macrophage-rich tissues should be considered as the samples of choice for detection of the virus or viral RNA in diagnostic settings. Detection of moderate to high levels of viral RNA from the sera of all but one infected possum in this study also supports the use of blood or serum samples for detection of WPDV. Finally, detection of moderately high levels of viral RNA in salivary glands raises the possibility of virus excretion in saliva. The latter two sample types (blood and saliva) may provide a means of diagnosis of active infection in live animals, without the necessity of a retrospective diagnosis following death or euthanasia.

Moderate to high levels of viral RNA in serum samples in all but one infected possum indicates that cell-free viraemia is a feature of WPD. High levels of viral RNA were still detected in the serum nearly four weeks post infection, suggesting a failure of the immune system to clear the virus within this time-frame. A feature of arteriviruses is the ability to evade the immune response and induce persistent infections (Kimman et al., 2009; Sun et al., 2012). For example, mice infected with lactate dehydrogenase-elevating virus (LDV) develop persistent life-long viraemia which is believed to be facilitated by the fact that the primary target cell for LDV infection is a renewable, continuously regenerated and apparently 'non-vital' subpopulation of macrophages (Plagemann et al., 1995). Persistent viraemia (months to years) has been also described for pigs infected with porcine reproductive and respiratory syndrome virus PRRSV (Benfield et al., 1999), and for patas monkeys persistently infected with low-virulence isolates (P-248 and P-741) of simian haemorrhagic fever virus SHFV (Gravell et al., 1986).

The presence of multi-organ lymphoplasmacytic inflammation in possums euthanased within eight to nine days following infection indicates that immune response to viral antigens begins early in the disease course. This response, however, appears to be ineffective at containing virus replication within the time-period studied, as high levels of viral RNA continued to be detected in tissues from possums that were euthanased approximately three weeks later. An on-going inflammatory response may promote egress of circulating monocytes into tissues. This may be an undesirable consequence as, in addition to contributing to tissue damage, they could serve as new targets for virus infection.

The presence of the virus in serum, urine and salivary glands provides indicators on possible routes of transmission of the virus. Fighting between possums with exchange of saliva and/or blood through bite wounds may facilitate transmission. Grooming of joeys by mothers, face-to-face contact, and hissing during encounters could also facilitate spread of the virus via saliva. Despite comparatively low levels of viral RNA in the urine, urinary excretion of WPDV could provide a source of virus contamination in the environment. Direct contact with urine from infected possums may also occur during sharing of dens by multiple possums. Moderately high levels of viral RNA in the serum also raise the possibility that WPDV could be spread by blood-sucking ectoparasites. This is supported by successful induction of WPD by injection of macerated hematophagus mites (*Trichosurolaelaps crassipes)* derived from WPD-affected possums into healthy possums in one of the previous studies (Perrott et al., 2000b). Further studies are required to provide experimental support for the main routes of transmission during natural infection as well as the infectious dose of the virus required to elicit compatible clinical signs.

Higher levels of viral RNA were consistently detected in the fore brain in comparison with the hind brain. One possible explanation of these results is that location-specific differences in the numbers of cells supportive of viral replication exist. For example, regional variations of the distribution of microglia have been described in the brain of placental species, with denser

populations of microglia in the forebrain as compared to the hindbrain (Lawson et al., 1990; Perry et al., 1985). Given that macrophages are the target cell of arteriviruses (Duan et al., 1997; Onyekaba et al., 1989; Plagemann and Moennig, 1992), it is possible that microglia which are similarly derived from myeloid precursors, may support WPDV replication. Whilst IHC for WPDV-antigen was positive in 8/12 brains from WPDV-infected possums, the cell types supporting viral replication is unclear. Further investigation into the cell types that support viral replication with the CNS is thus warranted.

The histological lesions observed in the WPD-affected possums were, in general, similar to those described previously (O'Keefe et al., 1997; Perrott, 1998). Lesions in the brain included varying degrees of non-suppurative mengingoencephalitis, choroiditis, gliosis and glial nodules, the number of which varied between possums. The cause of the glial nodules in one control possum is unknown, however, it is unlikely to be related to active WPDV infection as this possum was clinically normal and viral RNA was not detected in its brain. The preponderance of inflammatory infiltrates in the subependymal region of the brain in some infected possums is intriguing. Similar periventricular lesion location has been reported for neurotropic coronaviruses such as feline infectious peritonitis virus and mouse hepatitis virus (Diaz and Poma, 2009) and occasionally for neurotropic arterivirus variants, for example during field outbreaks of PRRSV (Thanawongnuwech et al., 1997).

Given the lack of overt endothelial cell damage and lack of immunopositivity for WPDV antigen within endothelial cells in the CNS and elsewhere, it is unlikely that WPDV traverses the blood-brain barrier within the CNS through infection of endothelial cells. Other possible mechanisms of transport into the CNS could include passive transfer across the endothelium at pinocytotic junctions of the choroid plexus or transport across endothelium within infected blood-borne monocytes (Whitley and Gnann, 2002).

Neurological signs are atypical with infection with other arteriviruses but can occur with neurotropic variants of PRRSV and LDV (Anderson et al., 1995; Rossow et al., 1999; Tian et al., 2007). Because of the multi-organ distribution of histological lesions and high viral RNA levels in multiple tissues, the nervous system is not the sole target for WPDV replication. Whilst the most clinically obvious signs in the experimentally infected possums were neurological, it is possible that disturbances in other organ systems, general malaise and severe acute wasting contributed in part to the clinical picture, both during the current and previous studies (Giles et al., 2016; Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott et al., 2000a; Perrott et al., 2000b)

In contrast to previous reports (O'Keefe et al., 1997; Perrott, 1998), renal lesions were consistently observed in the vast majority (11/12, 92%) of experimentally infected possums in the current study, which prompted further investigation into the aetiology of these lesions and their contribution to the clinical signs observed. Mild azotaemia was present in two and severe azotaemia in another two of 12 infected possums suggesting that despite gross and pathological changes observed in the kidneys, acute severe kidney injury was not a universal feature of WPDV infection. Whilst histological features of nephritis support the possibility that azotaemia in the five WPDV-infected possums was of renal origin, pre-renal causes such as dehydration cannot be ruled out, particularly in possums with milder azotaemia. The determination of urine specific gravity (USG) could have helped in the differentiation of pre-renal and renal azotaemia, but was not performed. Leptospirosis was also considered as a possible cause of the renal changes, Leptospirosis is endemic in New Zealand and infection with pathogenic serovars can result in kidney disease. However, only one control possum and one WPD-affected possum were positive for leptospiral DNA and it is unlikely that pre-existing infection with pathogenic *Leptospira* spp. resulted in the biochemical or histological changes observed in the kidneys of *Leptospira*-negative possums.

To help provide a further understanding of the pathogenesis of the renal lesions, IHC for WPDV antigen was performed. Immunopositivity was predominantly observed within the cytoplasm of macrophages in interstitial inflammatory aggregates and to a lesser extent in cells within glomeruli. Whilst the cellular populations supporting the virus replication within these locations are currently unknown, we speculate that these are cells of monocyte-macrophage lineage, such as interstitial macrophages and/or mesangial cells. This is based on the fact that arteriviruses are generally tissue-type and cell-type specific, primarily replicating in macrophages (Duan et al., 1997; Onyekaba et al., 1989; Plagemann and Moennig, 1992). However, expanded cell and tissue tropism has been described for highly pathogenic arteriviruses, including a highly pathogenic PRRSV that is capable of replication within distal renal tubular epithelium in pigs (Li et al., 2012). Thus, to further understand the pathogenesis of renal lesions the exact cell types supporting viral replication within kidney tissues need to be further investigated.

Conclusion

The preferred sites for replication of WPDV are splenic, hepatic and lymphoid tissue, making these samples of choice for diagnostic testing of deceased animals. Histological changes and moderate amounts of RNA were also present within other tissues including sera and salivary glands suggesting that blood and saliva may be used for diagnosis of infection in live animals. Apparent tropism for macrophage-rich tissues may reflect a generalized infection of the mononuclear-phagocyte system. Within the central nervous systems, there seem to be location-related differences in virus tropism. Levels of viral RNA remained high in all tissues tested throughout the duration of the study, suggesting that on-going virus replication and evasion of the immune responses may be important in the pathogenesis of a chronic disease state. The observations described in this paper may inform experimental design of further studies into mechanisms and duration of virus persistence following natural infection with WPDV.

Disclaimer

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Chapter 5

Development of an indirect ELISA for detection of antibody to wobbly possum disease virus

Summary

An indirect ELISA based on recombinant nucleocapsid protein (rN) of wobbly possum disease virus (WPDV) has been developed. A set of pre- and post-infection sera obtained from a previous experimental challenge study were used for ELISA development. Sera were characterised as positive or negative for WPDV antibody based on Western blot using rN as antigen. An additional 215 archival serum samples were used in a serological survey to estimate prevalence of WPDV infection among New Zealand possums. Bayesian estimates of parameters for a model of the ELISA data were used to establish ELISA cut-offs for WPDV antibody positive and negative samples, and to estimate the prevalence of WPDV infection. The archival serum samples were collected between the years 2000 and 2016 and obtained from five different regions of New Zealand. Based on the model, the estimated prevalence of WPDV infection among New Zealand possums was 30%, which fitted well with the 21% prevalence calculated by applying the established cut-offs to the ELISA data. The data presented indicate that WPD, or related, virus has circulated among possums in New Zealand, although the prevalence of infection seemed to vary between geographical regions. The presence of WPDV antibody did not seem to protect possums from disease following experimental infection, as approximately one third of WPD affected possums from the previous challenge study showed evidence of pre-existing antibody at the time of challenge. The exact determinants of protection against WPD

and epidemiology of infection in various regions of New Zealand remain to be established. Availability of the indirect ELISA test for WPDV antibody will facilitate such research.

Introduction

Based on clinical signs observed during the challenge study (chapter 3), we have hypothesized that WPDV-infected possums would be unlikely to survive in the wild. However, we have investigated only one source of the virus. Several lines of evidence support existence of different pathotypes of the virus. Firstly, a few experimentally infected possums housed in a captive environment appeared to have started recovering from disease at the time of euthanasia (Perrott, 1998). In addition, possums experimentally infected with inocula produced from two geographically distinct sources of the virus developed disease of different severity (Perrott et al., 2000), with different levels of WPDV RNA detected in comparable tissue samples (Dunowska et al., 2013). Moreover, the ultimate outcome of natural WPDV infection under New Zealand conditions remains unknown (Perrott, 1998).

Recent phylogenetic analysis of WPDV genome suggests that WPDV separated from the current members of *Arteriviridae* early in the process of evolution, and at present, WPDV is the closest known member to ancestral arterivirus (Gulyaeva et al., 2017). There are two plausible hypotheses with regard to the origin of WPDV. It is either a possum virus that was brought to New Zealand at the time when possums were introduced to this country from their native Australia in the late 1800's (King, 1990) or it emerged among possums in New Zealand, possibly via a cross-species transfer. Development of a serological assay would facilitate future epidemiological investigations to test these hypotheses. As such, the aim of the current project was to develop an indirect ELISA for the detection of WPDV antibody in possum sera. We also describe application of this test to a limited serological survey of New Zealand possums using archival serum samples.

Materials and Methods

Possum sera

Archival serum samples were used for the project (table 5.1). These included 12 serum samples from possums (possums E5-E16) experimentally infected with WPDV as part of the previous challenge study (chapter 3). These were collected at the time of death or euthanasia, between six and 27 days post-inoculation (post-sera). Pre-inoculation serum samples from 11/12 of the same possums (all with the exception of possum E11), as well as serum samples from four control possums (possums E1-E4) were also included. Possums E1 and E2 received nothing and possums E3 and E4 received control inoculum. Serum samples from control possums were collected twice: at the time of experimental inoculations of the challenged possums and 41-42 days later, more than two weeks after euthanasia of the last two challenged possums (possums E8 and E12). In addition to these experimental possums, archival serum samples (n=215) collected for various other unrelated projects were included in the study. There was limited information available on the origin of these samples, except for the geographical location of captured possums and dates (month/year) of sampling. All sera were stored at -80 ˚C until use.

Table 5.1: Description of possum serum samples used for ELISA development and serological survey

Possum ID	WPD status	Geographical origin	Sampling date	Description	n
E5-E16 post[a]	WPD+	Manawatu	July-August 2013	Possums experimentally infected with WPDV, sera collected at the time of euthanasia	12
E5-E16 pre[b]	Healthy	Manawatu	July 2013	Possum experimentally infected with WPDV, sera collected pre-challenge	11
E1-E4[c]	Healthy	Manawatu	July-August 2013	Control uninfected possums, sera collected twice 41-42 days apart.	8
Various[d]	Unknown	Waimea	March 2013	Field samples	81
Various	Unknown	Whiteman's Valley	Oct-Nov 2000	Field samples	42
Various	Unknown	Paraparumu	May-June 2000	Field samples	58
Various	Unknown	Kaikora	April 2014	Field samples	14
Various	Unknown	Manawatu	Feb-March 2015	Field samples	18
Various	Unknown	Manawatu	Oct-Dec 2015	Field samples	2

[a] Samples E5-E16 comprised sera collected before (pre-) and after (post-) experimental infection with WPDV.
[b] Post-infection samples were collected at the time of euthanasia or death, between 6 and 27 days post-inoculation.
[c] Samples E1-E4 comprise sera from uninfected controls maintained during the experimental study.
[d] Archival sera from various locations in New Zealand (n=215).

WPDV antigen

Open reading frame (ORF) 7 (nt 12424-12804) of the WPDV genome (GenBank accession JN116253.3) predicted to encode the putative WPDV N protein was commercially expressed in *Escherichia coli* (Lifeome Biolabs). The purified recombinant N protein (rN) had a molecular weight of 40 kDa including GST tag. The stock protein was stored at -80 ˚C at a concentration of 1 mg/mL in phosphate buffered saline (PBS), pH 7.4 with 0.02% NaN_3.

SDS PAGE and Western Blot

Purified rN protein (6 µg in 150 µL of H_2O) was mixed with 142.5 µL of Laemmli sample buffer (Sigma-Aldrich) and 7.5 µL 2-mercaptoethanol (Sigma-Aldrich), boiled at 100˚C for five minutes and then loaded into the single prep well of Criterion TGX pre-cast gel, 4 – 20% (Prep + 2 well comb, cat # 5671092 Biorad). Precision Plus protein dual colour standard (10 µL, Biorad) and MagicMark XP western protein standard (10µL, Thermo Scientific) were prepared according to the manufacturer's instructions and included as molecular weight markers in the remaining two wells. The gel was subjected to electrophoresis at 200 V for 45 minutes in Tris/glycine/SDS running buffer (Biorad) in Criterion electrophoresis cell (Cat # 165-6001, Biorad). Proteins were transferred onto 0.45 µm pore-size PVDF membrane (Immunoblot, cat #162-0262, Biorad) in Criterion blotter (Biorad) with Tris/glycine transfer buffer (Biorad) for 90 minutes at 70 V under cooling conditions. Detection of rN protein was performed using a commercial kit (Pierce Fast Western blot kit, supersignal west pico substrate, cat # 35061, Thermo Scientific) according to the manufacturer's instructions. Briefly, the PVDF membrane was cut into 4 mm strips, which were individually incubated with sera from possums E1 – E16 (1:100 dilution in Fast Western antibody diluent, 1 mL volume) overnight at 4˚C. Positive control strip was incubated with rabbit rN antiserum (1:500, 1 mL volume) and negative control strip with rabbit serum negative for rN antibody (1:500, 1 mL volume). The rabbit control sera were commercially sourced (Lifeome Biolabs). The following morning, strips were washed two

times in Fast Western wash buffer and incubated individually with 1 mL of rabbit anti-opossum polyclonal antibody (cat # A140-111A, Bethyl Laboratories Inc.) diluted 1:500 in Fast Western antibody diluent for 60 minutes with shaking. Strips were removed from antibody solutions and incubated at room temperature with Fast Western rabbit optimized HRP reagent for 15 minutes, washed four times in Fast Western wash buffer and incubated with SuperSignal West Pico Working solution for five minutes at room temperature before being transferred to a plastic sheet protector and imaged using C-DiGit blot scanner (LI-COR Biosciences) on high sensitivity settings. Samples were considered to be positive for anti-N IgG if a distinct band of approximately 40 kDa was detected on the blot. Altogether, 38 possum sera were tested including pre- and post- sera from possums E1-16, and an additional 6/215 archival serum samples from possums of unknown disease status. These were randomly selected to represent a range of OD_{450} values.

Development of indirect ELISA

Checker-board titration was used to optimise the concentration of primary and secondary antibody. Various coating buffers, rN protein concentrations, blocking buffers, washing protocols and incubation times were tested. Sera from possums experimentally infected with WPDV (post-sera from possums E5-E16) were considered as known positives for the purpose of ELISA optimisation. Due to the unavailability of known negative sera, the ELISA was optimized to obtain the clearest differentiation (highest difference between corrected OD_{450}) between pre- and post-inoculation sera from possums experimentally infected with WPDV.

Optimised indirect ELISA protocol

Each well in a flat-bottom 96-well microtitre plate (Nunc Immuno MaxiSorp, Thermo Scientific) was coated overnight at 4˚C with 100 µL (0.1 µg) purified rN protein diluted in carbonate-bicarbonate buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4,

pH 9.6). The following morning, plates were washed three times in PBS pH 7.5 containing 0.05% (v/v) Tween 20 (PBST), with a rest step of five minutes between each wash cycle, and blocked with 5% bovine serum albumin (BSA) in PBST (200 µL/well) for 90 minutes at room temperature. Plates were used immediately following blocking. After three washes with PBST, serum samples (100 µL/well) were added in duplicate to wells on the plate. All sera were diluted in PBST and tested at two dilutions: 1:10 and 1:100. On each plate, post-serum from possum 12 were included as a positive control and four wells were left as serum-free negative controls. Plates were incubated at room temperature with shaking for 60 minutes before washing three times with PBST as above. Plates were then incubated for 60 minutes with rabbit anti-opossum affinity-purified polyclonal antibody (cat # A140-111A, Bethyl Laboratories Inc.) diluted 1:5,000 in PBST (100 µL/well), washed as above, and incubated with goat anti-rabbit HRP-conjugated polyclonal antibody (ab97051, Abcam) diluted 1:40,000 in PBST (100 µL/well). The plate was washed again, and 100 µL of TMB ELISA substrate highest sensitivity (ab171522, Abcam) was added per well. After nine minutes, the reaction was stopped with 2 N H_2SO_4 (100 µL/well). The optical density of each well was measured at 450 nm using a microplate reader (Powerwave 340, Biotech). Results were presented as corrected OD_{450} values by subtracting the average OD_{450} of the blank wells from the average OD_{450} of each sample. The plate was considered valid if the corrected OD_{450} value of the positive control was ≥1.6 at 1:10 dilution and ≥0.6 at 1:100 dilution and the OD_{450} of blank wells were < 0.1.

Establishment of cut-off values for indirect ELISA

Corrected OD_{450} values for all sera tested either 1:10 or 1:100 dilution were transformed to ln(OD + 0.3) to achieve approximate normality conditional on infection status as determined by Western blot. These transformed values were assumed to be normally distributed with means mu1 and mu2, and variances sigma1 and sigma2, for positive and negative samples respectively. The transformed observations for non-experimental possums with unknown WPDV antibody status were modelled as a mixture of these two normal distributions, with

prevalence of infection as the mixing parameter. The model was restricted to satisfy mu2 > mu1 since it was believed that the average OD_{450} for WPDV seropositive possums would exceed that for seronegative possums. This restriction was also necessary for the model to be identifiable. Diffuse prior distributions (Christensen et al., 2011) were placed on the means, variances and prevalence. Using this model, Bayes estimates of the receiver operating characteristic (ROC) curve, area under the curve (AUC), prevalence (pi) and means and standard deviations of the normal distributions were obtained for each of the two serum dilutions. The model fit was checked by plotting the mixture model based predicted distribution of the data from non-experimental possums against a non-parametric estimate of the same.

Statistical analysis

ELISA data were analysed using descriptive statistics in GraphPad Prism version 5.04 (GraphPad Software, San Diego California USA, www.graphpad.com). Chi-squared test was used to determine the statistical significance of differences in prevalence of WPD antibody between regions. The significance level was set at $p<0.05$.

Development of blocking ELISA

Checker-board titration was used to optimise the concentration of primary and secondary antibody. Coating conditions, protein concentrations and wash buffers were as described for optimised indirect ELISA protocol. Sera from possums experimentally infected with WPDV (post-sera from possums E5-16) were considered as known positives for the purpose of ELISA optimisation. Due to the unavailability of known negative sera, the ELISA was optimized to obtain the clearest differentiation (highest difference between corrected OD450) between pre- and post-inoculation sera from possums experimentally infected with WPDV. The final protocol involved coating each well in a flat-bottom 96-well microtitre plate (Nunc Immuno MaxiSorp, Thermo Scientific) overnight at 4˚C with 100 µL (0.1 µg) purified rN protein diluted in

carbonate-bicarbonate buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 9.6). The following morning, plates were washed three times in PBST pH 7.5, with a rest step of five minutes between each wash cycle, and blocked with 3% BSA in PBST (200 µL/well) for 90 minutes at room temperature. After washing with PBST (three times with five minutes of rest between washing steps), pre and post sera from possums E1 – E16 diluted 1:5, 1:10, 1:100 and 1:500 in PBST were added to wells on the plate (100 µL/well) in duplicate. On each plate, four wells were left as serum-free controls. Plates were incubated at room temperature with shaking for 60 minutes, washed three times as above, and 100 µL of rabbit rN antiserum (0.49 mg/mL, Lifeome Biolabs) diluted 1:10,000 in PBST was then added to each well. Plates were incubated for 60 minutes at room temperature with shaking, washed as above, and incubated for 60 minutes with goat anti-rabbit HRP-conjugated polyclonal antibody (ab97051, Abcam) (100 µL/well) diluted 1:20,000 in PBST. After washing, plates were incubated with TMB ELISA substrate highest sensitivity (ab171522 , Abcam, 100 µL/well) for 10 minutes, and the reaction was stopped with 100 µL/well of 2 N H_2SO_4. The optical density of each well was measured at 450 nm in a microplate reader (Powerwave 340, Biotech, Winooski, VT, USA). Results were expressed as percentage inhibition (PI) according to the following calculation: PI (%) = (1 − ($OD_{450\ nm}$ of test serum/$OD_{450\ nm}$ of serum free controls)) × 100%.

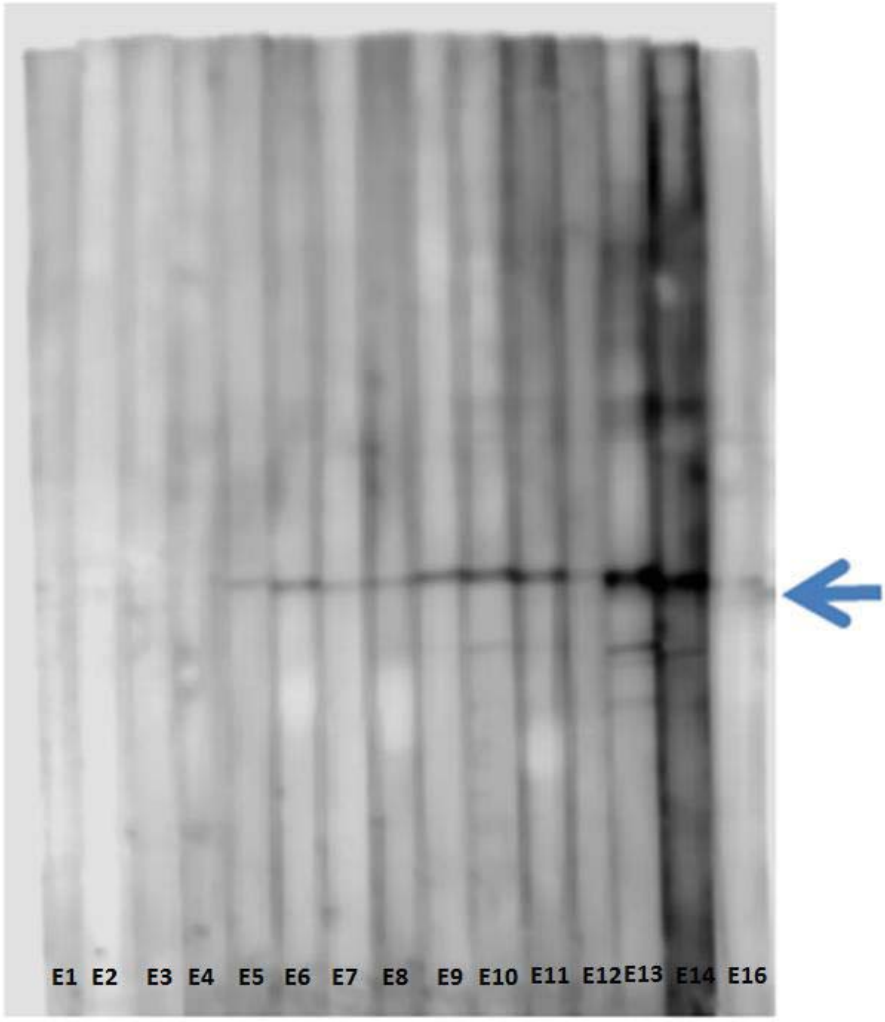
Results

Western blot analysis

Insufficient serum samples were available for analysis from possums E6 and E11 (pre-sera) and from possum E15 (post-serum). Recombinant N protein was recognised by 5/14 (36%) pre-sera (possums E5, E10, E13, E14, E15) and 11/11 (100%) of post-sera from WPDV-infected possums (Figure 5.1). An indistinct band present for possum 12 pre-sera was considered equivocal. An additional 2/6 (33%) archival sera from possums of unknown health status tested positive for anti-WPDV antibody. The corresponding corrected OD_{450} for these two samples

were 0.35 and 0.46. The OD_{450} values for the remaining four sera that were negative on Western blot ranged from 0.04 to 0.27.

Figure 5.1: Western blot to detect anti-WPDV IgG in sera collected at the time of euthanasia post-challenge with wobbly possum disease virus (WPDV)

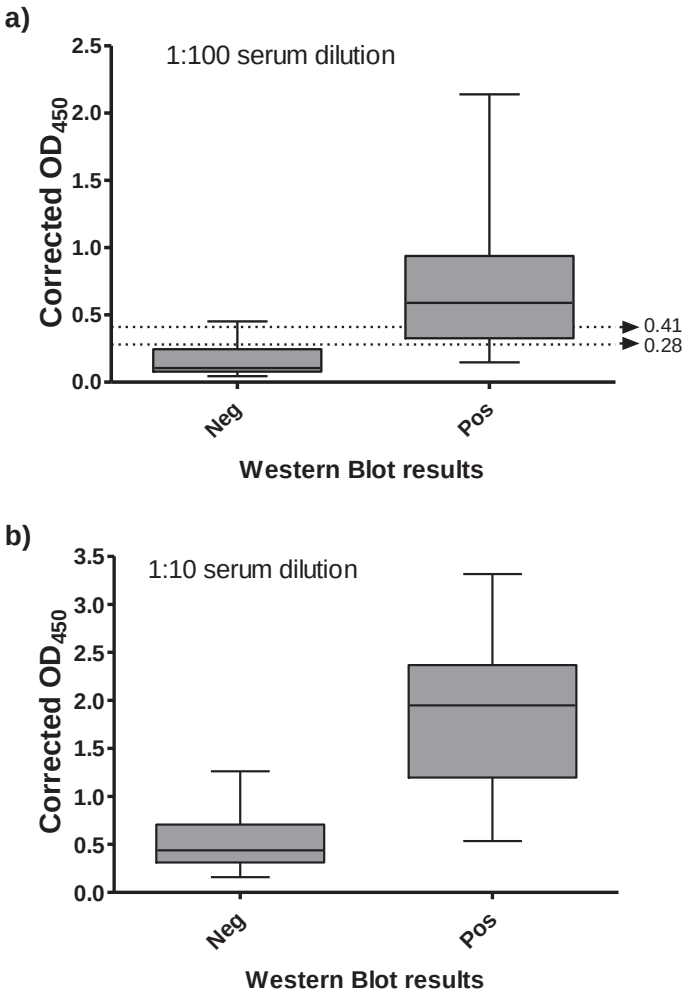


WPDV nucleocapsid (N) protein expressed in *E. coli* was used as antigen. Sera were considered positive for anti-WPDV IgG if a distinct band was present at the 40 kDa measure (arrow). The lines are labelled with possum numbers: possums E1-E4 were non-infected controls and possums E5-E16 were infected with WPDV. Serum from possum 15 was not available for testing.

Optimization of WPDV indirect ELISA

The optimised protocol for indirect WPDV ELISA is described in the Methods section. The reproducibility of the optimised ELISA was acceptable, with CV values for a known positive serum from possum E12 lower than 27% for both 1:10 and 1:100 serum dilutions (table 5.2). Corrected OD_{450} values for pre-sera from WPDV-infected possums (*n*=11) ranged from 0.32 to 2.12 with a mean of 0.8 (SD 0.60) at 1:10 dilution and from 0.08 to 0.82 with a mean 0.3 (SD 0.31) at 1:100 dilution (table 5.3). Corrected OD_{450} values for post-sera from WPDV-infected possums (*n*=12) ranged from 1.22 to 3.31 with a mean of 2.20 (SD 0.71) at 1:10 dilution and from 0.20 to 2.14 with a mean 0.79 (SD 0.57) at 1:100 dilution. Corrected OD_{450} values increased at least two-fold between pre- and post- serum samples for 8/11 (73%) paired samples from WPDV infected possums at 1:10 serum dilution and for 7/11 (64%) paired samples at 1:100 dilution. Mean corrected OD_{450} values for Western blot negative sera (*n*=16) were 0.51 (SD 0.29) at 1:10 and 0.16 (SD 0.12) at 1:100 dilution (figure 5.2). Mean corrected OD_{450} values for Western blot positive sera (*n*=18) were 1.87 (SD 0.83) at 1:10 dilution, and 0.70 (SD 0.51) at 1:100 dilution.

Figure 5.2: Box-and-whiskers graph showing distribution of corrected optical density values measured at 450 nm (OD_{450}) for Western blot negative and Western blot positive sera at 1:100 (a) and 1:10 (b) serum dilutions.



The low and high ELISA cut-off values are indicated for 1:100 serum dilution. Western blot and indirect ELISA were performed using rN protein as the antigen. Whiskers represent minimum and maximum values.

Table 5.2: Reproducibility of indirect ELISA for detection of antibody to wobbly possum disease (WPD) virus

Parameter[a]	OD_{450} blank	Possum E1 Corrected OD (1:10)	Possum E1 Corrected ID (1:100)	Possum E12 Corrected OD (1:10)	Possum E12 post Corrected OD (1:100)
Mean	0.07	0.42	0.15	2.54	0.83
SD	0.01	0.14	0.08	0.63	0.22
CV (%)	16	33	50	25	26

[a] Mean, standard deviation (SD) and coefficient of variation (CV) were calculated based on mean corrected OD_{450} values for duplicate samples of possum E12 post-sera from 13 plates.

Table 5.3: Results of indirect ELISA and Western blot using experimental possum sera

Possum ID[a]	Health status[b]	Euthanasia (days post infection)	Pre-inoculation sera			Post inoculation sera		
			Western blot [c] (+/-)	OD_{450}[d] 1:10	OD_{450}[d] 1:100	Western blot [c]	OD_{450}[d] 1:10	OD_{450}[d] 1:100
E1	Healthy	42	-	0.37	0.08	-	0.39	0.08
E2	Healthy	42	-	0.25	0.05	-	0.31	0.10
E4	Healthy	41	-	0.85	0.37	-	1.26	0.45
E5	WPD	20	+	0.53	0.15	+	1.65	1.41
E6	WPD	8	NT	0.52	0.16	+	3.20	0.20
E7	WPD	20	-	0.48	0.11	+	3.01	1.10
E8	WPD	27	-	0.72	0.25	+	2.50	0.61
E9	WPD	19	-	0.33	0.08	+	1.51	0.20
E10	WPD	9	+	2.12	1.01	+	2.18	0.79
E11	WPD	22	NT	NT	NT	+	3.32	2.14
E12	WPD	27	?	0.66	0.14	+	2.32	0.57
E13	WPD	23	+	0.63	0.38	+	1.94	0.91
E14	WPD	9	+	1.95	0.82	+	1.22	0.35
E15	WPD	6 (died)	+	0.86	0.26	NT	1.32	0.29
E16	WPD	20	_	0.60	0.22	+	2.28	0.87

[a] Samples E1-E4 comprised healthy controls. Samples E5 – E16 comprised sera obtained before (pre-inoculation) and after (post-inoculation) challenge with WPDV. [b] Healthy = clinically normal, WPD = clinical signs of wobbly possum disease. [c] Western blot was performed at serum dilution of 1:100 and considered positive if a distinct band was present at the 40 kDa measure. + positive for WPDV rN antibody; - negative for WPDV rN antibody; ? equivocal; NT not tested. [d] For indirect ELISA, sera were run at 1:10 and 1:100 dilutions in duplicate. Results of ELISA are presented as corrected OD_{450} values.

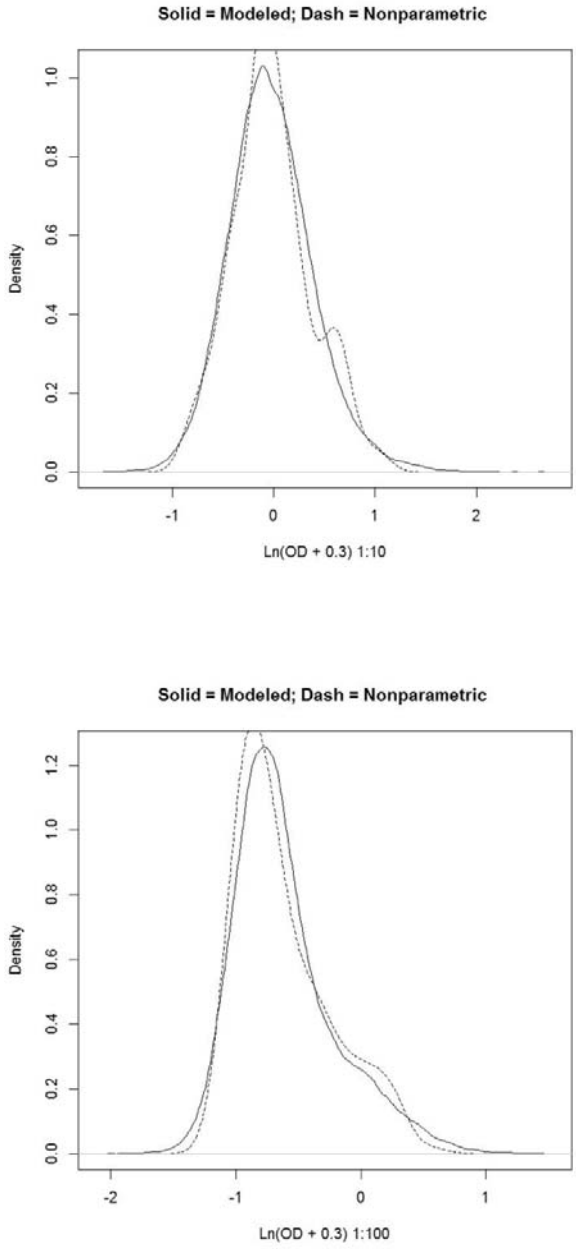
Bayesian modelling and establishment of the cut-off points

Since most of the archival possum sera were not tested with Western blot, the data were analysed using methods that assumed the absence of a gold-standard test to determine true WPDV antibody status of available possum sera (Choi et al., 2006). Bayesian modelling was used to determine performance of the test at both 1:10 and 1:100 serum dilutions (table 5.4). The model fit was considered good for both serum dilutions (figure 5.3), however Bayes estimates of the AUC for 1:100 dilution was higher (0.88) than the AUC estimate for 1:10 serum dilution (0.86) and the estimated ROC curve for 1:10 dilution was notably below the one for OD 1:100 data (figure 5.4). The data from 1:100 serum dilution also showed smaller overlap between predicted seropositive and seronegative samples. Based on better discriminatory performance for 1:100 data according to the model fits, serum dilution of 1:100 was selected as better than 1:10 dilution. The following cut-off values were established for 1:100 serum dilution: ≥ 0.41 for positive samples and <0.28 for negative samples. Samples with corrected OD_{450} of ≥ 0.28 and <0.41 were considered questionable.

Serological survey for WPDV antibody among New Zealand possums

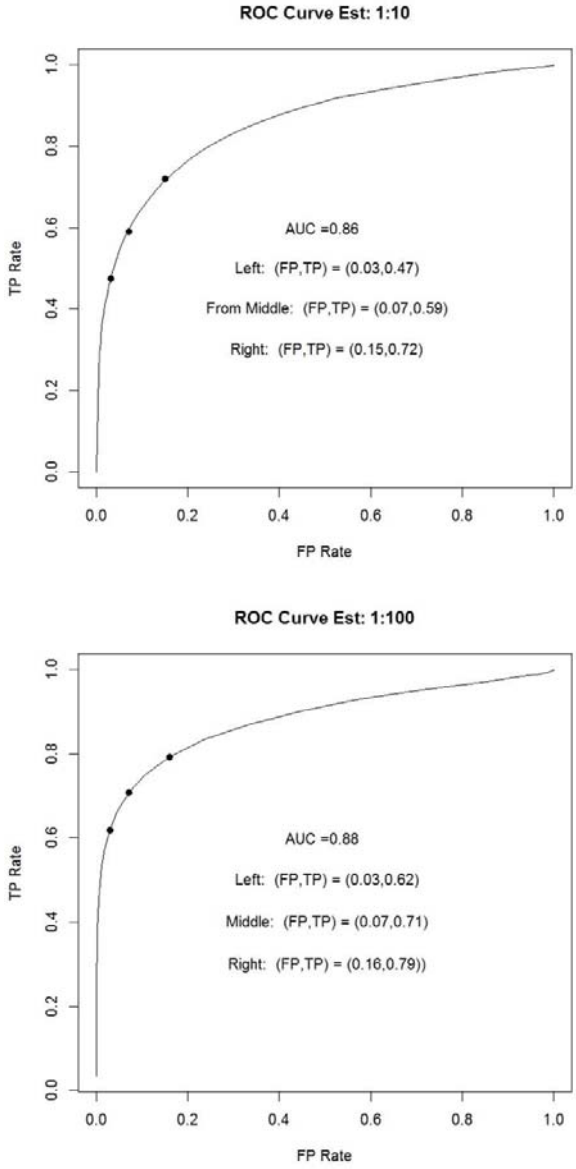
The optimized indirect ELISA protocol was used to test an additional 215 serum samples from various sources. Hence, the full dataset available for serological survey consisted of sera from 230 possums including 15 pre-challenge sera from experimental possums. The overall prevalence of possums positive for WPDV IgG was 48/230 (21%), with 155/230 (67%) negative and 27/230 (12%) equivocal samples. The prevalence of WPD antibody between regions (figure 5.5) were significantly different ($p<0.00001$).

Figure 5.3: Model fit for 1:10 and 1:100 indirect ELISA data



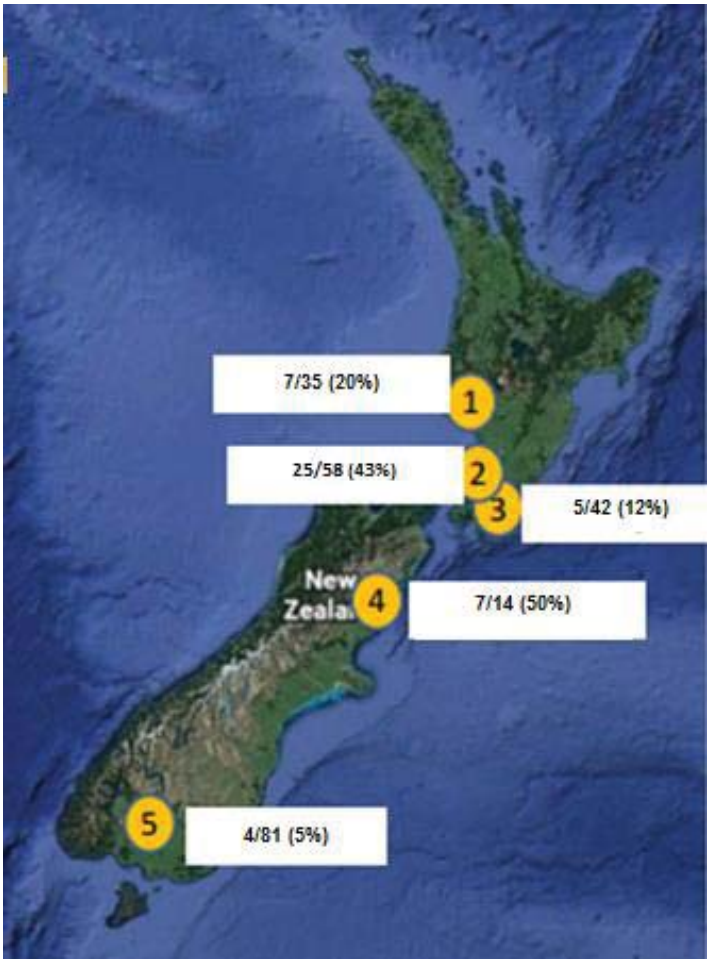
ELISA data was modelled using Bayesian estimates as described in the text. The model fit was checked by plotting the mixture model based predicted distribution of the data from non-experimental possums (solid line) against a non-parametric estimate of the same (dashed line) for 1:10 and 1:100 serum dilutions.

Figure 5.4: Bayes estimates (Est) of the receiver operating characteristic (ROC) curves for 1:10 and 1:100 serum dilutions based on indirect ELISA data



Bayes estimates (Est) of the receiver operating characteristic (ROC) curves for 1:10 and 1:100 serum dilutions based on indirect ELISA data, which have been log-transformed as specified on the graphs. ROC curve was generated using modelled data based on sera that had been classified as negative or positive for anti-N IgG by Western blot. AUC = area under the curve, FP = false positive, TP = true positive.

Figure 5.5: Prevalence of anti-WPDV IgG in archival possum serum samples by region



Indirect ELISA using rN protein as antigen was performed on 230 archival samples from five different regions of New Zealand (table 5.1). At 1:100 serum dilution, $OD_{450} \geq 0.41$ were considered positive, < 0.28 were considered negative, and in-between values were considered questionable. 1 = Manawatu, 2 = Paraparumu, 3 = Whiteman's Valley, 4 = Kaikoura, 5 = Waimea.

Table 5.4: Parameter estimates obtained with Bayesian Analysis for indirect ELISA data generated with 1:100 serum dilution

Parameter[a]	1:100 serum dilution		1:10 serum dilution	
	WPDV Ab negative	**WPDV Ab positive**	**WPDV Ab negative**	**WPDV Ab positive**
Mean	-0.792 (-0.839 , -0.741)	-0.199 (-0.335 ,-0.064)	-0.078 (-0.162 , -0.008)	0.583 (0.367 ,0.802)
Variance	0.238 (0.206 , 0.279)	0.426 (0.357, 0.512)	0.369 (0.312 , 0.421)	0.476 (0.368 , 0.657)
Prevalence		0.30 (0.196 , 0.418)		0.08 (0.004 , 0.234)

[a] Transformed values were modelled on known wobbly possum disease virus antibody (WPDV Ab) positive and known negative samples as determined by Western blot and normally distributed with means mu1 and mu2, and variances sigma1 and sigma2, respectively.

Blocking ELISA

The optimised protocol for indirect WPDV ELISA is described in the Methods section. The mean percentage inhibition value of Western blot negative sera was 30.9% +/- 5.6 SD at 1:5, 26.2% +/- 7.7 SD at 1:10, 4.4% +/-4.2 SD at 1:100 and -1.2% +/- 4.2 SD at 1:500 serum dilutions respectively. The mean percentage inhibition value of Western blot positive sera was 36.3% +/- 7.9 SD at 1:5 dilution, 33% +/- 6.7 SD at 1:10, 16.6 +/- 8.5 SD at 1:100 and 2% +/- 5.2 SD at 1:500 serum dilutions respectively. Percentage inhibition values for serum dilutions are presented in table 5.5.

Table 5.5: Results of blocking ELISA on experimental possum sera

		Percent inhibition[d]							
		Pre-inoculation sera[c]				Post inoculation sera[c]			
Possum ID[a]	Health status[b]	1:5	1:10	1:100	1:500	1:5	1:10	1:100	1:500
E1	Healthy	29.74	26.49	3.02	-5.90	24.26	7.52	3.25	-1.97
E2	Healthy	35.41	29.43	2.77	7.10	24.69	19.36	7.15	-6.45
E3	Healthy	36.29	27.55	2.67	-5.26	33.16	24.01	6.93	-4.78
E4	Healthy	33.33	27.96	6.07	1.85	22.99	14.31	8.18	1.75
E5	WPD	32.42	26.84	6.06	10.64	41.24	33.62	14.98	-0.07
E6	WPD	14.97	16.59	11.26	0.45	28.42	30.91	19.12	2.68
E7	WPD	30.73	33.48	6.24	-0.64	22.85	26.00	13.94	19.45
E8	WPD	40.31	34.80	4.92	-0.73	48.91	44.45	16.22	3.87
E9	WPD	27.51	34.24	7.94	-4.08	28.22	31.76	18.60	3.36
E10	WPD	35.14	35.44	8.15	2.23	27.75	23.43	10.94	4.99
E11	WPD	N/A	N/A	N/A	N/A	35.88	34.66	21.43	6.51
E12	WPD	33.96	29.03	1.67	-11.39	45.38	42.24	19.46	2.92
E13	WPD	32.20	27.40	3.59	-3.51	32.43	22.86	10.13	-4.61
E14	WPD	45.31	39.39	11.62	1.82	48.03	43.54	11.28	3.32
E15	WPD	47.12	37.87	20.04	-6.04	14.34	9.83	-3.54	-5.87
E16	WPD	33.12	29.88	4.26	-6.75	27.73	27.54	14.33	-0.37

[a] Samples E1-E4 comprised healthy controls. Samples E5 – E16 comprised samples obtained before (pre-inoculation) and after (post-inoculation) challenge with WPDV. [b]Healthy = clinically normal, WPD = clinical signs of wobbly possum disease, NA = not available
[c]Sera were run at 1:5, 1:10, 1:100 and 1:500 dilutions in duplicate. [d] Results of ELISA were obtained as OD450, and expressed as percentage inhibition of the mean OD450 of serum-free wells.

Discussion

Since its description some 20 years ago, there have been anecdotal reports that WPD continues to occur in wild possum populations in New Zealand. However, lack of disease surveillance and suitable detection methods has precluded epidemiological estimation of disease prevalence. The discovery of a novel marsupial arterivirus in archival tissues from clinically affected possums (Dunowska et al., 2012) and more recently, implication of WPDV as the causal agent of WPD has allowed for development of virus-specific tests such as quantitative reverse transcription PCR (RT-qPCR), in situ hybridisation (ISH) (Dunowska et al., 2013) and immunohistochemistry (IHC) (chapter 4). These tests however rely on detection of viral RNA and thus require collection of samples from actively infected animals. In addition, viral RNA may degrade in a field setting if processing or storage of biological samples was suboptimal. Thus, to provide evidence that the virus continues to circulate among possums in New Zealand and to estimate prevalence of infection within different possum populations, development of a serological assay was required. In the work presented here, an indirect ELISA using rN protein as antigen has been successfully developed for these purposes.

Receiver operating characteristic curves based on modelled data were used to assess the diagnostic performance of the ELISA and to determine cut-off OD_{450} values for positive and negative samples. This approach is frequently used as a measure of diagnostic effectiveness (Faraggi and Reiser, 2002) and due to lack of known negative controls we considered this to be a more appropriate approach than placing cut-off at two standard deviations from the mean OD_{450} of the known negative sera, which is a commonly used method (Singh, 2007). The decision to choose 1:100 serum dilutions for analysis of the survey data and any future testing was based on comparatively better performance of the modelled data at 1:100 than 1:10 serum dilutions. However, 4/18 Western blot positive sera were negative by ELISA at 1:100 dilution

(figure 5.1). Hence, further optimisation of 1:10 serum dilution to help lower the degree of non-specific binding may lead to improvements in the sensitivity of the assay.

Based on the serological survey data, 21% of possums were positive for anti-N IgG at the higher (0.42) and 33% at the lower (0.28) ELISA cut-offs. This corresponded well with the estimated prevalence of 30% based on the model, and confirmed that WPD or a similar virus circulates within wild possum populations in New Zealand. However, marked differences between local seroprevalences existed, even within possum populations that were geographically close and were sampled in the same year, albeit not at the same month (Paraparumu and Whiteman's Valley). Further epidemiological evaluation including a larger sample size and temporally-comparable samples would be required to investigate regional differences in seroprevalence of WPDV. Factors such as possum density, den sharing, landscape, urbanisation, or presence of other susceptible species may influence local transmission dynamics.

As the only cell type known to support growth of WPDV is primary possum macrophage (chapter 2), *E. coli* expressed antigen rather than cell-culture derived virus was used as ELISA antigen in order to reduce the possibility of non-specific binding to non-WPDV antigens that may have been co-purified with the cultured virus, and to reduce the cost of assay development. The decision to use rN protein was based on data available for other arteriviruses, as it is currently unknown which WPDV proteins provide the main target(s) for development of humoral immune responses. Differences in immunodominant proteins exist between current members of the family *Arteriviridae*. For example, the N protein of porcine reproductive and respiratory syndrome virus (PRRSV) is the most abundant and antigenic of the structural proteins (Dea et al., 2000a; Meulenberg, 2000). While conservation of nucleotide and amino acid sequences of N protein are low between the two prototypic PRRSV strains (North American and European) the major antigenic determinants of the N protein are generally well conserved amongst viruses from the same lineage (Mardassi et al., 1994; Meng et al., 1995; Murtaugh et al., 1995). For this reason, numerous N-protein based serological assays have been

developed for diagnosis of PRRSV (Dea et al., 2000b). In contrast, sera from equine arteritis virus (EAV) infected horses other than carrier stallions most consistently recognized the conserved membrane (M) protein, whilst the response to the membrane-associate glycoproteins and N protein were more variable (Hedges et al., 1998; MacLachlan et al., 1998; Wagner et al., 2003). Interestingly, EAV carrier stallions consistently reacted with the N protein (Hedges et al., 1998).

We have demonstrated that following experimental infection with WPDV, possums develop a detectable IgG response against WPDV N protein. It remains to be established whether or not possums develop humoral immune responses against other WPDV proteins and if so, if those proteins would provide a better antigen for detection of viral infection by ELISA. Likewise, information regarding the timing and magnitude of antibody response following natural or experimental infection with WPDV is lacking. All four Western blot negative possums for which paired sera were available seroconverted to WPDV within 19 to 27 days following experimental infection. Since only pre-infection and post-euthanasia serum samples were collected, the earliest time when WPDV antibodies were detectable following infection of these four possums remains unknown.

Two experimentally WPDV-infected possums (possums E10 and E14) did not appear to develop a humoral response, as the corrected OD_{450} values of sera collected post-euthanasia were slightly lower than corrected OD_{450} values of pre-challenge sera. One possible explanation for this observation is that these two possums did not have enough time to develop antibody response, as both were euthanased at only nine days post infection. However, the other two possums that were euthanased or died around the same time, on days six (possum E15) and eight (possum E6) showed an increase in corrected OD_{450} values at the time of death. For the related arterivirus, PRRSV, detection of IgG by ELISA has been described as early as nine days post-inoculation with the majority of pigs seroconverting by 14 days (Kittawornrat et al., 2013; Yoon et al., 1995), although maximum titres may take a further two to four weeks to be reached

(Yoon et al., 1995). By extrapolation, time to develop detectable seroconversion to WPDV may vary between individual possums. It is also possible that pre-existing antibody to a variant of WPDV masked detection of antibody raised in response to the challenge virus, as (in contrast to possums E6 and E15), pre-infection sera of possums E10 and E14 had high corrected OD_{450} values and were also positive on Western blot. Lastly, lack of detectable humoral immune response may reflect protection offered by pre-existing immunity and lack of, or very limited, viral replication. This is, however, unlikely as the levels of the viral RNA detected in spleens of possums E10 and E14 were similar to the levels of viral RNA detected in spleens of other challenged possums (Giles et al., 2016).

The main difficulty encountered during ELISA development was the sourcing of negative control sera. Whilst pre-infection sera were available from the challenge study, these sera were obtained from wild-caught possums. As such, their WPDV antibody status was unknown, as the ability to survive infection with WPDV and the prevalence of WPDV infection among possums in the catchment area (Manawatu) were unknown at the time. To help discriminate between non-specific binding and specific detection of pre-existing WPDV antibody, Western blots were performed using rN protein as the antigen. Pre-inoculation sera from five of 14 possums tested recognised WPDV rN protein, including two (E10 and E14) possums that consistently showed relatively high (>2 at 1:10 dilution and >0.8 at 1:100 dilution) corrected OD_{450} values, irrespective of the conditions used during the optimisation process. This confirmed our initial suspicion that some of the possums used for experimental challenge had pre-existing antibody to WPD or a similar virus.

Interestingly, presence of pre-existing antibody did not seem to offer protection from disease after experimental challenge, as all five possums developed WPD (Giles et al., 2016), with possums E10 and E14 being among the first three to develop clinical disease - both possums were euthanased on day nine post infection. Another possum with pre-existing WPD antibody (possum 6) died on day six before developing clinical signs of WPD, while the remaining two

possums (E5 and E13) survived for approximately three weeks following challenge (table 5.3). Comparatively fast development of disease in two possums with the highest pre-existing antibody levels suggests that immunopathology may have contributed to their disease. Antibody-dependent enhancement of disease has been proposed to occur during infection with a related arterivirus, PRRSV, where rapid development of non-neutralising antibodies early in the course of infection may enhance virus internalization by macrophages, thus facilitating infection of susceptible cells (Cancel-Tirado et al., 2004; Rascón-Castelo et al., 2015; Tirado and Yoon, 2003). Whether or not similar mechanisms operate during WPDV infection remains to be established.

The five WPDV antibody positive possums used for experimental infection appeared clinically healthy at the time of catchment. Although clinical data for other serologically positive possums were not available, this suggests that some possums can fully recover from natural infection with WPDV. In agreement with these conclusions, existence of WPDV variants of lower virulence in comparison with the virus used for the experimental challenge has been previously suggested (Dunowska et al., 2013; Perrott et al., 2000). In addition, limited experimental observations indicated that susceptibility to WPD may be age-dependent, with joeys less likely to develop severe disease than adults (Perrott, 1998). Altogether, further investigation is needed to determine what virus- and host-related factors influence clinical outcome of infection with WPDV.

Development of a non-species specific assay, such as blocking ELISA, could facilitate serological testing of animals which share the environment with infected possums. As such, it is one way that species specificity of WPDV could be explored. We have been unsuccessful in developing blocking ELISA using rN protein and polyclonal anti-rN rabbit sera, as evidenced by inefficient blocking by sera from experimentally-infected possums. Whilst failure to develop blocking ELISA may simply reflect insufficient optimisation, it is possible that differences exist between the antigenic domains of the rN protein recognized by sera from WPDV-infected

possums and anti-rN sera raised in rabbits. Should this be the case, differences in antibody affinity for shared antigenic epitopes between animals infected with WPDV and anti-rN serum may result in less competition by experimental possum sera. Development of blocking ELISA using monoclonal antibodies against specific epitopes may result in a more robust ELISA with longer-term application due to less variability between batches of polyclonal sera or antibodies.

Conclusion

Indirect rN ELISA can be used to detect anti-WPDV IgG in possum sera. Our data support circulation of WPDV in New Zealand possum populations with an estimated prevalence of 30%. This suggests that prolonged survival or recovery following infection with WPDV is possible and that field viruses of varied virulence are likely to exist. Presence of pre-existing WPDV antibody in archival sera from possums used in the previous challenge study did not confer cross-protection against experimental challenge. Further research is required to understand both host (e.g. age of the animal at the time of infection, or pre-existing immunity) and virus (e.g. dose or route of exposure) factors on disease outcome in a field setting.

Disclaimer

I have performed all experiments and analyses described in this chapter, with the exception of Bayesian modelling, which was done Dr Wesley Johnson of the Department of Statistics, University of California, Irvine, Dr Geoff Jones of the Institute of Fundamental Sciences, Massey University, and Dr Cord Heuer of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University. Manuscript describing work presented in this chapter has been submitted to the New Zealand Veterinary Journal.

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Chapter 6

Final discussion

The work presented in this thesis provides strong experimental evidence of the aetiological involvement of a possum arterivirus in development of WPD. This severe multisystemic disease was first described some 20 years ago (Mackintosh et al., 1995). There were several lines of evidence to suggest that the aetiological agent was a virus. Firstly, filtered tissue lysate derived from WPD-affected possums and administered to healthy possums reproduced the disease (O'Keefe et al., 1997; Perrott, 1998; Perrott et al., 2000). Secondly, virus-like particles were observed in preparations of livers from two experimentally-infected possums under electron microscopy (O'Keefe et al., 1997). Further investigation into the aetiology of WPD was halted until recently, when the partial genomic sequence of a novel arterivirus, termed WPDV, was discovered in archival tissue samples from WPD-affected possums by next generation sequencing (Dunowska et al., 2012). Aetiological involvement of the virus in the development of WPD was first suggested based on the results of WPDV-specific reverse transcriptase PCR (RT-PCR) using liver DNA from WPD-affected and healthy possums as a template (Dunowska et al., 2012) and was further supported by the demonstration of viral RNA in archival tissues from WPD-affected possums using in situ hybridisation (ISH) and quantitative reverse transcriptase PCR (RT-qPCR) (Dunowska et al., 2012; Dunowska et al., 2013).

Further support for the aetiological link between WPDV and WPD was provided by the development of disease in healthy possums following challenge with purified WPDV (chapter 3). Furthermore, convergence of our histological, serological and molecular data provides auxiliary evidence for the aetiological link. Firstly, histological changes in tissues from possums

challenged with purified WPDV were consistent with what has previously been described for WPD-affected possums (O'Keefe et al., 1997; Perrott, 1998). Secondly, using WPDV-specific RT-qPCR, we demonstrated high WPDV RNA levels in tissues from these possums, consistent with active viral replication at the time of death or euthanasia (chapters 3 and 4). Thirdly, we demonstrated that these possums developed detectable serological responses against WPDV N protein (chapter 5). Thus, our data supports a view that clinical disease and pathological changes in tissues are linked to active replication of WPDV and immune response to WPDV antigen. Altogether, this provides strong evidence for aetiological involvement of WPDV in the development of WPD.

Convergence of our data also provides insight into the biological behaviour of WPDV. Similar to other arteriviruses (Duan et al., 1997; Onyekaba et al., 1989; Plagemann and Moennig, 1992), it appears that WPDV has cellular tropism for macrophages. This is supported by the ability to grow WPDV in cultured primary possum macrophages (chapter 2), and the discovery of highest levels of WPDV RNA in macrophage rich tissues from experimentally infected possums, including liver, splenic and lymphoid tissue (chapter 4). Also similar to other arteriviruses (Kimman et al., 2009; Sun et al., 2012), it appears that WPDV may be able to evade the immune system. This is evidenced by high levels of WPDV RNA in tissues and sera from possums euthanased nearly four weeks post infection, suggesting on-going virus replication (chapter 5), in the presence of immune response to viral antigens. This has been demonstrated both serologically and histologically. The former by demonstration of a serological response to WPDV nucleocapsid (N) protein as early as six days following experimental infection with WPDV (chapter 5), and the latter by the presence of mononuclear inflammation in multiple tissues from WPD affected possums as early as eight days post infection (chapter 4).

Previously, lack of a serological assay precluded investigation of WPDV seroprevalence in New Zealand. Utilizing indirect ELISA for a serological survey of archival possum serum samples, we demonstrated that WPDV or a related virus has circulated, both spatially and temporally in

wild possum populations in New Zealand, with an overall prevalence estimate of 30% (chapter 5). Additionally, demonstration of anti-N IgG by Western-blot in 5/15 (33%) of pre-infection sera from healthy possums suggested that these possums have survived prior infection with field WPDV. One possible explanation for this is the existence of different WPDV pathotypes. The possibility of different pathotypes of WPDV has already been suggested by others and several lines of evidence exist to support this notion (Dunowska et al., 2013; Perrott, 1998). Firstly, infectious inocula derived from the tissues of WPD-affected possums from two geographically distinct outbreaks resulted in disease that differed in severity when administered to healthy possums (Perrott, 1998). Secondly, different levels of WPDV RNA were later detected in archival tissue samples from these possums (Dunowska et al., 2013). Further investigation into pathogenicity of field WPDV is thus warranted.

Development of indirect ELISA opens possibilities for further research. WPD has not been reported in Australia, however, due to the presence of predators in Australia, it is possible that possums may perish at an earlier stage of disease than possums in New Zealand. Therefore the neurological signs consistent with WPD may go unnoticed. Alternatively Australia may be WPDV free or harbour less pathogenic field variants. Investigation into the presence of WPDV in Australian possum populations may provide clues as to the origin of the virus. Specifically, serological survey among Australian possums may help to determine if the virus was co-introduced to New Zealand along with possums from Australia in the 1800's, or alternatively, was transmitted to possums from another host species after possums had become established within New Zealand. The discovery of WPDV in Australia would allow for studies of genetic divergence between New Zealand and Australian viruses. Closely related possum species in Australia, such as the mountain brushtail possum (*Trichosurus cunninghami*) and the short-eared possum (*Trichosurus caninus*) that are not present in New Zealand should also be investigated using indirect ELISA. This may provide some information regarding species specificity and evolution of the virus.

On the basis of transmission potential, WPDV has been suggested as a potential biocide in New Zealand (Perrott, 1998), where possums are an introduced pest that have continued to cause substantial destruction of New Zealand's unique ecosystem (Brown et al., 1993; King, 1990). Whilst complete evaluation of WPDV as a biocide was outside the scope of this thesis, the research presented here provides several lines of evidence that supports suitability of Invermay-origin WPDV for such purpose. Firstly, this virus is highly pathogenic. Extrapolating from clinical signs observed during the experimental challenge study (chapter 3), infected possums would be unlikely to survive in the wild. Secondly, serological data from challenged possums suggests that pre-existing immunity from infection with field WPDV was not protective against development of WPD in a captive setting (chapter 5). However, the ultimate outcomes of infection in a field setting following natural transmission remain to be established. Further research is also required to understand interplay between the host (e.g. age of animal, pre-existing immunity), virus (e.g. dose, route of exposure) and environment (e.g. possum density, terrain) on disease outcome in individual possums and on transmission within possum populations.

Should large scale release of WPDV be considered, there must be ability to mass produce the virus. We have successfully grown WPDV within primary possum macrophages (chapter 2). Unfortunately, these cultures require animal sacrifice to establish, and have short life span. Thus, generating sufficient virus stock in these culture systems would be expensive and time consuming. Unfortunately, attempts to grow WPDV in a number of commercial cell lines has so far been unsuccessful (Giles et al., 2015; Perrott, 1998). Consequently, further investigation into *in vitro* culture systems that support growth of WPDV is required. Should attempts to grow the virus within additional commercial cell lines also fail, investigation into cellular components that facilitate entry of WPDV into permissive cells could be investigated. This would enable genetic modification of cell lines to make them permissive to the virus, as has been described for the related arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV) (Delrue et al., 2010).

Importantly, to be considered as a biocide, WPDV must be species specific. One way this could be investigated is by the development of a non-species specific serological assay, such as blocking ELISA. Development of blocking ELISA for detection of WPDV antibody would enable testing of animals that share the same habitat as possums in the wild, including the only other marsupials present in New Zealand, the Dama wallaby (*Macropus eugenii*) and Bennetts wallaby (*Macropus rufogriseus rufogriseus)*. As such, it is one way that species specificity of WPDV could be explored. We have attempted to develop blocking ELISA using WPDV N protein as antigen and polyclonal rabbit anti-N sera as blocker/competitor (chapter 5). However, the initial results in the process of development of this assay were discouraging, as post-infection sera from experimentally infected possums did not appear to efficiently block reaction with the rabbit antisera, most likely due to differences between the range of epitopes recognised by rabbit and possum sera on rN antigen. Several additional options exist for development of a non-species specific serological assay going forward. Firstly, development of a monoclonal antibody-based blocking ELISA with the same rN protein as antigen. This may work better than the current blocking ELISA design due to the fact that monoclonal antibodies, unlike polyclonal sera, recognise one specific antigen. Alternatively, other WPDV antigens could be investigated for suitability for assay development. Extrapolating from other arteriviruses, these could include major and minor membrane glycoproteins, M protein and non-structural proteins (Brown et al., 2009; MacLachlan et al., 1998; Rascón-Castelo et al., 2015; Wagner et al., 2003). Suitability of viral proteins for assay development could be investigated by Western-blot analysis of extracts from the purified virus or virus-infected tissues/cultures using sera from WPDV infected possums as probes. Alternatively, newer generation immunoassay techniques such as the BLItz® (Pall ForteBio) system may allow for more rapid characterization of immunoglobulin binding affinity to different WPDV antigens.

Aside from biological control, further investigation into WPDV is interesting from a virus evolution perspective. This is because the results of recent phylogenetic analysis have placed WPDV in a basal position in the arteriviral phylogenetic tree, and as such, WPDV is currently

the closest known member to ancestral arterivirus (Gulyaeva et al., 2017; Kuhn et al., 2016). Further investigation is especially compelling as the arterivirus family contains important animal pathogens, including PRRSV which is one of the most economically important pathogens of swine (Perez et al., 2015) . The sudden transcontinental appearance of PRRSV in the 1980's is somewhat of an enigma, although there is evidence to suggest that it was transmitted to pigs from another unknown host-species around this time (Hanada et al., 2005). There is also concern that another arterivirus member that affects primates, simian haemorrhagic fever virus (SHFV), shares features in common with emergent RNA viruses (Bailey et al., 2014; Lauck et al., 2013), flagging concern for future zoonotic potential. Thus, investigation into this marsupial arterivirus that could shed light on arterivirus evolution is warranted. Further investigation into WPDV could include investigation into the expression of structural genes, which would allow comparison to other arteri- and nidoviruses. Whilst arteri- and coronavirus structural proteins are primarily produced by the discontinuous synthesis and transcription of 5' and 3' coterminal sets of subgenomic-length mRNAs (sg mRNAs) (Sawicki et al., 2001; Sawicki and Sawicki, 1995; Sawicki et al., 2005), this does not appear to be a conserved feature of all families within the order *Nidovirales* (Pasternak et al., 2006). Expression of structural genes could be investigated by Northern-blot analysis of RNA extracted from WPDV infected cell culture lysate or tissue samples from infected possums.

There are limitations to the research presented in this thesis. Firstly, we have investigated WPDV from only one source. The existence of different pathotypes of WPDV has previously been hypothesized (Dunowska et al., 2013; Perrott, 1998), and whilst the data presented in this thesis provides further support, additional research is required to confirm this hypothesis. This could be achieved by genetic comparison of RNA from field WPDV and Rotorua-origin WPDV with the Invermay-origin purified cell culture isolate used in this study. Secondly, a lack of diagnostic sampling between experimental challenge with WPDV and euthanasia precluded evaluation of the kinetics of antibody development. Finally, the use of archival serum samples for the serological survey in chapter 5 made direct comparison of prevalence between

geographical locations difficult due to substantial differences in sample collection dates (years) for some areas. While there did appear to be marked differences in seroprevalence for areas that were temporally comparable, a prospective serological survey of New Zealand possums would provide better data to compare seroprevalence between different possum populations. Collection of additional data, such as population and ecological data could assist in understanding dynamics which govern spread of the virus in the wild.

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Appendix 1

Reagents

Gey's balanced salt solution (GBSS)

Calcium Chloride (CaCl2)	0.166 g
Potassium Chloride (KCl)	0.97 g
Potassium Phosphate Monobasic (KH2PO4)	0.03 g
Magnesium Chloride (MgCl2-6H20)	0.21 g
Magnesium Sulfate (MgSO4-7H2O)	0.07 g
Sodium Chloride (NaCl)	8.00 g
Sodium Bicarbonate (NaHCO3)	0.227 g
Sodium Phosphate Dibasic (Na2HPO4)	0.12 g
Glucose	1.00 g

Made to a total volume of 1 litre and sterilised by filtering.

Phosphate buffered sodium (PBS)

Distilled H_2O	800 mL
NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Following adjustment to desired pH (7.2-7.4), distilled water was added to a total volume of 1 litre.

Carbonate-bicarbonate buffer

1. Prepare a 0.2-M solution of anhydrous sodium carbonate (2.2 g/100 mL).
2. Prepare a 0.2-M solution of sodium bicarbonate (1.68 g/100 mL).
3. Combine 4 mL of carbonate solution from Step 1 and 46 mL of bicarbonate solution from Step 2.
4. Bring to 200 mL with H_2O.

Final pH was 9.2.

Appendix 2

WPDV RNA in purified macrophage culture

[a] Cq = quantification cycle
[b] Qty = Viral copies/µL of template cDNA
[c] = Time post infection of cell cultures (hours) with purified WPDV at an moi of 0.5.
[d] Sample wells comprised cell free media with purified virus, primary possum macrophages infected with purified WPDV, supernatant from infected culture wells, uninfected cells and supernatant from uninfected wells.

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121215	19.2690781	19.0630805	0.2913246	536310.5166	594947.9634	82925.87253	0.5	Virus + cell-free media
06121215	18.8570829	19.0630805	0.2913246	653585.4102	594947.9634	82925.87253	0.5	Virus + cell-free media
06121219	21.8926746	21.718566	0.2462267	152227.0261	166074.124	19582.75366	0.5	Infected cells
06121219	21.5444574	21.718566	0.2462267	179921.2219	166074.124	19582.75366	0.5	Infected cells
06121220	21.7573547	21.5598212	0.2793545	162443.0157	179402.664	23984.5647	0.5	Infected cells
06121220	21.3622878	21.5598212	0.2793545	196362.3124	179402.664	23984.5647	0.5	Infected cells
06121221	21.1684289	21.1590296	0.0132925	215511.6141	216488.3358	1381.293156	0.5	Infected cells
06121221	21.1496304	21.1590296	0.0132925	217465.0576	216488.3358	1381.293156	0.5	Infected cells
06121214	0	0	0	0	0	0	0.5	Uninfected cells
06121214	0	0	0	0	0	0	0.5	Uninfected cells
06121213	0	0	0	0	0	0	0.5	Uninfected supernatant
06121213	0	0	0	0	0	0	0.5	Uninfected supernatant
06121216	20.5840649	20.3238823	0.3679538	285291.8303	325766.2544	57239.47939	0.5	Infected supernatant
06121216	20.0636997	20.3238823	0.3679538	366240.6784	325766.2544	57239.47939	0.5	Infected supernatant
06121217	19.6321242	19.8300739	0.2799431	450541.0635	411553.7588	55136.37503	0.5	Infected supernatant
06121217	20.0280236	19.8300739	0.2799431	372566.4541	411553.7588	55136.37503	0.5	Infected supernatant
06121218	20.0483539	19.8868755	0.228365	368948.3811	399881.4762	43746.00259	0.5	Infected supernatant

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121218	19.7253971	19.8868755	0.228365	430814.5713	399881.4762	43746.00259	0.5	Infected supernatant
06121224	18.4762414	18.7567537	0.3967043	784682.4933	692057.7866	130991.1165	3	Virus + cell-free media
06121224	19.037266	18.7567537	0.3967043	599433.0798	692057.7866	130991.1165	3	Virus + cell-free media
06121228	22.9873073	23.0621528	0.1058475	90012.04201	86891.6932	4412.839598	3	Infected cells
06121228	23.1369983	23.0621528	0.1058475	83771.3444	86891.6932	4412.839598	3	Infected cells
06121229	22.0448937	22.0655091	0.0291547	141501.0357	140114.571	1960.757238	3	Infected cells
06121229	22.0861246	22.0655091	0.0291547	138728.1062	140114.571	1960.757238	3	Infected cells
06121230	22.4981906	22.4036944	0.1336378	113831.82	119236.5265	7643.409275	3	Infected cells
06121230	22.3091981	22.4036944	0.1336378	124641.233	119236.5265	7643.409275	3	Infected cells
06121223	0	0	0	0	0	0	3	Uninfected cells
06121223	0	0	0	0	0	0	3	Uninfected cells
06121222	0	0	0	0	0	0	3	Uninfected supernatant
06121222	0	0	0	0	0	0	3	Uninfected supernatant
06121225	19.8701808	20.1472192	0.3917914	401890.949	354964.1417	66364.52734	3	Infected supernatant
06121225	20.4242576	20.1472192	0.3917914	308037.3344	354964.1417	66364.52734	3	Infected supernatant
06121226	20.1389225	20.0392866	0.1409064	353252.5911	370981.5529	25072.53825	3	Infected supernatant
06121226	19.9396507	20.0392866	0.1409064	388710.5147	370981.5529	25072.53825	3	Infected supernatant
06121227	20.2932962	20.1270902	0.2350508	328022.8172	356395.8184	40125.48312	3	Infected supernatant
06121227	19.9608842	20.1270902	0.2350508	384768.8197	356395.8184	40125.48312	3	Infected supernatant
06121233	19.6797292	19.7093644	0.0419106	440362.652	434186.731	8734.071187	6	Virus + cell-free media
06121233	19.7389997	19.7093644	0.0419106	428010.8101	434186.731	8734.071187	6	Virus + cell-free media
06121237	22.3053234	22.0699629	0.33285	124873.2705	140701.6559	22384.71737	6	Infected cells
06121237	21.8346024	22.0699629	0.33285	156530.0413	140701.6559	22384.71737	6	Infected cells
06121238	22.1352214	22.1085387	0.037735	135496.9671	137254.8061	2485.959807	6	Infected cells
06121238	22.081856	22.1085387	0.037735	139012.6451	137254.8061	2485.959807	6	Infected cells
06121239	21.6044673	21.6855664	0.1146915	174812.5132	168265.5746	9258.769341	6	Infected cells
06121239	21.7666655	21.6855664	0.1146915	161718.636	168265.5746	9258.769341	6	Infected cells
06121232	0	0	0	0	0	0	6	Uninfected cells

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121232	0	0	0	0	0	0	6	Uninfected cells
06121231	0	0	0	0	0	0	6	Uninfected supernatant
06121231	0	0	0	0	0	0	6	Uninfected supernatant
06121234	20.3077171	20.3324336	0.0349544	325760.0429	321940.7039	5401.361132	6	Infected supernatant
06121234	20.3571501	20.3324336	0.0349544	318121.3648	321940.7039	5401.361132	6	Infected supernatant
06121235	20.392531	20.3443006	0.0682081	312764.3208	320175.3276	10480.74633	6	Infected supernatant
06121235	20.2960702	20.3443006	0.0682081	327586.3344	320175.3276	10480.74633	6	Infected supernatant
06121236	20.7616969	20.237035	0.7419839	261974.8002	347745.5299	121298.1293	6	Infected supernatant
06121236	19.7123732	20.237035	0.7419839	433516.2597	347745.5299	121298.1293	6	Infected supernatant
06121242	19.0032122	19.0459469	0.060436	609311.9285	597066.1029	17318.21265	9	Virus + cell-free media
06121242	19.0886816	19.0459469	0.060436	584820.2773	597066.1029	17318.21265	9	Virus + cell-free media
06121246	17.4452318	17.6984301	0.3580765	1287128.75	1148256.699	196394.7376	9	Infected cells
06121246	17.9516284	17.6984301	0.3580765	1009384.649	1148256.699	196394.7376	9	Infected cells
06121247	16.3986402	16.9038266	0.7144415	2127151.045	1718426.213	578024.2007	9	Infected cells
06121247	17.4090131	16.9038266	0.7144415	1309701.381	1718426.213	578024.2007	9	Infected cells
06121248	17.5981954	17.1175269	0.679768	1196009.519	1546660.175	495894.913	9	Infected cells
06121248	16.6368584	17.1175269	0.679768	1897310.83	1546660.175	495894.913	9	Infected cells
06121241	0	0	0	0	0	0	9	Uninfected cells
06121241	0	0	0	0	0	0	9	Uninfected cells
06121240	0	0	0	0	0	0	9	Uninfected supernatant
06121240	0	0	0	0	0	0	9	Uninfected supernatant
06232343	20.228891	20.3438746	0.1626115	338321.9705	320642.7519	25002.19074	9	Infected supernatant
06232343	20.4588583	20.3438746	0.1626115	302963.5333	320642.7519	25002.19074	9	Infected supernatant
06121244	26.4489528	26.3846689	0.090911	17087.29454	17631.15906	769.1405679	9	Infected supernatant
06121244	26.3203851	26.3846689	0.090911	18175.02357	17631.15906	769.1405679	9	Infected supernatant
06121245	20.8270032	21.2693458	0.6255669	253889.9586	209966.5229	62117.11841	9	Infected supernatant

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121245	21.7116884	21.2693458	0.6255669	166043.0872	209966.5229	62117.11841	9	Infected supernatant
06121251	19.4924445	19.3180332	0.2466547	481784.184	525691.6777	62094.57308	12	Virus + cell-free media
06121251	19.143622	19.3180332	0.2466547	569599.1714	525691.6777	62094.57308	12	Virus + cell-free media
06121255	14.4243056	14.7872481	0.5132782	5487510.241	4680287.206	1141585.764	12	Infected cells
06121255	15.1501906	14.7872481	0.5132782	3873064.171	4680287.206	1141585.764	12	Infected cells
06121256	14.8319868	14.5696729	0.3709678	4512203.057	5158282.264	913693.9762	12	Infected cells
06121256	14.3073591	14.5696729	0.3709678	5804361.47	5158282.264	913693.9762	12	Infected cells
06121257	14.1252727	14.3881216	0.3717244	6334507.305	5628153.759	998934.7646	12	Infected cells
06121257	14.6509704	14.3881216	0.3717244	4921800.213	5628153.759	998934.7646	12	Infected cells
06121250	0	0	0	0	0	0	12	Uninfected cells
06121250	0	0	0	0	0	0	12	Uninfected cells
06121249	0	0	0	0	0	0	12	Uninfected supernatant
06121249	0	0	0	0	0	0	12	Uninfected supernatant
06121252	22.0822313	22.1752975	0.1316154	138987.6032	133047.9998	8399.86771	12	Infected supernatant
06121252	22.2683637	22.1752975	0.1316154	127108.3964	133047.9998	8399.86771	12	Infected supernatant
06121253	20.3967192	20.3493167	0.0670373	312136.1745	319402.4336	10276.0422	12	Infected supernatant
06121253	20.3019142	20.3493167	0.0670373	326668.6927	319402.4336	10276.0422	12	Infected supernatant
06121254	20.3950921	20.6941718	0.4229625	312380.0579	273398.4514	55128.31655	12	Infected supernatant
06121254	20.9932514	20.6941718	0.4229625	234416.8449	273398.4514	55128.31655	12	Infected supernatant
06121260	19.502763	19.330719	0.2433069	479403.8372	522451.0934	60878.0135	18	Virus + cell-free media
06121260	19.158675	19.330719	0.2433069	565498.3496	522451.0934	60878.0135	18	Virus + cell-free media
06121264	13.9443864	13.7898809	0.2185038	6909093.445	7461448.142	781147.504	18	Infected cells
06121264	13.6353753	13.7898809	0.2185038	8013802.839	7461448.142	781147.504	18	Infected cells
06121265	14.0005928	14.2257051	0.3183568	6725182.451	6071651.15	924232.8293	18	Infected cells
06121265	14.4508174	14.2257051	0.3183568	5418119.849	6071651.15	924232.8293	18	Infected cells
06121266	13.3888456	13.410143	0.030119	9020510.863	8929232.058	129087.7239	18	Infected cells
06121266	13.4314403	13.410143	0.030119	8837953.253	8929232.058	129087.7239	18	Infected cells
06121259	0	0	0	0	0	0	18	Uninfected cells

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121259	0	0	0	0	0	0	18	Uninfected cells
06121258	0	0	0	0	0	0	18	Uninfected supernatant
06121258	0	0	0	0	0	0	18	Uninfected supernatant
06121261	17.7686932	17.6747331	0.1328797	1102026.5	1154040.006	73558.20643	18	Infected supernatant
06121261	17.580773	17.6747331	0.1328797	1206053.513	1154040.006	73558.20643	18	Infected supernatant
06121262	17.5018769	17.4443572	0.0813452	1252603.323	1288160.092	50284.86436	18	Infected supernatant
06121262	17.3868374	17.4443572	0.0813452	1323716.86	1288160.092	50284.86436	18	Infected supernatant
06121263	18.3697533	18.1831255	0.2639316	825834.1776	906855.8523	114581.9513	18	Infected supernatant
06121263	17.9964976	18.1831255	0.2639316	987877.5271	906855.8523	114581.9513	18	Infected supernatant
06121269	20.102054	19.5278092	0.8121048	359559.7736	491783.3847	186992.4241	24	Virus + cell-free media
06121269	18.9535644	19.5278092	0.8121048	624006.9958	491783.3847	186992.4241	24	Virus + cell-free media
06121273	13.0589552	13.4114482	0.4985003	10568206.78	9051205.138	2145364.291	24	Infected cells
06121273	13.7639412	13.4114482	0.4985003	7534203.5	9051205.138	2145364.291	24	Infected cells
06121274	12.523921	12.7631096	0.3382638	13662698.96	12261129.58	1982118.43	24	Infected cells
06121274	13.0022982	12.7631096	0.3382638	10859560.2	12261129.58	1982118.43	24	Infected cells
06121275	12.5866098	12.8557949	0.3806852	13257698.91	11748113.43	2134876.256	24	Infected cells
06121275	13.1249801	12.8557949	0.3806852	10238527.96	11748113.43	2134876.256	24	Infected cells
06121268	0	0	0	0	0	0	24	Uninfected cells
06121268	0	0	0	0	0	0	24	Uninfected cells
06121267	0	0	0	0	0	0	24	Uninfected supernatant
06121267	0	0	0	0	0	0	24	Uninfected supernatant
06121270	16.8485626	17.2201566	0.5255133	1713980.393	1456847.757	363640.4616	24	Infected supernatant
06121270	17.5917507	17.2201566	0.5255133	1199715.121	1456847.757	363640.4616	24	Infected supernatant
06121271	17.4187825	17.4088988	0.0139777	1303574.06	1309787.941	8787.755359	24	Infected supernatant
06121271	17.3990151	17.4088988	0.0139777	1316001.823	1309787.941	8787.755359	24	Infected supernatant
06121272	16.9795788	16.3383787	0.9067939	1609510.212	2294107.94	968167.3923	24	Infected supernatant

Sample ID#	Cq[a]	Cq Mean	Cq Std. Dev.	Qty.[b]	Qty. Mean	Qty. Std. Dev.	Time[c]	Sample type[d]
06121272	15.6971785	16.3383787	0.9067939	2978705.669	2294107.94	968167.3923	24	Infected supernatant
06121278	19.2291706	19.5161157	0.4058017	546682.9941	480866.4438	93078.65794	48	Virus + cell-free media
06121278	19.8030608	19.5161157	0.4058017	415049.8936	480866.4438	93078.65794	48	Virus + cell-free media
06121282	18.9985772	17.7911661	1.7075371	610669.0714	1278481.893	944429.9496	48	Infected cells
06121282	16.583755	17.7911661	1.7075371	1946294.715	1278481.893	944429.9496	48	Infected cells
06121283	18.8861471	18.2071237	0.9602842	644530.5537	940732.8693	418893.332	48	Infected cells
06121283	17.5281002	18.2071237	0.9602842	1236935.185	940732.8693	418893.332	48	Infected cells
06121284	17.7791224	17.8597715	0.114055	1096523.463	1055676.546	57766.26326	48	Infected cells
06121284	17.9404205	17.8597715	0.114055	1014829.63	1055676.546	57766.26326	48	Infected cells
06121277	0	0	0	0	0	0	48	Uninfected cells
06121277	0	0	0	0	0	0	48	Uninfected cells
06121276	0	0	0	0	0	0	48	Uninfected supernatant
06121276	0	0	0	0	0	0	48	Uninfected supernatant
06121279	15.7545117	16.1741011	0.593389	2897848.703	2417441.781	679397.9838	48	Infected supernatant
06121279	16.5936904	16.1741011	0.593389	1937034.86	2417441.781	679397.9838	48	Infected supernatant
06121280	15.1144746	15.4181584	0.4294737	3940036.053	3441842.871	704551.5544	48	Infected supernatant
06121280	15.7218421	15.4181584	0.4294737	2943649.689	3441842.871	704551.5544	48	Infected supernatant
06121281	16.0578126	16.0777153	0.0281466	2505235.13	2481528.851	33525.74156	48	Infected supernatant
06121281	16.0976179	16.0777153	0.0281466	2457822.572	2481528.851	33525.74156	48	Infected supernatant

Appendix 3

Quantity of Viral RNA in Tissues and Body Fluids From WPDV-Challenge Study

[a] Possums E1 & E3 = uninfected controls, Possums E5-E16 = WPDV-infected
[b] Qty. = Quantity of viral RNA/µL template cDNA
[c] = Concentration of extracted RNA determined by NanoDrop™.
[d] Viral copy numbers/µg RNA
[e] Copies/ng RNA
[f] Amount of RNA per qPCR reaction
[g] Sampling date, days post-inoculation with WPDV (possums E5-E16) or nothing (possums E1, E2) or control inoculum (possums E3, E4).

Possum ID#[a]	Tissue Type	Qty.[b]	Qty. Mean	RNA concentration (ng/uL)[c]	RNA for cDNA synthesis (uL)	copies/ug[d]	copies/ng RNA[e]	average copies/ug RNA	RNA/RT rxn [ng/uL][f]	Days post inoculation[g]
E1	Bladder	0	0	50	8.0	0.00	0.00	0.00	40	42
E1	Bladder	0		50	8.0	0.00	0.00		40	42
E3	Bladder	0	0	48.7	8.0	0.00	0.00	0.00	38.96	41
E3	Bladder	0		48.7	8.0	0.00	0.00		38.96	41
E5	Bladder	805.72	751.94	75.9	8.0	13269.43	13.27	12383.73	60.72	20
E5	Bladder	698.16		75.9	8.0	11498.02	11.50		60.72	20
E7	Bladder	666.6	669.435	17.2	8.0	48444.77	48.44	48650.80	13.76	20
E7	Bladder	672.27		17.2	8.0	48856.83	48.86		13.76	20
E8	Bladder	2052.08	2201.255	42	8.0	61073.81	61.07	65513.54	33.6	27
E8	Bladder	2350.43		42	8.0	69953.27	69.95		33.6	27
E9	Bladder	1986.25	1784.6	68.1	8.0	36458.33	36.46	32756.98	54.48	19
E9	Bladder	1582.95		68.1	8.0	29055.62	29.06		54.48	19
E10	Bladder	326.79	344.565	10.2	8.0	40047.79	40.05	42226.10	8.16	9
E10	Bladder	362.34		10.2	8.0	44404.41	44.40		8.16	9
E12	Bladder	1484.45	1331.68	33.6	8.0	55225.07	55.23	49541.67	26.88	27

Possum ID#[a]	Tissue Type	Qty.[b]	Qty. Mean	RNA concentration (ng/uL)[c]	RNA for cDNA synthesis (uL)	copies/ug[d]	copies/ng RNA[e]	average copies/ug RNA	RNA/RT rxn [ng/uL][f]	Days post inoculation[g]
E12	Bladder	1178.91		33.6	8.0	43858.26	43.86		26.88	27
E13	Bladder	392.88	343.605	37.5	8.0	13096.00	13.10	11453.50	30	23
E13	Bladder	294.33		37.5	8.0	9811.00	9.81		30	23
E14	Bladder	341.35	328.31	26.6	8.0	16040.88	16.04	15428.10	21.28	9
E14	Bladder	315.27		26.6	8.0	14815.32	14.82		21.28	9
E15	Bladder	646.58	651.68	43.9	8.0	18410.59	18.41	18555.81	35.12	6
E15	Bladder	656.78		43.9	8.0	18701.03	18.70		35.12	6
E16	Bladder	83.36	93.815	15.9	8.0	6553.46	6.55	7375.39	12.72	20
E16	Bladder	104.27		15.9	8.0	8197.33	8.20		12.72	20
E1	HindBrain	0	0	77	8.0	0.00	0.00	0.00	61.6	42
E1	HindBrain	0		77	8.0	0.00	0.00		61.6	42
E3	HindBrain	0	0	51.4	8.0	0.00	0.00	0.00	41.12	41
E3	HindBrain	0		51.4	8.0	0.00	0.00		41.12	41
E5	HindBrain	2.52	3.8	11.9	8.0	264.71	0.26	399.16	9.52	20
E5	HindBrain	5.08		11.9	8.0	533.61	0.53		9.52	20
E7	HindBrain	7.55	4.935	11.6	8.0	813.58	0.81	531.79	9.28	20
E7	HindBrain	2.32		11.6	8.0	250.00	0.25		9.28	20
E8	HindBrain	0	0	48.9	8.0	0.00	0.00	0.00	39.12	27
E8	HindBrain	0		48.9	8.0	0.00	0.00		39.12	27
E9	HindBrain	151.62	147.87	165.7	6.0	1525.05	1.53	1487.33	99.42	19
E9	HindBrain	144.12		165.7	6.0	1449.61	1.45		99.42	19
E10	HindBrain	72.43	70.365	90.6	8.0	999.31	1.00	970.82	72.48	9
E10	HindBrain	68.3		90.6	8.0	942.33	0.94		72.48	9
E12	HindBrain	5.28	5.71	34.6	8.0	190.75	0.19	206.29	27.68	27
E12	HindBrain	6.14		34.6	8.0	221.82	0.22		27.68	27

Possum ID#[a]	Tissue Type	Qty.[b]	Qty. Mean	RNA concentration (ng/uL)[c]	RNA for cDNA synthesis (uL)	copies/ug[d]	copies/ng RNA[e]	average copies/ug RNA	RNA/RT rxn [ng/uL][f]	Days post inoculation[g]
E13	HindBrain	31.62	29.44	25.6	8.0	1543.95	1.54	1437.50	20.48	23
E13	HindBrain	27.26		25.6	8.0	1331.05	1.33		20.48	23
E14	HIndBrain	42.86	38.375	63.5	8.0	843.70	0.84	755.41	50.8	9
E14	HindBrain	33.89		63.5	8.0	667.13	0.67		50.8	9
E15	HindBrain	2.76	2.685	107.1	8.0	32.21	0.03	31.34	85.68	6
E15	HindBrain	2.61		107.1	8.0	30.46	0.03		85.68	6
E16	HindBrain	32.87	33.585	72.5	8.0	566.72	0.57	579.05	58	20
E16	HindBrain	34.3		72.5	8.0	591.38	0.59		58	20
E1	Kidney	0	0	175.2	5.0	0.00	0.00	0.00	87.6	42
E1	Kidney	0		175.2	5.0	0.00	0.00		87.6	42
E3	Kidney	0	0	400.4	2.0	0.00	0.00	0.00	80.08	41
E3	Kidney	0		400.4	2.0	0.00	0.00		80.08	41
E5	Kidney	711.63	702.3	97.2	8.0	9151.62	9.15	9031.64	77.76	20
E5	Kidney	692.97		97.2	8.0	8911.65	8.91		77.76	20
E7	Kidney	1456	1477.615	509.9	1.5	19036.41	19.04	19319.02	76.485	20
E7	Kidney	1499.23		509.9	1.5	19601.62	19.60		76.485	20
E8	Kidney	4133.85	4082.755	215.2	4.0	48023.35	48.02	47429.77	86.08	27
E8	Kidney	4031.66		215.2	4.0	46836.20	46.84		86.08	27
E9	Kidney	1809.37	1779.315	384.9	2.5	18803.53	18.80	18491.19	96.225	19
E9	Kidney	1749.26		384.9	2.5	18178.85	18.18		96.225	19
E10	Kidney	761.55	787.78	460.7	2.0	8265.14	8.27	8549.82	92.14	9
E10	Kidney	814.01		460.7	2.0	8834.49	8.83		92.14	9
E12	Kidney	8943.61	8885.24	508.7	1.5	117208.70	117.21	116443.75	76.305	27
E12	Kidney	8826.87		508.7	1.5	115678.79	115.68		76.305	27
E13	Kidney	550.11	549.535	370.8	2.1	7064.65	7.06	6493.27	77.868	23

Possum ID#[a]	Tissue Type	Qty.[b]	Qty. Mean	RNA concentration (ng/uL)[c]	RNA for cDNA synthesis (uL)	copies/ug[d]	copies/ng RNA[e]	average copies/ug RNA	RNA/RT rxn [ng/uL][f]	Days post inoculation[g]
E13	Kidney	548.96		370.8	2.5	5921.90	5.92		92.7	23
E14	Kidney	121.3	122.38	345.1	2.5	1405.97	1.41	1418.49	86.275	9
E14	Kidney	123.46		345.1	2.5	1431.01	1.43		86.275	9
E15	Kidney	1640.53	1469.6	132	7.0	17754.65	17.75	15904.76	92.4	6
E15	Kidney	1298.67		132	7.0	14054.87	14.05		92.4	6
E16	Kidney	2647.29	2742.36	149.3	6.5	27278.99	27.28	28258.64	97.045	20
E16	Kidney	2837.43		149.3	6.5	29238.29	29.24		97.045	20
E1	Liver	0	0	247	4.0	0.00	0.00	0.00	98.8	42
E1	Liver	0		247	7.0	0.00	0.00		172.9	42
E3	Liver	0	0	329.2	3.0	0.00	0.00	0.00	98.76	41
E3	Liver	0		329.2	3.0	0.00	0.00		98.76	41
E5	Liver	388655.26	400017.385	98.6	8.0	4927171.15	4927.17	5071214.31	78.88	20
E5	Liver	411379.51		98.6	8.0	5215257.48	5215.26		78.88	20
E6	Liver	54129.13	54745.73	357.8	2.5	605132.81	605.13	612026.05	89.45	8
E6	Liver	55362.33		357.8	2.5	618919.28	618.92		89.45	8
E7	Liver	779589.38	745627.095	260.9	3.5	8537363.85	8537.36	8165439.36	91.315	20
E7	Liver	711664.81		260.9	3.5	7793514.87	7793.51		91.315	20
E8	Liver	580282	529719.365	221.2	4.5	5829636.33	5829.64	5321673.35	99.54	27
E8	Liver	479156.73		221.2	4.5	4813710.37	4813.71		99.54	27
E9	Liver	190688.81	192302.415	323	3.0	1967892.78	1967.89	1984545.05	96.9	19
E9	Liver	193916.02		323	3.0	2001197.32	2001.20		96.9	19
E10	Liver	29763.53	29796.82	330.8	3.0	299914.65	299.91	300250.10	99.24	9
E10	Liver	29830.11		330.8	3.0	300585.55	300.59		99.24	9
E11	Liver	248614.7	243497.61	284	3.5	2501153.92	2501.15	2449674.14	99.4	22
E11	Liver	238380.52		284	3.5	2398194.37	2398.19		99.4	22

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E12	Liver	1739034.24	1691957.77	441.1	2.0	19712471.55	19712.47	19178845.73	88.22	27
E12	Liver	1644881.3		441.1	2.0	18645219.90	18645.22		88.22	27
E13	Liver	145890.41	144426.595	322.8	3.0	1506509.81	1506.51	1491394.00	96.84	23
E13	Liver	142962.78		322.8	3.0	1476278.19	1476.28		96.84	23
E14	Liver	23845.25	23959.96	255.1	3.5	267068.94	267.07	268353.70	89.285	9
E14	Liver	24074.67		255.1	3.5	269638.46	269.64		89.285	9
E15	Liver	33044.91	33994.585	57.6	8.0	717120.44	717.12	737729.71	46.08	6
E15	Liver	34944.26		57.6	8.0	758338.98	758.34		46.08	6
E16	Liver	118245.22	115698.22	124.6	8.0	1186248.19	1186.25	1160696.43	99.68	20
E16	Liver	113151.22		124.6	8.0	1135144.66	1135.14		99.68	20
E1	Lymph Node	0	0	509.2	1.5	0.00	0.00	0.00	76.38	42
E1	Lymph Node	0		509.2	1.5	0.00	0.00		76.38	42
E3	Lymph Node	0	0	35.8	8.0	0.00	0.00	0.00	28.64	41
E3	Lymph Node	0		35.8	8.0	0.00	0.00		28.64	41
E5	Lymph Node	105174.55	103391.66	141	7.0	1065598.28	1065.60	1047534.55	98.7	20
E5	Lymph Node	101608.77		141	7.0	1029470.82	1029.47		98.7	20
E7	Lymph Node	292918.32	285322.99	248.7	4.0	2944494.57	2944.49	2868144.25	99.48	20
E7	Lymph Node	277727.66		248.7	4.0	2791793.93	2791.79		99.48	20
E8	Lymph Node	10108.81	7849.525	303.7	3.0	110951.71	110.95	86154.37	91.11	27
E8	Lymph Node	5590.24		303.7	3.0	61357.04	61.36		91.11	27

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E9	Lymph Node	4159179.87	2738058.83	66.8	8.0	77828964.63	77828.96	51236130.80	53.44	19
E9	Lymph Node	1316937.79		66.8	8.0	24643296.97	24643.30		53.44	19
E10	Lymph Node	27467.05	27512.105	270.2	3.5	290441.47	290.44	290917.89	94.57	9
E10	Lymph Node	27557.16		270.2	3.5	291394.31	291.39		94.57	9
E12	Lymph Node	10926.57	11772.51	73.5	8.0	185826.02	185.83	200212.76	58.8	27
E12	Lymph Node	12618.45		73.5	8.0	214599.49	214.60		58.8	27
E13	Lymph Node	50520.03	49006.485	155.4	6.0	541827.86	541.83	525595.08	93.24	23
E13	Lymph Node	47492.94		155.4	6.0	509362.29	509.36		93.24	23
E15	Lymph Node	17374.84	12401.535	126	7.0	196993.65	196.99	140606.97	88.2	6
E15	Lymph Node	7428.23		126	7.0	84220.29	84.22		88.2	6
E16	Lymph Node	702781.09	1180856.79	161	6.0	7275166.56	7275.17	12224190.37	96.6	20
E16	Lymph Node	1658932.49		161	6.0	17173214.18	17173.21		96.6	20
E1	Salivary	0	0	270.3	3.5	0.00	0.00	0.00	94.605	42
E1	Salivary	0		270.3	3.5	0.00	0.00		94.605	42
E3	Salivary	0	0	51.62	8.0	0.00	0.00	0.00	41.296	41
E3	Salivary	0		51.62	8.0	0.00	0.00		41.296	41
E5	Salivary	25062.53	23999.4	256.4	3.5	279279.36	279.28	267432.58	89.74	20
E5	Salivary	22936.27		256.4	3.5	255585.80	255.59		89.74	20

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E7	Salivary	30596.29	27888.775	185.7	5.0	329523.86	329.52	300363.76	92.85	20
E7	Salivary	25181.26		185.7	5.0	271203.66	271.20		92.85	20
E8	Salivary	30589.16	30016.09	592.1	1.5	344414.34	344.41	337961.94	88.815	27
E8	Salivary	29443.02		592.1	1.5	331509.54	331.51		88.815	27
E9	Salivary	1694.48	1589.13	395.5	2.5	17137.60	17.14	16072.11	98.875	19
E9	Salivary	1483.78		395.5	2.5	15006.62	15.01		98.875	19
E10	Salivary	10581.37	11508.455	198.2	5.0	106774.67	106.77	116129.72	99.1	9
E10	Salivary	12435.54		198.2	5.0	125484.76	125.48		99.1	9
E12	Salivary	29763.51	30057.855	261.4	3.5	325319.82	325.32	328537.05	91.49	27
E12	Salivary	30352.2		261.4	3.5	331754.29	331.75		91.49	27
E13	Salivary	3101.32	3710.595	299.9	3.0	34470.60	34.47	41242.58	89.97	23
E13	Salivary	4319.87		299.9	3.0	48014.56	48.01		89.97	23
E14	Salivary	7904.24	8332.31	367.5	2.5	86032.54	86.03	92025.39	91.875	9
E14	Salivary	8760.38		357.5	2.5	98018.24	98.02		89.375	9
E15	Salivary	7282.43	7291.205	243.4	4.0	74798.99	74.80	74889.12	97.36	6
E15	Salivary	7299.98		243.4	4.0	74979.25	74.98		97.36	6
E16	Salivary	3735.45	3498.66	133.7	7.0	39912.92	39.91	37382.84	93.59	20
E16	Salivary	3261.87		133.7	7.0	34852.76	34.85		93.59	20
E1	Serum	0	0	1.5	8.0	0.00	0.00	0.00	1.2	42
E1	Serum	0		1.5	8.0	0.00	0.00		1.2	42
E3	Serum	0	0	1.3	8.0	0.00	0.00	0.00	1.04	41
E3	Serum	0		1.3	8.0	0.00	0.00		1.04	41
E5	Serum	9318.96	9679.355	0.6	8.0	19414500.00	19414.50	20165322.92	0.48	20
E5	Serum	10039.75		0.6	8.0	20916145.83	20916.15		0.48	20
E6	Serum	0	0	4.02	8.0	0.00	0.00	0.00	3.216	8

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E6	Serum	0		4.02	8.0	0.00	0.00		3.216	8
E7	Serum	21887.65	22581.055	0	8.0	0	0	0	0	20
E7	Serum	23274.46		0	8.0	0	0		0	20
E8	Serum	134338.97	133188.02	1.1	8.0	152657920.45	152657.92	151350022.73	0.88	27
E8	Serum	132037.07		1.1	8.0	150042125.00	150042.13		0.88	27
E9	Serum	10062.2	9809.27	0.8	8.0	15722187.50	15722.19	15326984.38	0.64	19
E9	Serum	9556.34		0.8	8.0	14931781.25	14931.78		0.64	19
E10	Serum	525.3	545.425	6	8.0	109437.50	109.44	113630.21	4.8	9
E10	Serum	565.55		6	8.0	117822.92	117.82		4.8	9
E11	Serum	14836.41	14366.415	0.9	8.0	20606125.00	20606.13	19953354.17	0.72	22
E11	Serum	13896.42		0.9	8.0	19300583.33	19300.58		0.72	22
E12	Serum	2747883.55	3065938.81	1.1	8.0	0.00	3122594.94	3484021375	0.88	27
E12	Serum	3383994.07		1.1	8.0	0.00	3845447.81		0.88	27
E13	Serum	2952.34	3033.915	0.8	8.0	4613031.25	4613.03	4740492.19	0.64	23
E13	Serum	3115.49		0.8	8.0	4867953.13	4867.95		0.64	23
E14	Serum	7268.08	7107.51	0.4	8.0	0.00	22712.75	22210968.75	0.32	9
E14	Serum	6946.94		0.4	8.0	0.00	21709.19		0.32	9
E15	Serum	28059.29	26941.475	14.5	8.0	0.00	2418.90	2322540.95	11.6	6
E15	Serum	25823.66		14.5	8.0	0.00	2226.18		11.6	6
E16	Serum	16478.55	16073.79	1.1	8.0	0.00	18725.63	18265670.45	0.88	20
E16	Serum	15669.03		1.1	8.0	0.00	17805.72		0.88	20
E1	Spinal Cord	0	0	6.6	8.0	0.00	0.00	0.00	5.28	42
E1	Spinal Cord	0		6.6	8.0	0.00	0.00		5.28	42
E3	Spinal Cord	0	0	34.6	8.0	0.00	0.00	0.00	27.68	41
E3	Spinal Cord	0		34.6	8.0	0.00	0.00		27.68	41

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E5	Spinal Cord	3.02	2.68	31.9	8.0	118.34	0.12	105.02	25.52	20
E5	Spinal Cord	2.34		31.9	8.0	91.69	0.09		25.52	20
E7	Spinal Cord	0	0	24.8	8.0	0.00	0.00	0.00	19.84	20
E7	Spinal Cord	0		24.8	8.0	0.00	0.00		19.84	20
E8	Spinal Cord	6.25	7.835	10.4	8.0	751.20	0.75	941.71	8.32	27
E8	Spinal Cord	9.42		10.4	8.0	1132.21	1.13		8.32	27
E9	Spinal Cord	6.88	7.775	20	8.0	430.00	0.43	485.94	16	19
E9	Spinal Cord	8.67		20	8.0	541.88	0.54		16	19
E10	Spinal Cord	4.62	5.16	18.3	8.0	315.57	0.32	352.46	14.64	9
E10	Spinal Cord	5.7		18.3	8.0	389.34	0.39		14.64	9
E12	Spinal Cord	9.38	9.685	28.6	8.0	409.97	0.41	423.30	22.88	27
E12	Spinal Cord	9.99		28.6	8.0	436.63	0.44		22.88	27
E13	Spinal Cord	0.17	0.42	17.9	8.0	11.87	0.01	29.33	14.32	23
E13	Spinal Cord	0.67		17.9	8.0	46.79	0.05		14.32	23
E14	Spinal Cord	0.71	0.645	31.3	8.0	28.35	0.03	25.76	25.04	9
E14	Spinal Cord	0.58		31.3	8.0	23.16	0.02		25.04	9
E15	Spinal Cord	1.41	1.46	20.6	8.0	85.56	0.09	88.59	16.48	6
E15	Spinal Cord	1.51		20.6	8.0	91.63	0.09		16.48	6
E16	Spinal Cord	20.28	17.76	15.5	8.0	1635.48	1.64	1432.26	12.4	20
E16	Spinal Cord	15.24		15.5	8.0	1229.03	1.23		12.4	20
E1	Spleen	0	0	38.7	8.0	0.00	0.00	0.00	30.96	42
E1	Spleen	0		38.7	8.0	0.00	0.00		30.96	42
E3	Spleen	0	0	91.7	8.0	0.00	0.00	0.00	73.36	41
E3	Spleen	0		91.7	8.0	0.00	0.00		73.36	41
E5	Spleen	300013.45	300367.225	222	4.5	3003137.64	3003.14	3006678.93	99.9	20

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E5	Spleen	300721		222	4.5	3010220.22	3010.22		99.9	20
E6	Spleen	470316.51	474566.915	318.8	3.0	4917571.20	4917.57	4962012.91	95.64	8
E6	Spleen	478817.32		318.8	3.0	5006454.62	5006.45		95.64	8
E7	Spleen	132616.08	115929.81	53.5	8.0	3098506.54	3098.51	2708640.42	42.8	20
E7	Spleen	99243.54		53.5	8.0	2318774.30	2318.77		42.8	20
E8	Spleen	710872.3	700880.8	291.2	3.0	8137274.50	8137.27	8022902.87	87.36	27
E8	Spleen	690889.29		291.2	3.0	7908531.25	7908.53		87.36	27
E9	Spleen	674385.51	644144.485	188.1	5.0	7170499.84	7170.50	6848957.84	94.05	19
E9	Spleen	613903.46		188.1	5.0	6527415.84	6527.42		94.05	19
E10	Spleen	41721.8	42204.34	352.8	2.5	473036.28	473.04	478507.26	88.2	9
E10	Spleen	42686.88		352.8	2.5	483978.23	483.98		88.2	9
E11	Spleen	156930.2	162331.535	123	8.0	1594819.11	1594.82	1649710.72	98.4	22
E11	Spleen	167732.87		123	8.0	1704602.34	1704.60		98.4	22
E12	Spleen	870064.53	884526.81	201.3	4.5	9604951.48	9604.95	9764605.73	90.585	27
E12	Spleen	898989.09		201.3	4.5	9924259.98	9924.26		90.585	27
E13	Spleen	165863.91	175209.62	97.5	8.0	2126460.38	2126.46	2246277.18	78	23
E13	Spleen	184555.33		97.5	8.0	2366093.97	2366.09		78	23
E14	Spleen	27045.52	31010.365	469.3	2.0	288147.45	288.15	330389.57	93.86	9
E14	Spleen	34975.21		469.3	2.0	372631.69	372.63		93.86	9
E15	Spleen	269092.55	267579.315	291.9	3.0	3072885.12	3072.89	3055604.83	87.57	6
E15	Spleen	266066.08		291.9	3.0	3038324.54	3038.32		87.57	6
E16	Spleen	768994.94	742412.155	247	4.0	7783349.60	7783.35	7514293.07	98.8	20
E16	Spleen	715829.37		247	4.0	7245236.54	7245.24		98.8	20
E1	Urine	0	0	21	8.0	0.00	0.00	0.00	16.8	42
E1	Urine	0		21	8.0	0.00	0.00		16.8	42

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E3	Urine	0	0	1.3	8.0	0.00	0.00	0.00	1.04	41
E3	Urine	0		1.3	8.0	0.00	0.00		1.04	41
E5	Urine	4.01	3.785	0.3	8.0	16708.33	16.71	15770.83	0.24	20
E5	Urine	3.56		0.3	8.0	14833.33	14.83		0.24	20
E7	Urine	50.59	48.67	1.1	8.0	57488.64	57.49	55306.82	0.88	20
E7	Urine	46.75		1.1	8.0	53125.00	53.13		0.88	20
E8	Urine	27.41	28.8	0.9	8.0	38069.44	38.07	40000.00	0.72	27
E8	Urine	30.19		0.9	8.0	41930.56	41.93		0.72	27
E9	Urine	2265.51	2322.535	33.7	8.0	84032.27	84.03	86147.44	26.96	19
E9	Urine	2379.56		33.7	8.0	88262.61	88.26		26.96	19
E12	Urine	901.85	951.155	20.7	8.0	54459.54	54.46	57436.90	16.56	27
E12	Urine	1000.46		20.7	8.0	60414.25	60.41		16.56	27
E13	Urine	3807.18	3488.26	1.4	8.0	3399267.86	3399.27	3114517.86	1.12	23
E13	Urine	3169.34		1.4	8.0	2829767.86	2829.77		1.12	23
E14	Urine	1235.24	1314.78	4.7	8.0	328521.28	328.52	349675.53	3.76	9
E14	Urine	1394.32		4.7	8.0	370829.79	370.83		3.76	9
E15	Urine	4.19	5.84	1.9	8.0	2756.58	2.76	3842.11	1.52	6
E15	Urine	7.49		1.9	8.0	4927.63	4.93		1.52	6
E1	ForeBrain	0	0	77.3	8.0	0	0	0	0	42
E1	ForeBrain	0		77.3	8.0	0	0		0	42
E3	ForeBrain	0	0	162.3	6.0	0	0	0	0	41
E3	ForeBrain	0		162.3	6.0	0	0		0	41
E5	ForeBrain	564.97	542.37	134.5	7.0	6000.74	6.00	5760.70	94.15	20
E5	ForeBrain	519.77		134.5	7.0	5520.66	5.52		94.15	20
E7	ForeBrain	4790.99	4839.03	118	6.0	67669.35	67.67	68347.88	70.8	20

Possum ID#[a]	Tissue Type	Qty.[b]	Qty. Mean	RNA concentration (ng/uL)[c]	RNA for cDNA synthesis (uL)	copies/ug[d]	copies/ng RNA[e]	average copies/ug RNA	RNA/RT rxn [ng/uL][f]	Days post inoculation[g]
E7	ForeBrain	4887.07		118	6.0	69026.41	69.03		70.8	20
E8	ForeBrain	90529.17	87439.805	186.5	5.0	970822.20	970.82	937692.28	93.25	27
E8	ForeBrain	84350.44		186.5	5.0	904562.36	904.56		93.25	27
E9	ForeBrain	1577.4	1502.66	49.9	8.0	39514.03	39.51	37641.78	39.92	19
E9	ForeBrain	1427.92		49.9	8.0	35769.54	35.77		39.92	19
E10	ForeBrain	0	0.03	7.5	8.0	0.00	0.00	5.00	6	9
E10	ForeBrain	0.06		7.5	8.0	10.00	0.01		6	9
E12	ForeBrain	2310.09	2298.105	76.8	8.0	37599.12	37.60	37404.05	61.44	27
E12	ForeBrain	2286.12		76.8	8.0	37208.98	37.21		61.44	27
E13	ForeBrain	9461.72	9471.23	93.5	8.0	126493.58	126.49	126620.72	74.8	23
E13	ForeBrain	9480.74		93.5	8.0	126747.86	126.75		74.8	23
E14	ForeBrain	55.64	46.78	23.2	8.0	2997.84	3.00	2520.47	18.56	9
E14	ForeBrain	37.92		23.2	8.0	2043.10	2.04		18.56	9
E15	ForeBrain	907.08	904.415	52.9	8.0	21433.84	21.43	21370.86	42.32	6
E15	ForeBrain	901.75		52.9	8.0	21307.89	21.31		42.32	6
E16	ForeBrain	9429.12	9483.02	62.6	8.0	188281.15	188.28	189357.43	50.08	20
E16	ForeBrain	9536.92		62.6	8.0	190433.71	190.43		50.08	20

Appendix 4

Statistical Analysis of Viral RNA Levels From Experimental Challenge Study

Statistical analysis kindly performed by Dr Patrick Morel, Institute of Veterinary, Animal and Biomedical Sciences, Massey University

1) Values were log transformed to be normally distributed
2) Two possums ID 6 and 11 were removed for the analysis as they had many missing data points.
3) Two values with a 0 were removed from the analysis.
4) Proc Genmod with a tissue fix effect and possum random effect was fitted to the data.

The SAS System

The Mixed Procedure

Model Information

Data Set	WORK.RNA
Dependent Variable	LRNA
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
ID	10	5 7 8 9 10 12 13 14 15 16

TISSUE	9	Bladder Forebrai Hindbrai Kidney Liver Lymphnod Salivary Spinalco Spleen

Dimensions

Covariance Parameters	2
Columns in X	10
Columns in Z	10
Subjects	1
Max Obs Per Subject	90

Number of Observations

Number of Observations Read	90
Number of Observations Used	87
Number of Observations Not Used	3

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	192.71950785	
1	2	186.78619253	0.00000091
2	1	186.78617269	0.00000000

Convergence criteria met

Covariance Parameter Estimates

Cov Parm	Estimate
ID	0.09862
Residual	0.4361

Fit Statistics

-2 Res Log Likelihood	186.8
AIC (smaller is better)	190.8
AICC (smaller is better)	190.9
BIC (smaller is better)	191.4

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TISSUE	8	69	46.25	<.0001

Least Squares Means

Effect	TISSUE	Estimate	Standard Error	DF	t Value	Pr > |t|
TISSUE	Bladder	4.3816	0.2312	69	18.95	<.0001
TISSUE	Forebrain	4.2605	0.2312	69	18.43	<.0001
TISSUE	Hindbrain	2.7122	0.2423	69	11.19	<.0001
TISSUE	Kidney	4.1800	0.2312	69	18.08	<.0001
TISSUE	Liver	6.2936	0.2312	69	27.22	<.0001
TISSUE	Lymph node	5.9407	0.2423	69	24.52	<.0001
TISSUE	Salivary gland	5.0248	0.2312	69	21.73	<.0001
TISSUE	Spinal column	2.3431	0.2423	69	9.67	<.0001
TISSUE	Spleen	6.4552	0.2312	69	27.92	<.0001

Differences of Least Squares Means

Effect	TISSUE	_TISSUE	Estimate	Standard Error	DF	t Value	Pr > |t|
TISSUE	Bladder	Forebrain	0.1210	0.2953	69	0.41	0.6832
TISSUE	Bladder	Hindbrain	1.6694	0.3041	69	5.49	<.0001
TISSUE	Bladder	Kidney	0.2015	0.2953	69	0.68	0.4972
TISSUE	Bladder	Liver	-1.9120	0.2953	69	-6.47	<.0001
TISSUE	Bladder	Lymph node	-1.5591	0.3041	69	-5.13	<.0001
TISSUE	Bladder	Salivary gland	-0.6432	0.2953	69	-2.18	0.0328
TISSUE	Bladder	Spinal column	2.0385	0.3041	69	6.70	<.0001
TISSUE	Bladder	Spleen	-2.0736	0.2953	69	-7.02	<.0001
TISSUE	Forebrain	Hindbrain	1.5484	0.3041	69	5.09	<.0001
TISSUE	Forebrain	Kidney	0.08050	0.2953	69	0.27	0.7860
TISSUE	Forebrain	Liver	-2.0331	0.2953	69	-6.88	<.0001
TISSUE	Forebrain	Lymph node	-1.6801	0.3041	69	-5.53	<.0001
TISSUE	Forebrain	Salivary gland	-0.7642	0.2953	69	-2.59	0.0118
TISSUE	Forebrain	Spinal column	1.9175	0.3041	69	6.31	<.0001
TISSUE	Forebrain	Spleen	-2.1946	0.2953	69	-7.43	<.0001
TISSUE	Hindbrain	Kidney	-1.4679	0.3041	69	-4.83	<.0001
TISSUE	Hindbrain	Liver	-3.5814	0.3041	69	-11.78	<.0001
TISSUE	Hindbrain	Lymph node	-3.2285	0.3127	69	-10.32	<.0001
TISSUE	Hindbrain	Salivary gland	-2.3126	0.3041	69	-7.61	<.0001
TISSUE	Hindbrain	Spinal column	0.3691	0.3127	69	1.18	0.2419
TISSUE	Hindbrain	Spleen	-3.7430	0.3041	69	-12.31	<.0001
TISSUE	Kidney	Liver	-2.1136	0.2953	69	-7.16	<.0001
TISSUE	Kidney	Lymph node	-1.7606	0.3041	69	-5.79	<.0001
TISSUE	Kidney	Salivary gland	-0.8447	0.2953	69	-2.86	0.0056
TISSUE	Kidney	Spinal column	1.8370	0.3041	69	6.04	<.0001
TISSUE	Kidney	Spleen	-2.2751	0.2953	69	-7.70	<.0001
TISSUE	Liver	Lymph node	0.3529	0.3041	69	1.16	0.2498
TISSUE	Liver	Salivary	1.2688	0.2953	69	4.30	<.0001

		gland					
TISSUE	**Liver**	**Spinal column**	3.9505	0.3041	69	12.99	<.0001
TISSUE	**Liver**	**Spleen**	-0.1616	0.2953	69	-0.55	0.5861
TISSUE	**Lymph node**	**Salivary gland**	0.9159	0.3041	69	3.01	0.0036
TISSUE	**Lymph node**	**Spinal column**	3.5976	0.3127	69	11.51	<.0001
TISSUE	**Lymph node**	**Spleen**	-0.5145	0.3041	69	-1.69	0.0951
TISSUE	**Salivary gland**	**Spinal column**	2.6817	0.3041	69	8.82	<.0001
TISSUE	**Salivary gland**	**Spleen**	-1.4304	0.2953	69	-4.84	<.0001
TISSUE	**Spinal column**	**Spleen**	-4.1121	0.3041	69	-13.52	<.0001

Appendix 5

Statistical Analysis of Renal Lesions From Experimental Challenge Study

Statistical analysis kindly performed by Dr Patrick Morel, Institute of Veterinary, Animal and Biomedical Sciences, Massey University

Kruskal-Wallis Test: Interstitial nephritis

```
Kruskal-Wallis Test on Interstitial nephritis

                     Ave
inoc      N  Median  Rank      Z
NINC      4  0.5000   4.3  -2.06
YINC     12  2.0000   9.9   2.06
Overall  16           8.5

H = 4.25  DF = 1  P = 0.039
H = 4.61  DF = 1  P = 0.032  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: Tubular lesions

```
Kruskal-Wallis Test on Tubular lesions

inoc      N       Median  Ave Rank      Z
NINC      4  0.000000000       3.5  -2.43
YINC     12  1.000000000      10.2   2.43
Overall  16                    8.5

H = 5.88  DF = 1  P = 0.015
H = 6.84  DF = 1  P = 0.009  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: Glomerular

```
Kruskal-Wallis Test on Glomerular

                          Ave
inoc      N       Median  Rank      Z
NINC      4  0.000000000   6.0  -1.21
YINC     12  0.000000000   9.3   1.21
Overall  16                8.5

H = 1.47  DF = 1  P = 0.225
H = 2.20  DF = 1  P = 0.138  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: Interstitial

```
Kruskal-Wallis Test on Interstitial

                          Ave
inoc      N       Median  Rank      Z
NINC      4  0.000000000   6.5  -0.97
YINC     12  0.000000000   9.2   0.97
Overall  16                8.5

H = 0.94  DF = 1  P = 0.332
H = 1.63  DF = 1  P = 0.201  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: Total score

```
Kruskal-Wallis Test on Total score

inoc      N  Median  Ave Rank      Z
NINC      4  0.5000       3.5  -2.43
YINC     12  3.5000      10.2   2.43
Overall  16               8.5

H = 5.88  DF = 1  P = 0.015
H = 6.01  DF = 1  P = 0.014  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: IHC

```
Kruskal-Wallis Test on IHC

inoc      N       Median  Ave Rank      Z
NINC      4  0.000000000       3.5  -2.43
YINC     12  1.000000000      10.2   2.43
Overall  16                    8.5

H = 5.88  DF = 1  P = 0.015
H = 8.33  DF = 1  P = 0.004  (adjusted for ties)

* NOTE * One or more small samples
```

Kruskal-Wallis Test: qPCR Leptospira

```
14 cases were used
2 cases contained missing values


Kruskal-Wallis Test on qPCR Leptospira

                           Ave
inoc      N       Median  Rank      Z
NINC      4  0.000000000   8.3   0.42
YINC     10  0.000000000   7.2  -0.42
Overall  14                7.5

H = 0.18  DF = 1  P = 0.671
H = 0.49  DF = 1  P = 0.485  (adjusted for ties)

* NOTE * One or more small samples
```

Appendix 6

Indirect ELISA and Western-Blot Data

[a] E1- E4 = uninfected controls and E5-E16 = WPDV-infected possums from experimental challenge study. All other samples = archival serum samples for serological survey
[b] Pre/post = samples collected prior to (pre) and after (post) experimental challenge with WPDV(possums E5-E16) or nothing/control inoculum (possums E1-E4)
[c] average OD450 of samples at 1:10 and
[d] 1:100 serum dilutions.
[e] ELISA plate number
[f] average OD450 of serum-free wells for respective ELISA plate
[g] Possum E12 was run as positive control on each plate at 1:10 and 1:100 serum dilutions
[h] Samples and positive controls were "corrected" by subtraction of OD450 of blank wells from the OD450 of sample wells for each serum dilution
[i] - = negative and + = positive for WPDV-antibody by Western blot, ? = equivocal
[j] Region where sample was collected from; Man = Manawatu, Wai = Waimea, Whi = Whiteman's Valley, Par = Paraparumu, Kai = Kaikoura

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
E1	Pre	17.07.13	0.4575	0.174	1	0.09	2.412	0.657	0.3675	0.084	2.322	0.567	-	Man
E1	Post	28.8.13	0.4825	0.1675	1	0.09	2.412	0.657	0.3925	0.0775	2.322	0.567	-	Man
E2	Pre	17.07.13	0.3365	0.1395	1	0.09	2.412	0.657	0.2465	0.0495	2.322	0.567	-	Man
E2	Post	28.8.13	0.402	0.191	1	0.09	2.412	0.657	0.312	0.101	2.322	0.567	-	Man
E3	Pre	17.07.13	0.342	0.1345	1	0.09	2.412	0.657	0.252	0.0445	2.322	0.567	-	Man
E3	Post	27.8.13	0.4045	0.1675	1	0.09	2.412	0.657	0.3145	0.0775	2.322	0.567	-	Man
E4	Pre	17.07.13	0.925	0.446	2	0.0755	1.6495	0.5965	0.8495	0.3705	1.574	0.521	-	Man
E4	Post	27.8.13	1.3355	0.5275	2	0.0755	1.6495	0.5965	1.26	0.452	1.574	0.521	-	Man
E5	Pre	17.07.13	0.6245	0.2365	1	0.09	2.412	0.657	0.5345	0.1465	2.322	0.567	+	Man
E5	Post	6.8.13	1.742	1.501	1	0.09	2.412	0.657	1.652	1.411	2.322	0.567	+	Man
E6	Pre	17.07.13	0.607	0.2475	1	0.09	2.412	0.657	0.517	0.1575	2.322	0.567		Man

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
E6	Post	23.7.13	3.2895	0.2925	1	0.09	2.412	0.657	3.1995	0.2025	2.322	0.567	-	Man
E7	Pre	17.07.13	0.5715	0.198	1	0.09	2.412	0.657	0.4815	0.108	2.322	0.567	-	Man
E7	Post	6.8.13	3.095	1.1875	1	0.09	2.412	0.657	3.005	1.0975	2.322	0.567	+	Man
E8	Pre	17.07.13	0.811	0.3395	1	0.09	2.412	0.657	0.721	0.2495	2.322	0.567	-	Man
E8	Post	13.8.13	2.596	0.7045	1	0.09	2.412	0.657	2.506	0.6145	2.322	0.567	+	Man
E9	Pre	17.07.13	0.4155	0.168	1	0.09	2.412	0.657	0.3255	0.078	2.322	0.567	-	Man
E9	Post	5.8.13	1.6045	0.291	1	0.09	2.412	0.657	1.5145	0.201	2.322	0.567	+	Man
E10	Pre	17.07.13	2.214	1.0995	1	0.09	2.412	0.657	2.124	1.0095	2.322	0.567	+	Man
E10	Post	26.7.13	2.2685	0.878	1	0.09	2.412	0.657	2.1785	0.788	2.322	0.567	+	Man
E11	Post	8.8.13	3.405	2.2305	1	0.09	2.412	0.657	3.315	2.1405	2.322	0.567	+	Man
E12	Pre	17.07.13	0.751	0.2275	1	0.09	2.412	0.657	0.661	0.1375	2.322	0.567	?	Man
E12	Post	13.8.13	2.412	0.657	1	0.09	2.412	0.657	2.322	0.567	2.322	0.567	+	Man
E13	Pre	17.07.13	0.705	0.4525	2	0.0755	1.6495	0.5965	0.6295	0.377	1.574	0.521	+	Man
E13	Post	9.08.13	2.0155	0.99	2	0.0755	1.6495	0.5965	1.94	0.9145	1.574	0.521	+	Man
E14	Pre	17.07.13	2.0285	0.8915	2	0.0755	1.6495	0.5965	1.953	0.816	1.574	0.521	+	Man
E14	Post	26.7.13	1.292	0.426	2	0.0755	1.6495	0.5965	1.2165	0.3505	1.574	0.521	+	Man
E15	Pre	17.07.13	0.935	0.3305	2	0.0755	1.6495	0.5965	0.8595	0.255	1.574	0.521	+	Man
E15	Post	23.7.13	1.3995	0.367	2	0.0755	1.6495	0.5965	1.324	0.2915	1.574	0.521		Man
E16	Pre	17.07.13	0.6735	0.2945	2	0.0755	1.6495	0.5965	0.598	0.219	1.574	0.521	+	Man
E16	Post	7.8.13	2.3545	0.9495	2	0.0755	1.6495	0.5965	2.279	0.874	1.574	0.521	+	Man
M48		31.03.13	0.763	0.248	5	0.078	3.5445	1.1765	0.685	0.17	3.4665	1.0985		Wai
M49		31.03.13	0.869	0.3565	5	0.078	3.5445	1.1765	0.791	0.2785	3.4665	1.0985		Wai
M50		31.03.13	0.215	0.1205	5	0.078	3.5445	1.1765	0.137	0.0425	3.4665	1.0985		Wai
M51		31.03.13	0.6735	0.202	5	0.078	3.5445	1.1765	0.5955	0.124	3.4665	1.0985		Wai
J2		31.03.13	1.832	0.7395	5	0.078	3.5445	1.1765	1.754	0.6615	3.4665	1.0985		Wai

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
J3		31.03.13	0.5315	0.1635	5	0.078	3.5445	1.1765	0.4535	0.0855	3.4665	1.0985		Wai
J5		31.03.13	0.3785	0.134	5	0.078	3.5445	1.1765	0.3005	0.056	3.4665	1.0985		Wai
J6		31.03.13	0.928	0.291	5	0.078	3.5445	1.1765	0.85	0.213	3.4665	1.0985		Wai
J7		31.03.13	0.797	0.1775	5	0.078	3.5445	1.1765	0.719	0.0995	3.4665	1.0985		Wai
J8		31.03.13	0.227	0.0975	5	0.078	3.5445	1.1765	0.149	0.0195	3.4665	1.0985		Wai
J9		31.03.13	0.692	0.2045	5	0.078	3.5445	1.1765	0.614	0.1265	3.4665	1.0985		Wai
J10		31.03.13	0.7425	0.2875	5	0.078	3.5445	1.1765	0.6645	0.2095	3.4665	1.0985		Wai
J12		31.03.13	0.6595	0.191	5	0.078	3.5445	1.1765	0.5815	0.113	3.4665	1.0985		Wai
J13		31.03.13	1.1825	0.6195	5	0.078	3.5445	1.1765	1.1045	0.5415	3.4665	1.0985		Wai
J14		31.03.13	0.4495	0.1485	5	0.078	3.5445	1.1765	0.3715	0.0705	3.4665	1.0985		Wai
J15		31.03.13	0.7855	0.204	5	0.078	3.5445	1.1765	0.7075	0.126	3.4665	1.0985		Wai
J16		31.03.13	0.255	0.134	5	0.078	3.5445	1.1765	0.177	0.056	3.4665	1.0985		Wai
J17		31.03.13	0.3765	0.151	5	0.078	3.5445	1.1765	0.2985	0.073	3.4665	1.0985		Wai
J18		31.03.13	0.3035	0.1105	5	0.078	3.5445	1.1765	0.2255	0.0325	3.4665	1.0985		Wai
J19		31.03.13	0.694	0.2115	5	0.078	3.5445	1.1765	0.616	0.1335	3.4665	1.0985		Wai
J21		31.03.13	0.3695	0.1345	5	0.078	3.5445	1.1765	0.2915	0.0565	3.4665	1.0985		Wai
1W		16.10.00	0.909	0.316	7	0.0645	3.0095	0.652	0.8445	0.2515	2.945	0.5875		Whi
3W		16.10.00	0.574	0.241	7	0.0645	3.0095	0.652	0.5095	0.1765	2.945	0.5875		Whi
4W		16.10.00	0.4245	0.1575	7	0.0645	3.0095	0.652	0.36	0.093	2.945	0.5875		Whi
5W		16.10.00	1.4445	0.8385	7	0.0645	3.0095	0.652	1.38	0.774	2.945	0.5875		Whi
6W		16.10.00	1.749	0.9325	7	0.0645	3.0095	0.652	1.6845	0.868	2.945	0.5875		Whi
7W		16.10.00	0.6665	0.2805	7	0.0645	3.0095	0.652	0.602	0.216	2.945	0.5875		Whi
8W		16.10.00	0.7905	0.2495	7	0.0645	3.0095	0.652	0.726	0.185	2.945	0.5875		Whi

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
9W		16.10.00	0.8685	0.2925	7	0.0645	3.0095	0.652	0.804	0.228	2.945	0.5875		Whi
10W		16.10.00	0.439	0.139	7	0.0645	3.0095	0.652	0.3745	0.0745	2.945	0.5875		Whi
11W		16.10.00	0.615	0.281	7	0.0645	3.0095	0.652	0.5505	0.2165	2.945	0.5875		Whi
12W		16.10.00	1.697	0.797	7	0.0645	3.0095	0.652	1.6325	0.7325	2.945	0.5875		Whi
13		16.10.00	1.1825	0.327	7	0.0645	3.0095	0.652	1.118	0.2625	2.945	0.5875		Whi
14		16.10.00	0.376	0.135	7	0.0645	3.0095	0.652	0.3115	0.0705	2.945	0.5875		Whi
15		16.10.00	0.779	0.374	7	0.0645	3.0095	0.652	0.7145	0.3095	2.945	0.5875		Whi
16		16.10.00	0.4645	0.1555	7	0.0645	3.0095	0.652	0.4	0.091	2.945	0.5875		Whi
17W		16.10.00	1.5565	0.8705	7	0.0645	3.0095	0.652	1.492	0.806	2.945	0.5875		Whi
18W		16.10.00	1.9685	0.468	7	0.0645	3.0095	0.652	1.904	0.4035	2.945	0.5875		Whi
19		16.10.00	0.5865	0.217	7	0.0645	3.0095	0.652	0.522	0.1525	2.945	0.5875		Whi
20		16.10.00	0.696	0.213	7	0.0645	3.0095	0.652	0.6315	0.1485	2.945	0.5875		Whi
690		?.11.00	0.223	0.11	7	0.0645	3.0095	0.652	0.1585	0.0455	2.945	0.5875	-	Whi
689		24.11.00	0.489	0.1705	7	0.0645	3.0095	0.652	0.4245	0.106	2.945	0.5875		Whi
691		15.11.00	0.6345	0.212	8	0.072	2.8565	0.746	0.5625	0.14	2.7845	0.674		Whi
692		16.11.00	0.618	0.264	8	0.072	2.8565	0.746	0.546	0.192	2.7845	0.674		Whi
693		20.11.00	0.6265	0.1865	8	0.072	2.8565	0.746	0.5545	0.1145	2.7845	0.674		Whi
694		21.11.00	0.4405	0.132	8	0.072	2.8565	0.746	0.3685	0.06	2.7845	0.674		Whi
21		16.10.00	1.227	0.5215	8	0.072	2.8565	0.746	1.155	0.4495	2.7845	0.674		Whi
22		16.10.00	0.948	0.3035	8	0.072	2.8565	0.746	0.876	0.2315	2.7845	0.674		Whi
699		28.11.00	0.2135	0.0945	8	0.072	2.8565	0.746	0.1415	0.0225	2.7845	0.674		Whi
695		24.11.00	0.384	0.176	8	0.072	2.8565	0.746	0.312	0.104	2.7845	0.674		Whi
6022		19.11.00	0.5005	0.213	8	0.072	2.8565	0.746	0.4285	0.141	2.7845	0.674		Whi
697		27.11.00	0.69	0.225	8	0.072	2.8565	0.746	0.618	0.153	2.7845	0.674		Whi
696		27.11.00	0.6355	0.1845	8	0.072	2.8565	0.746	0.5635	0.1125	2.7845	0.674		Whi

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
33T		29.11.00	0.68	0.202	8	0.072	2.8565	0.746	0.608	0.13	2.7845	0.674		Whi
B0		29.11.00	0.4045	0.1555	8	0.072	2.8565	0.746	0.3325	0.0835	2.7845	0.674		Whi
74T		29.11.00	0.6475	0.26	8	0.072	2.8565	0.746	0.5755	0.188	2.7845	0.674		Whi
3ET		29.11.00	0.778	0.193	8	0.072	2.8565	0.746	0.706	0.121	2.7845	0.674		Whi
CBT		29.11.00	0.6985	0.201	8	0.072	2.8565	0.746	0.6265	0.129	2.7845	0.674		Whi
CAT		29.11.00	0.2565	0.108	8	0.072	2.8565	0.746	0.1845	0.036	2.7845	0.674		Whi
B-25T		29.11.00	0.337	0.174	8	0.072	2.8565	0.746	0.265	0.102	2.7845	0.674		Whi
B7T		29.11.00	0.463	0.162	8	0.072	2.8565	0.746	0.391	0.09	2.7845	0.674		Whi
BOTSBR		29.11.00	0.3025	0.129	8	0.072	2.8565	0.746	0.2305	0.057	2.7845	0.674		Whi
26		30.06.00	0.4315	0.1325	10	0.063	2.652	0.709	0.3685	0.0695	2.589	0.646		Par
27		30.06.00	0.881	0.3495	10	0.063	2.652	0.709	0.818	0.2865	2.589	0.646		Par
28		30.06.00	0.7515	0.3145	10	0.063	2.652	0.709	0.6885	0.2515	2.589	0.646		Par
29		30.06.00	0.6375	0.2495	10	0.063	2.652	0.709	0.5745	0.1865	2.589	0.646		Par
30		30.06.00	1.163	0.4825	10	0.063	2.652	0.709	1.1	0.4195	2.589	0.646		Par
31		30.06.00	0.854	0.283	10	0.063	2.652	0.709	0.791	0.22	2.589	0.646		Par
32		30.06.00	0.5935	0.2015	10	0.063	2.652	0.709	0.5305	0.1385	2.589	0.646		Par
33		30.06.00	0.585	0.2535	10	0.063	2.652	0.709	0.522	0.1905	2.589	0.646		Par
34		30.06.00	0.7125	0.2605	10	0.063	2.652	0.709	0.6495	0.1975	2.589	0.646		Par
35		30.06.00	0.72	0.294	10	0.063	2.652	0.709	0.657	0.231	2.589	0.646	-	Par
36		30.06.00	0.4675	0.1765	10	0.063	2.652	0.709	0.4045	0.1135	2.589	0.646		Par
37		30.06.00	0.6135	0.233	10	0.063	2.652	0.709	0.5505	0.17	2.589	0.646		Par
38		30.06.00	1.445	0.443	10	0.063	2.652	0.709	1.382	0.38	2.589	0.646		Par
39		30.06.00	1.0035	0.674	10	0.063	2.652	0.709	0.9405	0.611	2.589	0.646		Par

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
40		30.06.00	0.733	0.3525	10	0.063	2.652	0.709	0.67	0.2895	2.589	0.646		Par
41		30.06.00	0.923	0.313	10	0.063	2.652	0.709	0.86	0.25	2.589	0.646		Par
42		30.06.00	0.4325	0.182	10	0.063	2.652	0.709	0.3695	0.119	2.589	0.646		Par
337		?.04.14	0.596	0.211	13	0.062	2.7765	0.971	0.534	0.149	2.7145	0.909		Kai
384		?.04.14	0.751	0.5465	13	0.062	2.7765	0.971	0.689	0.4845	2.7145	0.909		Kai
392		?.04.14	1.711	0.853	13	0.062	2.7765	0.971	1.649	0.791	2.7145	0.909		Kai
409		?.04.14	1.551	0.392	13	0.062	2.7765	0.971	1.489	0.33	2.7145	0.909		Kai
3108		?.04.14	1.6965	0.3415	13	0.062	2.7765	0.971	1.6345	0.2795	2.7145	0.909		Kai
4578		?.04.14	1.483	0.5285	13	0.062	2.7765	0.971	1.421	0.4665	2.7145	0.909		Kai
7404		?.04.14	1.468	0.6715	13	0.062	2.7765	0.971	1.406	0.6095	2.7145	0.909		Kai
7471		?.04.14	0.604	0.2495	13	0.062	2.7765	0.971	0.542	0.1875	2.7145	0.909		Kai
7616		?.04.14	0.871	0.379	13	0.062	2.7765	0.971	0.809	0.317	2.7145	0.909		Kai
7623		?.04.14	2.555	1.53	13	0.062	2.7765	0.971	2.493	1.468	2.7145	0.909		Kai
15s		?.04.14	1.5505	1.008	13	0.062	2.7765	0.971	1.4885	0.946	2.7145	0.909		Kai
1s		?.04.14	1.144	0.454	13	0.062	2.7765	0.971	1.082	0.392	2.7145	0.909		Kai
36s		?.04.14	1.8755	0.939	13	0.062	2.7765	0.971	1.8135	0.877	2.7145	0.909		Kai
possum 3		?.04.14	0.97	0.435	13	0.062	2.7765	0.971	0.908	0.373	2.7145	0.909		Kai
10A		30.06.00	1.6055	1.1255	11	0.0565	1.7665	0.8485	1.549	1.069	1.71	0.792		Par
10bry		26.06.00	0.6235	0.543	11	0.0565	1.7665	0.8485	0.567	0.4865	1.71	0.792		Par
11A		30.06.00	1.0175	0.382	11	0.0565	1.7665	0.8485	0.961	0.3255	1.71	0.792		Par
11bry		26.06.00	1.6995	1.044	11	0.0565	1.7665	0.8485	1.643	0.9875	1.71	0.792		Par
12A		30.06.00	0.65	0.299	11	0.0565	1.7665	0.8485	0.5935	0.2425	1.71	0.792		Par
14bry		26.06.00	0.7105	0.6805	11	0.0565	1.7665	0.8485	0.654	0.624	1.71	0.792		Par
15bry		26.06.00	1.0695	0.626	11	0.0565	1.7665	0.8485	1.013	0.5695	1.71	0.792		Par
17bry		26.06.00	1.008	1.14	11	0.0565	1.7665	0.8485	0.9515	1.0835	1.71	0.792		Par

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
18bry		26.06.00	1.7345	0.901	11	0.0565	1.7665	0.8485	1.678	0.8445	1.71	0.792		Par
19bry		26.06.00	0.6215	0.484	11	0.0565	1.7665	0.8485	0.565	0.4275	1.71	0.792		Par
1A		30.06.00	0.8995	0.2775	11	0.0565	1.7665	0.8485	0.843	0.221	1.71	0.792		Par
20bry		26.06.00	1.2665	0.7295	11	0.0565	1.7665	0.8485	1.21	0.673	1.71	0.792		Par
21 bry		26.06.00	0.7595	0.3965	11	0.0565	1.7665	0.8485	0.703	0.34	1.71	0.792		Par
22bry		30.06.00	1.001	0.413	11	0.0565	1.7665	0.8485	0.9445	0.3565	1.71	0.792		Par
23bry		30.06.00	1.0695	0.825	11	0.0565	1.7665	0.8485	1.013	0.7685	1.71	0.792		Par
25bry		30.06.00	2.1165	1.058	11	0.0565	1.7665	0.8485	2.06	1.0015	1.71	0.792		Par
2A		30.06.00	1.6	1.096	11	0.0565	1.7665	0.8485	1.5435	1.0395	1.71	0.792		Par
3A		30.06.00	0.8785	0.2755	11	0.0565	1.7665	0.8485	0.822	0.219	1.71	0.792		Par
4A		30.06.00	0.433	0.144	11	0.0565	1.7665	0.8485	0.3765	0.0875	1.71	0.792		Par
8A		30.06.00	1.4365	0.983	11	0.0565	1.7665	0.8485	1.38	0.9265	1.71	0.792		Par
9bry		26.06.00	1.319	0.855	11	0.0565	1.7665	0.8485	1.2625	0.7985	1.71	0.792		Par
20/03/15		20.03.15	0.539	0.1885	12	0.05775	1.878	0.941	0.48125	0.13075	1.82025	0.88325	-	Man
16.12.15		16.12.15	0.2745	0.109	12	0.05775	1.878	0.941	0.21675	0.05125	1.82025	0.88325		Man
26.3.15		26.03.15	0.847	0.5815	12	0.05775	1.878	0.941	0.78925	0.52375	1.82025	0.88325		Man
joey		10.10.15	1.0125	0.425	12	0.05775	1.878	0.941	0.95475	0.36725	1.82025	0.88325		Man
p26		18.02.15	1.1005	0.6075	12	0.05775	1.878	0.941	1.04275	0.54975	1.82025	0.88325		Man
p27		18.02.15	1.3565	0.5975	12	0.05775	1.878	0.941	1.29875	0.53975	1.82025	0.88325		Man
p30		18.02.15	1.0115	0.542	12	0.05775	1.878	0.941	0.95375	0.48425	1.82025	0.88325		Man
p32		18.02.15	1.11	0.463	12	0.05775	1.878	0.941	1.05225	0.40525	1.82025	0.88325		Man
p33		18.02.15	0.6515	0.1915	12	0.05775	1.878	0.941	0.59375	0.13375	1.82025	0.88325		Man
p34		18.02.15	0.608	0.1855	12	0.05775	1.878	0.941	0.55025	0.12775	1.82025	0.88325		Man

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
p35		27.02.15	1.034	0.4525	12	0.05775	1.878	0.941	0.97625	0.39475	1.82025	0.88325		Man
p37		27.02.15	0.6645	0.185	12	0.05775	1.878	0.941	0.60675	0.12725	1.82025	0.88325		Man
p40		27.02.15	0.9445	0.443	12	0.05775	1.878	0.941	0.88675	0.38525	1.82025	0.88325		Man
p41		27.02.15	0.7395	0.3395	12	0.05775	1.878	0.941	0.68175	0.28175	1.82025	0.88325		Man
p42		27.02.15	0.7805	0.384	12	0.05775	1.878	0.941	0.72275	0.32625	1.82025	0.88325		Man
p43		27.02.15	0.394	0.1465	12	0.05775	1.878	0.941	0.33625	0.08875	1.82025	0.88325		Man
p44		27.02.15	0.413	0.2595	12	0.05775	1.878	0.941	0.35525	0.20175	1.82025	0.88325		Man
p45		27.02.15	0.356	0.1185	12	0.05775	1.878	0.941	0.29825	0.06075	1.82025	0.88325		Man
p46		27.02.15	0.417	0.1975	12	0.05775	1.878	0.941	0.35925	0.13975	1.82025	0.88325		Man
p47		27.02.15	0.1965	0.0785	12	0.05775	1.878	0.941	0.13875	0.02075	1.82025	0.88325		Man
M1		31.03.13	0.6495	0.1525	3	0.06075	3.3085	1.2285	0.58875	0.09175	3.24775	1.16775		Wai
M10		31.03.13	0.3455	0.124	3	0.06075	3.3085	1.2285	0.28475	0.06325	3.24775	1.16775		Wai
M11		31.03.13	0.925	0.244	3	0.06075	3.3085	1.2285	0.86425	0.18325	3.24775	1.16775		Wai
M12		31.03.13	0.5615	0.2605	3	0.06075	3.3085	1.2285	0.50075	0.19975	3.24775	1.16775		Wai
M14		31.03.13	0.5475	0.204	3	0.06075	3.3085	1.2285	0.48675	0.14325	3.24775	1.16775		Wai
M15		31.03.13	0.85	0.2455	3	0.06075	3.3085	1.2285	0.78925	0.18475	3.24775	1.16775		Wai
M16		31.03.13	0.612	0.175	3	0.06075	3.3085	1.2285	0.55125	0.11425	3.24775	1.16775		Wai
M17		31.03.13	0.7125	0.252	3	0.06075	3.3085	1.2285	0.65175	0.19125	3.24775	1.16775		Wai
M18		31.03.13	0.861	0.2485	3	0.06075	3.3085	1.2285	0.80025	0.18775	3.24775	1.16775		Wai
M19		31.03.13	0.505	0.1685	3	0.06075	3.3085	1.2285	0.44425	0.10775	3.24775	1.16775		Wai
M2		31.03.13	0.5505	0.182	3	0.06075	3.3085	1.2285	0.48975	0.12125	3.24775	1.16775		Wai
M20		31.03.13	0.4705	0.154	3	0.06075	3.3085	1.2285	0.40975	0.09325	3.24775	1.16775		Wai
M3		31.03.13	0.8775	0.2055	3	0.06075	3.3085	1.2285	0.81675	0.14475	3.24775	1.16775		Wai
M4		31.03.13	0.445	0.1415	3	0.06075	3.3085	1.2285	0.38425	0.08075	3.24775	1.16775		Wai
M5		31.03.13	1.1715	0.2655	3	0.06075	3.3085	1.2285	1.11075	0.20475	3.24775	1.16775		Wai

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
M6		31.03.13	1.473	0.497	3	0.06075	3.3085	1.2285	1.41225	0.43625	3.24775	1.16775		Wai
M7		31.03.13	0.4705	0.13	3	0.06075	3.3085	1.2285	0.40975	0.06925	3.24775	1.16775		Wai
M8		31.03.13	0.8255	0.3285	3	0.06075	3.3085	1.2285	0.76475	0.26775	3.24775	1.16775	-	Wai
M9		31.03.13	0.647	0.2135	3	0.06075	3.3085	1.2285	0.58625	0.15275	3.24775	1.16775		Wai
M21		31.03.13	0.7915	0.28	4	0.0545	2.0475	0.8735	0.737	0.2255	1.993	0.819		Wai
M22		31.03.13	0.678	0.291	4	0.0545	2.0475	0.8735	0.6235	0.2365	1.993	0.819		Wai
M24		31.03.13	1.4065	0.4065	4	0.0545	2.0475	0.8735	1.352	0.352	1.993	0.819	+	Wai
M25		31.03.13	0.666	0.279	4	0.0545	2.0475	0.8735	0.6115	0.2245	1.993	0.819		Wai
M26		31.03.13	0.723	0.2455	4	0.0545	2.0475	0.8735	0.6685	0.191	1.993	0.819		Wai
M28		31.03.13	0.3855	0.133	4	0.0545	2.0475	0.8735	0.331	0.0785	1.993	0.819		Wai
M29		31.03.13	0.5455	0.157	4	0.0545	2.0475	0.8735	0.491	0.1025	1.993	0.819		Wai
M31		31.03.13	0.6015	0.201	4	0.0545	2.0475	0.8735	0.547	0.1465	1.993	0.819		Wai
M32		31.03.13	1.5835	0.4735	4	0.0545	2.0475	0.8735	1.529	0.419	1.993	0.819		Wai
M33		31.03.13	0.618	0.151	4	0.0545	2.0475	0.8735	0.5635	0.0965	1.993	0.819		Wai
M36		31.03.13	0.5515	0.1185	4	0.0545	2.0475	0.8735	0.497	0.064	1.993	0.819		Wai
M38		31.03.13	0.688	0.241	4	0.0545	2.0475	0.8735	0.6335	0.1865	1.993	0.819		Wai
M39		31.03.13	0.7445	0.3785	4	0.0545	2.0475	0.8735	0.69	0.324	1.993	0.819		Wai
M40		31.03.13	0.249	0.1135	4	0.0545	2.0475	0.8735	0.1945	0.059	1.993	0.819		Wai
M41		31.03.13	0.6755	0.186	4	0.0545	2.0475	0.8735	0.621	0.1315	1.993	0.819		Wai
M42		31.03.13	0.549	0.178	4	0.0545	2.0475	0.8735	0.4945	0.1235	1.993	0.819		Wai
M43		31.03.13	0.453	0.1425	4	0.0545	2.0475	0.8735	0.3985	0.088	1.993	0.819		Wai
M44		31.03.13	0.583	0.2025	4	0.0545	2.0475	0.8735	0.5285	0.148	1.993	0.819		Wai
M45		31.03.13	0.675	0.199	4	0.0545	2.0475	0.8735	0.6205	0.1445	1.993	0.819		Wai

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
M46		31.03.13	0.267	0.0985	4	0.0545	2.0475	0.8735	0.2125	0.044	1.993	0.819		Wai
M47		31.03.13	0.6065	0.163	4	0.0545	2.0475	0.8735	0.552	0.1085	1.993	0.819		Wai
2W		16.10.00	0.9885	0.3765	6	0.05675	2.491	1.034	0.93175	0.31975	2.43425	0.97725		Whi
J22		31.03.13	0.6595	0.1935	6	0.05675	2.491	1.034	0.60275	0.13675	2.43425	0.97725		Wai
J23		31.03.13	0.5045	0.1125	6	0.05675	2.491	1.034	0.44775	0.05575	2.43425	0.97725		Wai
J24		31.03.13	0.406	0.1535	6	0.05675	2.491	1.034	0.34925	0.09675	2.43425	0.97725		Wai
J25		31.03.13	0.4135	0.152	6	0.05675	2.491	1.034	0.35675	0.09525	2.43425	0.97725		Wai
J27		31.03.13	0.8025	0.232	6	0.05675	2.491	1.034	0.74575	0.17525	2.43425	0.97725		Wai
J28		31.03.13	0.857	0.251	6	0.05675	2.491	1.034	0.80025	0.19425	2.43425	0.97725		Wai
J29		31.03.13	0.544	0.148	6	0.05675	2.491	1.034	0.48725	0.09125	2.43425	0.97725		Wai
J32		31.03.13	0.7475	0.3255	6	0.05675	2.491	1.034	0.69075	0.26875	2.43425	0.97725		Wai
J33		31.03.13	0.7085	0.3865	6	0.05675	2.491	1.034	0.65175	0.32975	2.43425	0.97725		Wai
J34		31.03.13	0.4965	0.1615	6	0.05675	2.491	1.034	0.43975	0.10475	2.43425	0.97725		Wai
J35		31.03.13	0.44	0.1265	6	0.05675	2.491	1.034	0.38325	0.06975	2.43425	0.97725		Wai
J36		31.03.13	0.7965	0.237	6	0.05675	2.491	1.034	0.73975	0.18025	2.43425	0.97725		Wai
J37		31.03.13	0.158	0.0685	6	0.05675	2.491	1.034	0.10125	0.01175	2.43425	0.97725		Wai
J38		31.03.13	0.271	0.105	6	0.05675	2.491	1.034	0.21425	0.04825	2.43425	0.97725		Wai
J39		31.03.13	0.7655	0.1625	6	0.05675	2.491	1.034	0.70875	0.10575	2.43425	0.97725		Wai
J40		31.03.13	0.8685	0.2035	6	0.05675	2.491	1.034	0.81175	0.14675	2.43425	0.97725		Wai
J41		31.03.13	0.522	0.1415	6	0.05675	2.491	1.034	0.46525	0.08475	2.43425	0.97725		Wai
J44		31.03.13	0.3965	0.116	6	0.05675	2.491	1.034	0.33975	0.05925	2.43425	0.97725		Wai
J45		31.03.13	0.3175	0.105	6	0.05675	2.491	1.034	0.26075	0.04825	2.43425	0.97725		Wai
J51		31.03.13	0.561	0.167	6	0.05675	2.491	1.034	0.50425	0.11025	2.43425	0.97725		Wai
10P		?.05.00	0.7135	0.4585	9	0.0655	3.4545	1.159	0.648	0.393	3.389	1.0935		Par
11P		?.05.00	2.365	0.7755	9	0.0655	3.4545	1.159	2.2995	0.71	3.389	1.0935		Par

Possum ID[a]	Pre/Post[b]	Date of sampling	Sample OD450 (1:10)[c]	Sample OD450 (1:100)[d]	Plate #[e]	OD450 blank[f]	E12 OD450 (1:10)[g]	E12 OD450 (1:100)[g]	Sample Corrected OD450 (1:10)[h]	Corrected OD450 (1:100)[h]	E12 Corrected OD450 (1:10)[h]	E12 Corrected OD450 (1:100)[h]	Western Blot[i]	Region[j]
12P		?.05.00	2.7815	1.112	9	0.0655	3.4545	1.159	2.716	1.0465	3.389	1.0935		Par
17P		?.05.00	1.0085	0.3395	9	0.0655	3.4545	1.159	0.943	0.274	3.389	1.0935		Par
18P		?.05.00	0.903	0.3305	9	0.0655	3.4545	1.159	0.8375	0.265	3.389	1.0935		Par
1bry		26.06.00	0.6865	0.355	9	0.0655	3.4545	1.159	0.621	0.2895	3.389	1.0935		Par
1P		?.05.00	1.3465	0.578	9	0.0655	3.4545	1.159	1.281	0.5125	3.389	1.0935		Par
2bry		26.06.00	0.5945	0.1425	9	0.0655	3.4545	1.159	0.529	0.077	3.389	1.0935		Par
2P		?.05.00	1.6645	0.671	9	0.0655	3.4545	1.159	1.599	0.6055	3.389	1.0935		Par
3bry		26.06.00	0.749	0.606	9	0.0655	3.4545	1.159	0.6835	0.5405	3.389	1.0935		Par
3P		?.05.00	0.3245	0.089	9	0.0655	3.4545	1.159	0.259	0.0235	3.389	1.0935		Par
4bry		26.06.00	0.9455	0.51	9	0.0655	3.4545	1.159	0.88	0.4445	3.389	1.0935		Par
4P		?.05.00	0.845	0.2195	9	0.0655	3.4545	1.159	0.7795	0.154	3.389	1.0935		Par
5bry		26.06.00	0.9815	0.458	9	0.0655	3.4545	1.159	0.916	0.3925	3.389	1.0935		Par
6bry		26.06.00	1.14	0.7805	9	0.0655	3.4545	1.159	1.0745	0.715	3.389	1.0935		Par
6P		?.05.00	0.926	0.23	9	0.0655	3.4545	1.159	0.8605	0.1645	3.389	1.0935		Par
7bry		26.06.00	0.771	0.247	9	0.0655	3.4545	1.159	0.7055	0.1815	3.389	1.0935		Par
7P		?.05.00	2.0215	0.75	9	0.0655	3.4545	1.159	1.956	0.6845	3.389	1.0935		Par
8P		?.05.00	1.757	1.023	9	0.0655	3.4545	1.159	1.6915	0.9575	3.389	1.0935		Par
9P		?.05.00	1.2005	0.5305	9	0.0655	3.4545	1.159	1.135	0.465	3.389	1.0935	+	Par

Appendix 7

Source Code For Analysis of Indirect ELISA Data

Analysis kindly performed by Dr Wesley Johnson of the Department of Statistics, University of California, Irvine, Dr Geoff Jones of the Institute of Fundamental Sciences, Massey University, and Dr Cord Heuer of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University.

```
model{
    # the first 15 results are assumed to be true negative, the next
    # 20 true positive, and the rest unknown
    for (i in 1:15) {
        lnod100[i] ~ dnorm(mmu[i] , tau[1])
        mmu[i] <- mu[1]
    }

    for (i in 16:35)  {
        lnod100[i] ~ dnorm(mmu[i] , tau[2])
        mmu[i] <- mu[2]
    }

    for (i in 36:244) {
        z[i] ~ dbern(pi)
        w[i] <- z[i] +1
        lnod100[i] ~ dnorm(mu[w[i]], tau[w[i]] )
    }

    #  Prior
    mu[1] ~ dunif(-1.5,1)
    mu[2] ~ dunif(mu[1], 1)

    for (i in 1:2) {
        tau[i] ~ dgamma(1,1)  #<- 1/pow(sig[i],2)
        sig[i] <- 1/sqrt(tau[i])  # ~ dunif(0,1)
    }

    pi ~ dbeta(1,1)   # prob infected

    # for AUC and ROC
    for (i in 1:2) {
        y[i] ~ dlnorm(mu[i], tau[i])
        yy[i] <- y[i] -0.3

        #since lod = log(od + .3) ~ N(mu,1/tau), od+.3 ~ ln(mu,1/tau)
        # so yy gets back to od scale
        prob[i] <- 1- step(yy[i] - 1.5)
        zz[i] ~ dnorm(mu[i], tau[i])
    }
```

```
    ww ~ dbern(pi)
    yy[3] <- ww*yy[2] + (1-ww)*yy[1]
    zz[3] <-  ww*zz[2] + (1-ww)*zz[1]
    prob[3] <-  1- step(yy[3] - 2)
    AUC <- step(yy[2] - yy[1])
    l <- 0.3
    u <- 2.3

    for (i in 1:50)  {
        c[i] <- -0.3 +  (51- i)*1.8/50 + v[i]
        v[i] ~ dnorm(0,10000)
        se[i] <- step(yy[2] - c[i])
        fp[i] <- step(yy[1] -c[i])
    }
}
```

Appendix 8

Blocking ELISA and Western-Blot Data From WPDV-infected Possums

[a] E1 – E4 = uninfected controls, E5 – E6 = WPDV-infected possums
[b] pre/post = samples collected prior to (pre) and after (post) experimental challenge with WPDV(possums E5-E16) or nothing/control inoculum (possums E1-E4)
[c] OD450 of serum samples at 1:5, 1:10, 1:100 and 1:500 serum dilutions
[d] OD450 of serum-free well on respective ELISA plate
[e] Percentage inhibition of serum samples calculated by: $1 - (OD_{450\ nm}$ of test serum/$OD_{450\ nm}$ of serum free controls)) × 100%.
[f] - = negative and + = positive for WPDV antibody by Western-blot. NT = not tested, ? = equivocal

Possum[a]	Sample[b]	1:5 OD450[c]	1:10 OD450[c]	1:100 OD450[c]	1:500 OD450[c]	No serum OD450[d]	% inhibition 1:5[e]	%inhibition 1:10[e]	%inhibition 1:100[e]	%inhibition 1:500[e]	Western[f]
E1	pre	0.9295	0.9725	1.283	1.401	1.323	29.74300831	26.49281935	3.023431595	-5.89569161	-
E1	post	1.002	1.2235	1.28	1.349	1.323	24.26303855	7.520786092	3.250188964	-1.965230537	-
E2	pre	0.648	0.708	0.9755	0.932	1.00325	35.40991777	29.4293546	2.766010466	7.101918764	-
E2	post	0.7555	0.809	0.9315	1.068	1.00325	24.69474209	19.36207326	7.15175679	-6.454024421	-
E3	pre	0.936	1.0645	1.43	1.5465	1.46925	36.29402757	27.54806874	2.671431002	-5.257784584	-
E3	post	0.982	1.1165	1.3675	1.5395	1.46925	33.16317849	24.00884805	6.925302025	-4.781351029	-
E4	pre	0.9795	1.0585	1.38	1.442	1.46925	33.33333333	27.95644036	6.07452782	1.854687766	-
E4	post	1.1315	1.259	1.349	1.4435	1.46925	22.98791901	14.31002212	8.184447848	1.752594861	-
E5	pre	0.678	0.734	0.9425	0.8965	1.00325	32.41963618	26.83777722	6.055320209	10.64041864	-
E5	post	0.5895	0.666	0.853	1.004	1.00325	41.24096686	33.61574882	14.97632694	-0.07475704	-
E6	pre	1.125	1.1035	1.174	1.317	1.323	14.96598639	16.59108088	11.26228269	0.453514739	NT
E6	post	0.947	0.914	1.07	1.2875	1.323	28.42025699	30.91458806	19.12320484	2.68329554	+
E7	pre	0.9165	0.88	1.2405	1.3315	1.323	30.72562358	33.48450491	6.235827664	-0.642479214	-
E7	post	0.9185	0.881	1.0245	0.959	1.1905	22.84754305	25.99748005	13.94372113	19.44561109	+

E8	pre	0.877	0.958	1.397	1.48	1.46925	40.30968181	34.79666497	4.917474902	-0.731665816	-
E8	post	0.7565	0.8225	1.2405	1.4235	1.48075	48.91102482	44.45382408	16.22488604	3.866283978	+
E9	pre	0.959	0.87	1.218	1.377	1.323	27.51322751	34.24036281	7.936507937	-4.081632653	-
E9	post	1.0035	0.954	1.138	1.351	1.398	28.21888412	31.75965665	18.59799714	3.361945637	+
E10	pre	0.953	0.9485	1.3495	1.4365	1.46925	35.13697465	35.44325336	8.150416879	2.229028416	+
E10	post	1.0615	1.125	1.3085	1.396	1.46925	27.75225455	23.43032159	10.94095627	4.985536839	+
E11	post	1.158	1.18	1.419	1.6885	1.806	35.88039867	34.66223699	21.42857143	6.506090808	+
E12	pre	0.6625	0.712	0.9865	1.1175	1.00325	33.964615	29.03065039	1.669573885	-11.38798904	?
E12	post	0.548	0.5795	0.808	0.974	1.00325	45.37752305	42.23772739	19.46174931	2.915524545	+
E13	pre	0.897	0.9605	1.2755	1.3695	1.323	32.19954649	27.39984883	3.590325019	-3.514739229	+
E13	post	0.894	1.0205	1.189	1.384	1.323	32.42630385	22.86470144	10.12849584	-4.610733182	+
E14	pre	0.8035	0.8905	1.2985	1.4425	1.46925	45.31223413	39.39084567	11.62157563	1.820656798	+
E14	post	0.7635	0.8295	1.3035	1.4205	1.46925	48.03471159	43.54262379	11.28126595	3.318019398	+
E15	pre	0.955	1.122	1.444	1.915	1.806	47.12070875	37.87375415	20.04429679	-6.035437431	+
E15	post	1.547	1.6285	1.87	1.912	1.806	14.34108527	9.828349945	-3.543743079	-5.869324474	NT
E16	pre	0.671	0.7035	0.9605	1.071	1.00325	33.11736855	29.87789684	4.261151258	-6.753052579	-
E16	post	0.725	0.727	0.8595	1.007	1.00325	27.7348617	27.53550959	14.32843259	-0.373785198	+

Appendix 9

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