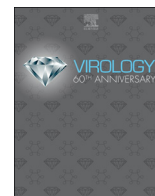ELSEVIER

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Virology

Viral RNA load and histological changes in tissues following experimental infection with an arterivirus of possums (wobbly possum disease virus)

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ABSTRACT

Tissues from Australian brushtail possums (*Trichosurus vulpecula*) that had been experimentally infected with wobbly possum disease (WPD) virus (WPDV) were examined to elucidate pathogenesis of WPDV infection. Mononuclear inflammatory cell infiltrates were present in livers, kidneys, salivary glands and brains of WPD-affected possums. Specific staining was detected by immunohistochemistry within macrophages in the livers and kidneys, and undefined cell types in the brains. The highest viral RNA load was found in macrophage-rich tissues. The detection of viral RNA in the salivary gland, serum, kidney, bladder and urine is compatible with transmission via close physical contact during encounters such as fighting or grooming, or by contact with an environment that has been contaminated with saliva or urine. Levels of viral RNA remained high in all tissues tested throughout the study, suggesting that on-going virus replication and evasion of the immune responses may be important in the pathogenesis of disease.

1. Introduction

Wobbly possum disease (WPD) is a fatal neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*) that has been thus far reported only in New Zealand, both in captive and free-living possums (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott, 1998; Perrott et al., 2000a, 2000c). The partial genomic sequence of WPD virus (WPDV) was first identified in archival tissues from WPD-affected possums (Dunowska et al., 2012), and a full genomic sequence has been reported recently (Gulyaeva et al., 2017). Based on the analysis of this sequence, WPDV has been classified within the family *Arteriviridae* as a sole member of the newly established genus *Dipartevirus* (Anonymus, 2017; Kuhn et al., 2016). Aetiological involvement of the virus in the development of WPD has been confirmed by induction of disease by experimental infection of wild-caught possums with a purified WPDV isolate (Giles et al., 2016).

Whilst possums are native to Australia and protected in some states, in New Zealand they are an introduced pest species causing substantial destruction of the country's unique ecosystem and wildlife (Cowan, 1990; Cowan and Waddington, 1990; King, 1990). As such, interest in understanding pathogens of possums was originally driven by their potential to be used as biological control in New Zealand (Perrott, 1998), and conversely, from conservation efforts in Australia. Very

limited numbers of viruses have been identified from the possum host. In addition to WPDV, these include possum enteroviruses (Zheng, 2007), possum adenovirus (Thomson et al., 2002), and possum papillomavirus (Perrott et al., 2000b). Herpesvirus- and coronavirus-like particles have also been observed in fecal samples from possums by electron microscopy (Rice and Wilks, 1996). Of these, only WPDV has been linked to severe systemic disease characterised by signs of multifocal neurological disturbances, clinically appearing to involve the vestibular system and resulting in ataxia, a decreased ability to climb, head tremor, and behavioural changes (Giles et al., 2016; Perrott et al., 2000c). Under experimental conditions, decreased appetite precedes the development of neurological signs and significant weight loss is also observed (Giles et al., 2016). The histological hallmark of the disease is mononuclear perivascular infiltrates in multiple organs, including the liver, spleen, brain and kidney (O'Keefe et al., 1997; Perrott, 1998).

The disease has been reproduced following the inoculation of healthy wild-caught possums with blood, urine, tissue homogenates and homogenized hematophagus skin mites (*Trichosurolaelaps crassipes*) derived from WPD-affected possums and administered by multiple routes (O'Keefe et al., 1997; Perrott, 1998; Perrott et al., 2000c). However, the infectious dose and mechanisms of natural transmission of WPDV are currently unknown. Under experimental conditions, healthy wild-caught possums sharing an enclosure with WPD-affected

possums developed WPD whereas possums housed in adjacent separate cages remained healthy (Perrott, 1998; Perrott et al., 2000c). This suggests that the virus is transmitted via direct contact or fomites and that aerosol or droplet transmission does not occur.

The interest in arteriviruses has been driven predominantly by the desire to improve control measures against porcine reproductive and respiratory syndrome virus (PRRSV), which is considered to be one of the most economically important pathogens of swine (Perez et al., 2015). 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 arteriviruses 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. Alternatively, the separation between WPDV and other arterivirus sequences may reflect a convergent evolution of their hosts, marsupials and placental mammals (Luo et al., 2011). Hence, deciphering pathogenesis and biological properties of WPDV is interesting not only from the point of view of possum health, but also from the evolutionary perspective.

The aim of the current study was to describe histological lesions and viral RNA levels in tissues from possum experimentally infected with WPDV.

2. Materials and methods

2.1. 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 5–8), infected cell culture lysate (possums 9–12), or standard inoculum that comprised a filtered 10% tissue suspension of brain, liver and spleen from seven WPD-affected possums (possums 13–16). Possums 1 and 2 received nothing and possums 3 and 4 received control inoculum. The latter comprised Iodixanol medium (Optiprep, Sigma-Aldrich) equivalent to the position of the virus band from density-gradient purification, but obtained after processing of uninfected cultures of primary possum macrophages. 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 pH 7.4 with added lysine (0.15 M) and sodium-m-periodate (0.01 M) 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 1500 × g for 10 min and stored at − 80 °C.

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

2.2. 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 lysates. Full sets of tissues were available for seven WPD-

affected possums. Urine was the only missing sample for two possums (possums 10 and 16) and lymph node was missing for one possum (possum 14). For the remaining two possums (6 and 11) 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 min (uracil DNA glycosylase activation), 95 °C for 10 min (initial denaturation and activation of DNA polymerase), 40 cycles of 95 °C for 5 s, 60 °C for 5 s and 72 °C for 15 s, 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 1 and 3 were included as negative controls.

Viral loads 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 for infected possums were determined by least mean squares using SAS software. Two possums (possums 6 and 11) were removed from the analysis due to multiple missing data points. Two negative data points from WPD-affected possums (spinal cord from possum 7 and hindbrain from possum 8) 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 $P < 0.05$.

2.3. 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 9,10,14 and 15. All tissue samples were fixed in paraformaldehyde in phosphate buffer 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 of the authors (JG, MP, WR, DA) blinded. The severity of lesions within the renal interstitium, glomeruli and tubules were individually graded on a scale of 0–3, 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 $P < 0.05$.

2.4. Serum biochemistry

Testing for urea and creatinine levels in stored serum samples was performed by IDEXX laboratories (Palmerston North, New Zealand). Sera from all four control possums and 11/12 WPDV-infected possums were tested. Insufficient serum from possum 15 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.

2.5. Leptospira qPCR

To further investigate the aetiology of the rental lesions, *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 6 and 11 were not available.

2.6. 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 min and rinsed in water for two minutes. Heat-based antigen retrieval (Tris EDTA pH 8.5 for 30 min at 95 °C) was followed by permeabilization (0.3% Triton X in PBS pH 7.0 for five minutes). Slides were then washed 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 previously been shown to be recognised by sera from WPDV infected possums by enzyme-linked immunosorbent assay and Western blot (Giles et al., 2018). 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.

3. Results

3.1. Determination of WPDV RNA levels in tissues

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 (Fig. 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, forebrain, hindbrain, 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 forebrain was significantly higher than the mean viral load in the hindbrain and spinal cord ($P < 0.05$).

Moderately high levels of viral RNA were detected in sera from all but one infected possums (average 2.76×10^5 copies/μL), however, there were marked differences between individual possums. Lower
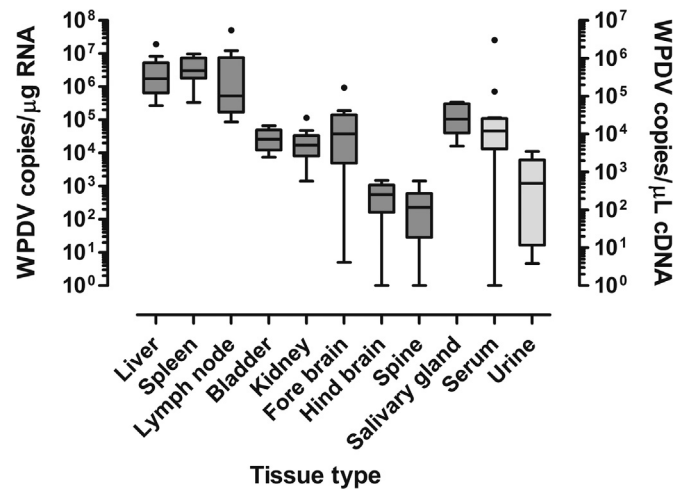

Fig. 1. Quantity of viral RNA in tissues and body fluids from wobbly possum disease virus (WPDV)-infected possums. Viral RNA was extracted and quantified using a previously described WPDV-specific RT-qPCR (Dunowska et al., 2013). The results were expressed as WPDV copies/μg RNA for tissues (liver, spleen, lymph node, bladder, kidney, forebrain, hindbrain, spine and salivary gland) or as WPDV copies/μL cDNA for body fluids (serum and urine).

levels of viral RNA were detected in the urine than the serum (1.02×10^3 copies/μL), with similar variability between individual animals.

3.2. Histology

Histological examination was performed on possums 1–4 (controls) and all WPDV-infected possums (possums 5–16).

3.2.1. Liver

Small, multifocal inflammatory aggregates were disseminated throughout the hepatic parenchyma in 11/12 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 (Fig. 2A) and centrilobular regions. Inflammatory aggregates were not obvious in the liver from possum 15, which died on day 6 post infection. There was a marked increase in inflammatory cells in the hepatic parenchyma and sinusoids observed in the liver of this possum, but these changes were more diffuse/uniform than those observed in other WPD-affected possums. Small foci of perivascular mononuclear inflammation around the central veins were also observed in sections of liver from two control possums (possums 1 and 3), however, control possums lacked mononuclear cell infiltration in the hepatic parenchyma.

3.2.2. Kidney

Kidney lesions are summarised in Table 1. Mild to severe interstitial nephritis was present in 2/4 (50%) control possums and 11/12 (91.6%) WPDV-infected possums. Additionally, mild to severe tubular lesions were present in 10/12 (83.3%), mild to moderate glomerular lesions were present in 5/12 (41.7%) and mild to severe interstitial fibrosis were present in 4/12 (33.3%) WPDV-infected possums. Scores for tubular lesions ($P = 0.009$), interstitial nephritis ($P = 0.032$) and total scores ($P = 0.014$) 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 3 and 4). In 10/11 (90.9%) WPDV-infected possums and 2/4 (50%) control possums, the interstitial infiltrate was
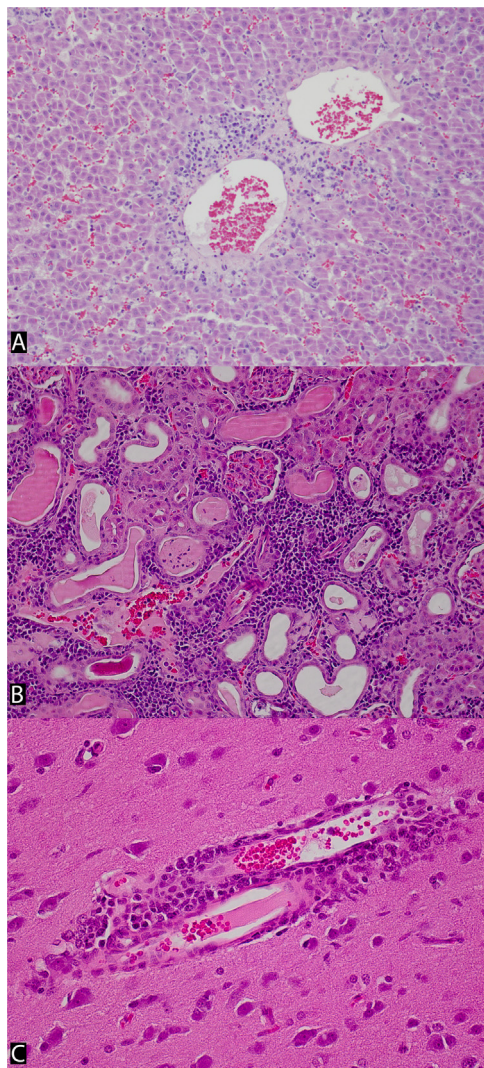
Fig. 2. Histology of brain, kidney and liver tissue from wobbly possum disease virus (WPDV)-infected possums. A) Possum 16 liver viewed under 10 × objective. Inflammatory aggregates surrounding hepatic veins. Aggregates comprise predominantly plasma cells with some macrophages. B) Possum 12 kidney, viewed under 20 × 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. C) Possum 13 brain, viewed under 20 × objective. Mononuclear cell perivascular cuff in the forebrain. Slides were observed using a light microscope (Olympus BX 51) and images captured using an Olympus XC 50 digital camera.

lymphoplasmacytic, while a mixed infiltrate (predominantly lymphocytes and plasma cells with fewer neutrophils) was present in one (8.3%) WPDV infected animal (possum 5). 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) (Fig. 2B). Glomerular changes were variable and mild in 3/12 (25%) WPDV-infected possums and moderate in 2/12 (16.7%) 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 (8%) cases there were perivascular aggregates of moderate numbers of mononuclear cells in the peri-renal adipose tissue (possum 5), but these were also present in one control animal (possum 3). Masson's trichrome was strongly positive for collagen in the interstitium of both of these possums.

3.2.3. Brain

Lesions are summarised in Table 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 (Fig. 2C). The inflammatory infiltrate was partially (possum 9) or predominantly (possum 7) neutrophilic in two possums. In 2/12 (16.7%) infected possums (possums 5 and 7) 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 5, 7, 12, 13, 14 and 16). Multifocal dense aggregates of glial cells (glial nodules) involving both grey or white matter were present in 5/12 (41.7%) infected possums (possums 5, 7, 8, 12, 16) and 1/4 (25%) control possum. Occasional foci of malacia were present in the forebrain of possum 9, while multiple suppurative foci and necrotising vasculitis were present within the forebrain of possum 7.

3.2.4. 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.

3.2.5. Lymph node

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

3.2.6. Salivary gland

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

3.2.7. Bladder

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

3.3. Immunohistochemistry

The WPDV antigen appeared as granular staining within the cytoplasm of cells. It was detected in macrophages in the livers of all experimentally infected possums (Fig. 3A), including possum 15 with atypical histological changes, but not in 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 1, Fig. 3C). Here, immunostaining was detected in macrophages within inflammatory aggregates in the renal interstitium. Immunostaining was also detected in the brains of 8/12 WPDV-infected possums and none of the control possums. Where present, immunostaining with a predominantly cytoplasmic distribution was seen in one or more of the following locations: glial nodules (Fig. 3E), the choroid plexus, the subarachnoid space and various subependymal locations.

3.4. Biochemistry

Mild azotaemia was present in two WPD-affected possums (possums 6 and 8) and severe azotaemia in another two possums (possums 10 and

Table 1
Description of histological and biochemical indices of renal disease in wobbly possum disease virus (WPDV)-infected (possum number 5 – 16) and uninfected control (possum number 1 – 4) possums.

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		
1 (M)	Control	42	10.3	47	0	0	0	0	0	−	−
2 (M)	Control	42	5.6	48	0	0	0	0	0	−	+
3 (M)	Control	41	6.2	48	1	0	0	0	1	−	−
4 (F)	Control	41	6.8	82	1	0	0	0	1	−	−
5 (M)	WPDV	20	6.4	34	1	1	0	0	2	+	−
6 (F)	WPDV	8	17.1	136	0	0	0	0	0	−	N/A
7 (M)	WPDV	20	17.8	53	1	1	0	0	2	+	−
8 (M)	WPDV	27	43.9	145	2	1	1	0	4	+	+
9 (M)	ICL	19	10.2	42	2	1	1	0	4	+	−
10 (F)	ICL	9	109.9	1108	3	3	2	3	11	+	−
11 (M)	ICL	22	6.4	43	1	1	0	0	2	+	N/A
12 (F)	ICL	27	3.1	23	3	3	0	1	7	+	−
13 (F)	SI	23	5.6	35	2	1	1	1	5	+	−
14 (M)	SI	9	107.8	951	3	3	2	3	11	+	−
15 (M)	SI	6 (died)	N/A	N/A	1	0	0	0	1	+	−
16 (F)	SI	20	8.8	26	2	1	0	0	3	−	−

[a] F = female, M = male.
[b] CI = control inoculum, WPDV = purified WPD virus isolate, ICL = infected cell culture lysates, SI = standard inoculum.
[c] Above 15.8 mmol/L = elevated, N/A = serum not available for testing.
[d] Above 100 µmol/L = elevated, N/A = serum not available for testing.
[e] 0 = no significant lesions, 1 = mild lesions, 2 = moderate lesions, 3 = severe lesions.
[f] = - = no significant signal, + = specific signal.
[g] = + = PCR positive, - = PCR negative, N/A = tissues not available for testing.

Table 2
Description of histological brain lesions in wobbly possum disease virus (WPDV)-infected (possum number 5 – 16) and uninfected control (possum number 1 – 4) possums.

Possum ID # (M/F)[a]	Inoculum Type[b]	Euthanasia (days post inoculation)	Histologic features of brain lesions (+/-)[c]								IHC (+/-)[d]
			Encephalitis	Meningitis	Inflammatory infiltrate	Cuffing	Gliosis	Glial nodules	Endothelial Hypertrophy	Choroiditis	
1 (M)	Control	42	−	−	−	−	−	−	−	−	−
2 (M)	Control	42	−	−	−	−	−	−	−	−	−
3 (M)	Control	41	−	−	−	−	−	−	−	−	−
4 (F)	Control	41	+	−	Nonsuppurative	−	−	+	−	−	−
5 (M)	WPDV	20	+	+	Nonsuppurative	+	−	+	+	+	+
6 (F)	WPDV	8	−	−	−	−	−	−	−	−	−
7 (M)	WPDV	20	+	+	Neutrophilic	+	+	+	−	+	+
8 (M)	WPDV	27	+	+	Nonsuppurative	−	−	+	−	−	+
9 (M)	ICL	19	+	+	Nonsuppurative	+	+	−	+	−	+
10 (F)	ICL	9	+	+	Nonsuppurative	+	−	−	+	−	+
11 (M)	ICL	22	+	+	Nonsuppurative	−	−	−	−	−	−
12 (F)	ICL	27	+	+	Nonsuppurative	+	+	+	−	+	+
13 (F)	SI	23	+	+	Nonsuppurative	+	+	−	−	+	+
14 (M)	SI	9	+	+	Nonsuppurative	+	+	−	+	+	−
15 (M)	SI	6 (died)	−	−	−	−	−	−	−	−	−
16 (F)	SI	20	+	+	Nonsuppurative	+	+	+	−	+	+

[a] F = female, M = male.
[b] CI = control inoculum, WPDV = purified WPD virus isolate, ICL = infected cell culture lysates, SI = standard inoculum.
[c] + = presence of lesion, - = absence of lesion.
[d] + = immunopositive, - = immunonegative, N/A = not available.

14). Urea was mildly elevated in one additional possum (possum 7), and both values were within references ranges in the remaining experimental possums including control possums (Table 1).

3.5. Leptospira qPCR

Two of the 14 possum kidneys tested were positive for *Leptospira* DNA by qPCR (Table 1) with Ct values of 19.89 (possum 1, uninoculated control) and 31.56 (possum 8, WPD).

4. Discussion

Whilst RNA levels in selected tissues (liver, spleen, brain and kidney) from WPD-infected 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.

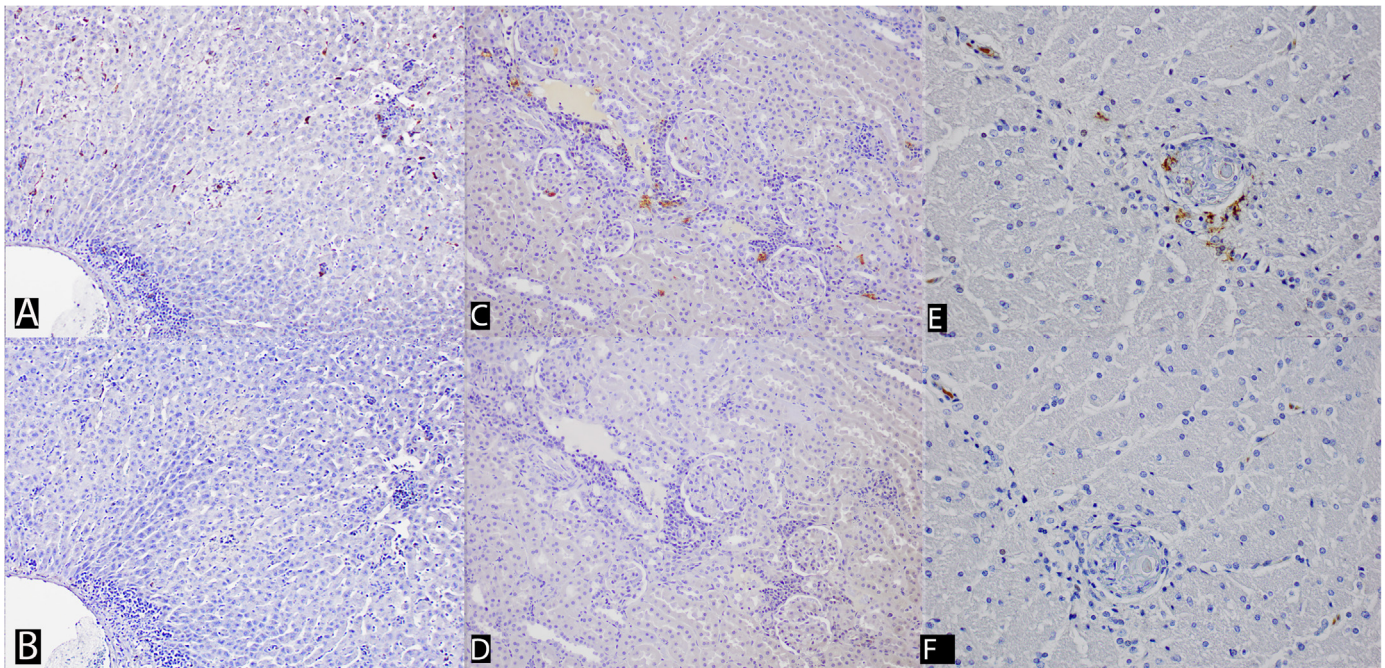To allow for comparison of RNA levels between tissues, qPCR results

Fig. 3. Specific immunostaining of wobbly possum disease virus (WPDV) antigen in macrophages in the hepatic parenchyma of possum 13. A smaller number of immunopositive macrophages also surround a sublobular vein (A); macrophages within inflammatory aggregates in the renal interstitium of possum 16 (C); and a glial nodule in the forebrain of possum 7 (E). Irrelevant antibody staining of respective tissue sections are presented for liver (B), kidney (D) and brain (F).

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 the 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 (Giles et al., 2015).

Based on these 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 indicate 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 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 (Gravell et al., 1986).

The presence of multi-organ lymphoplasmacytic inflammation in four possums that were euthanased or died within six to nine days following infection indicates that immune response to viral antigens begins early in the disease course. This is supported by detection of rising levels of WPDV antibody in two (possums 6 and 15) of those possums. The WPDV antibody levels in the remaining two possums (10 and 14) were already high at the time of experimental infection (Giles et al., 2018). 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 juvenile possums (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 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., 2000c).

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 forebrain in comparison with the hindbrain. One possible explanation of these results is that location-specific differences in the numbers of cells supportive of viral replication exist. Regional variations in the distribution of microglia have been described in the brain of mice, 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 were not unequivocally identified. Further investigation into the cell types that support viral replication within the CNS is thus warranted.

The histological lesions observed in the WPD-infected 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 meningoencephalitis, 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 central nervous system (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, 1998; Perrott et al., 2000c).

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 four 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. This disease 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.

One important limitation of this study is that possums were inoculated with WPDV via an unnatural route, using a high dose of the infectious virus (Giles et al., 2016). Hence, the pathogenicity of the virus following a natural exposure to a potentially lower infectious dose may differ from that described in this paper. However, healthy possums infected with various infectious inocula during one of the previous studies (Perrott et al., 2000c) developed disease with indistinguishable clinical and pathological features irrespective of the type of inoculum and the route of exposure used. The latter included IP, intra-gastric, intra-tracheal and intra-dermal infections. In addition to the SI, blood, urine and minced mites collected from WPD-affected possum were used as infectious inocula. Importantly, five possums infected via direct contact with two experimentally infected possums also developed WPD. With the exception of an apparently longer incubation period, suggesting that it took some time for in-contact possums to acquire WPDV infection, the clinical disease and pathological changes were the same for all affected possums. Altogether, this suggests that the tissue distribution of the virus and histopathological features observed in experimentally infected possums in the current study are likely to be relevant for the natural infection.

5. Conclusion

The results of this study contribute to our understanding of the pathogenesis of WPD and virus-host interactions. The data presented add to results of previously reported studies (Dunowska et al., 2013; O'Keefe et al., 1997; Perrott et al., 2000c) by increasing the number of diseased and healthy possums examined and by inclusion of additional tissue types. In addition, we have detected WPDV in diseased tissues using immunohistochemistry (IHC). The preferred sites for replication of WPDV were splenic, hepatic and lymphoid tissues, 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 system, 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.

Conflicts of interest

The authors declare that they have no conflict of interest.

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