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PATHOLOGY OF WEST NILE VIRUS LINEAGES 1 AND 2 IN MICE AND HORSES

BY

JUNE HEATHER WILLIAMS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

MAGISTER SCIENTIAE
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IN THE
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Declaration

I, June Heather Williams, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of Pretoria. It has not been submitted before for any degree or examination in any other Technikon or University.

June H Williams

This 22nd day of January 2014

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PRESENTATIONS AND PUBLICATIONS

Publications

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Posters

S Human, T Gerdes, C Potgieter, P Leman, A Kemp, J Paweska, J C Stroebel, J Steyl, R Last, **J Williams**, M Venter. Molecular characterisation of zoonotic flavi- and alphaviruses in sentinel animals in South Africa. National Institute for Communicable Diseases, Academic Day poster 23 November 2010.

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SUMMARY

PATHOLOGY OF WEST NILE VIRUS LINEAGES 1 AND 2 IN MICE AND HORSES

by

JUNE HEATHER WILLIAMS

SUPERVISOR: PROF M VENTER
DEPARTMENT: MEDICAL VIROLOGY
CO-SUPERVISOR: PROF J LAWRENCE
DEPARTMENT: VETERINARY PARACLINICAL SCIENCES
DEGREE: MAGISTER SCIENTIAE (MEDICAL VIROLOGY)

West Nile virus (WNV) is a widespread emerging zoonotic neurotropic flavivirus cycling naturally between mosquitos and birds. WNV causes disease in 20% of infections in the most susceptible incidental hosts which are horses and humans. Up to 40% of affected horses and 1- to approximately 50% of affected humans develop neurological signs and/or flaccid paralysis, in some cases fatal or severely debilitating, due to variable encephalitis, meningitis and poliomyelitis. Two predominant genetic lineages exist, 1 and 2, with neurovirulent lineage 1 strains recorded in the northern and western hemispheres, the milder lineage 1 Kunjin strain in Australia, and the lineage 2 strain endemic to southern Africa and Madagascar and considered, until recently, to have mainly mildly pathogenic strains. Since 2002 investigations into South African lineage 2 WNV strains showed that they resulted in severe neurological disease in horses and humans. From 2004 lineage 2 strains were recorded for the first time in southern Europe as a cause of neurological signs and death in birds, and increasingly, in horses and humans. In 2011 the

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mild lineage 1 Kunjin strain mutated to an equine neurovirulent strain in New South Wales, Australia, and in 2010 the first South African case of lineage 1 WNV was reported from the western Cape in a mare which showed severe neurological signs, abortion and death.

Laboratory strains of mice are extremely susceptible to WNV and have been mostly used in experimental studies since the 1937 discovery of the virus in Uganda. In the early 2000s studies in mice showed that field strains of lineage 1 and 2 WNV ranged from mildly pathogenic to highly neurovirulent, however, the associated pathology of the lineage 2 infections was not studied. In the current study, the macroscopic and microscopic pathology of a South African human-neurovirulent field strain of lineage 2 WNV (SPU93/01) and the neurovirulent lineage 1 (NY99/385) strain were investigated and compared in mice used as controls in 2 WNV vaccine studies. The clinical signs, CNS and extra-CNS pathology were indistinguishable between the lineages and some lesions were comparable to those previously reported.

Lineage 1 WNV equine pathology has been well described but that of lineage 2 only briefly previously described. The pathology in 6 naturally-occurring fatal lineage 2 WNV-infected horses with severe neurological signs, was investigated and compared with that of the single South African lineage 1 WNV field infection. Diagnoses were confirmed by real-time RT-PCR. Similarities and some slight differences in lesions were found in both mouse and horse studies when compared with lineage 1 pathology cases and with previous reports, and the neurovirulence of the lineage 2 field strains was confirmed. WNV immunohistochemistry (IHC) of all mouse tissues allowed speculation as to pathogenesis of intestinal lesions, but in equine CNS lesions was mostly negative. Ultrastructure of IHC positive cells showed rare WNV particles. In the horse cases rabies, equine herpes virus, and other arboviral co-infections were excluded and similarities and implications of gross lesions of African horsesickness to those often seen in WNV infections were discussed.

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KEY WORDS:

BALBc mice, histopathology, horses, immunohistochemistry, lineages 1 and 2, neurovirulent, pathology, RT-PCR, ultrastructure, West Nile virus

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ABBREVIATIONS

AHS	= African horsesickness
BBB	= blood brain barrier
BSL3	= biosafety level 3
CNS	= central nervous system
CSF	= cerebro-spinal fluid
DRG	= dorsal root ganglion
EEV	= equine encephalosis virus
EHV	= equine herpes virus
ELISA	= enzyme-linked immunosorbent assay
EM	= electron microscopy
GI	= gastrointestinal
HI	= haemagglutination inhibition
IFA	= immunofluorescent assay
IFN	= interferon
IFN-α	= interferon-alpha
Ig	= immunoglobulin
IHC/IMH	= immunohistochemistry
ISH	= *in situ* hybridisation
JE	= Japanese encephalitis
JEV	= Japanese encephalitis virus
MAC-ELISA	= IgM antibody capture – ELISA
MVE	= Murray Valley encephalitis
NHLS	= National Health Laboratory Service
NICD	= National Institute for Communicable Diseases
NY99/386	= New York 1999 isolate 385
OP	= Onderstepoort
OVI	= Onderstepoort Veterinary Institute
PBS	= phosphate buffered saline
PFU	= plaque forming units

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PI /pi	= post infection
PNS	= peripheral nervous system
PRNT	= plaque reduction neutralisation assay
rER	= rough endoplasmic reticulum
RT-PCR	= reverse transcriptase polymerase chain reaction
rRT-PCR	= real-time RT-PCR
SA	= South Africa
SCID	= severe combined immunodeficiency
SINV	= Sindbis virus
SLE	= St Louis encephalitis
SLEV	= St Louis encephalitis virus
SPF	= specific pathogen free
Spp	= species
SPU93/01	= Special Pathogens Unit isolate 93 of 2001
TEM	= transmission electron microscopy
UP	= University of Pretoria
USA	= United States of America
WESSV/WSLV	= Wesselsbron virus
WNND	= West Nile neurological disease
WNVD	= West Nile virus disease
WNV	= West Nile virus

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CHAPTER 1

Literature Review

1.1 INTRODUCTION

WNV is a widespread, single-stranded, positive sense RNA arbovirus of the genus *Flavivirus*, family *Flaviviridae*. It is zoonotic, has a predominantly bird – ornithophilic mosquito life-cycle, and *Culex* species (spp) mosquitoes are the most preferred vectors (Jupp, 2001; McLean *et al*., 2002). Of many mammalian species reported with incidental naturally-acquired WNV infection, horses and humans appear to be the most susceptible. Approximately 80% of human and equine infections are considered subclinical with low or absent viraemia, 20% manifest as mild transient fever and/or flu-like symptoms, and <1% develop severe neurological disease which manifests as encephalo-myelitis, meningitis and/or acute flaccid paralysis in humans. In horses however, of the 20% of symptomatic cases, up to 40% or more are neurological and might die or have to be euthenised (Bunning *et al*., 2002; MacKenzie *et al*., 2004; MMWR, 2005; Snook *et al*., 2001; Venter & Swanepoel, 2010). Laboratory mice of all ages and many strains are mildly to highly susceptible to neuroinvasion by WNV of many different strains of both lineages 1 and 2 and via various infection routes (Beasley *et al*., 2002; Burt *et al*., 2002; Eldadah & Nathanson, 1967; Smithburn *et al*., 1940; Venter *et al*., 2005).

The WNV lineage 1 New York 1999 strain that arrived by uncertain means in New York from mid-1999 was most closely related to an isolate from a dead goose in Israel from 1998 (Lanciotti *et al*., 1999). Avian deaths had been recorded for the first time in the known history of WNV in Israel in commercial geese in 1997 (Glavits *et al*., 2005) and 1999 (Swayne *et al*., 2001), and migratory storks in 1998 (Malkinson *et al*., 2002).

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From 1999 to 2004 during the WNV outbreak peak in North America, between 16000 and 17000 cases of WNV disease were reported in humans in the USA (Hayes & Gubler, 2006). In 2002 alone, approximately 15000 equine and 15000 human cases of WNV disease were reported in the USA (Beasley, 2005). Since this epidemic, there was a marked decline in growth rate of the predominant WNV strain, WN02, suggesting that WNV had reached its peak prevalence in North America, and the incidence of human cases decreased to its lowest level in 2009 (Lindsey, 2010). This changed in 2012 however, with the largest WNV outbreak since the early 2000s occurring in southeastern USA states, with the epicentre in Texas, and in which the incidence of human neurological disease rose to over 50% (Chung *et al.*, 2013).

WNV lineage 1, is considered an emerging pathogen with an increasing frequency of disease outbreaks and incidence of neurological disease with fatal outcome since 1996 (Petersen & Roehrig, 2001). Such outbreaks were recorded in birds, especially in Israel and the USA (Hubalek & Halouzka, 1999; Komar, 2000; Malkinson *et al.*, 2002; Petersen & Roehrig, 2001; Rappole & Hubalek, 2003); and in horses in Morocco (Schuffenecker *et al.*, 2005; Tber Abdelhaq, 1996), Italy (Angelini *et al.*, 2009; Antorino *et al.*, 2002; Calistri *et al.*, 2010a; Cantile *et al.*, 2000; Macini *et al.*, 2008; Monaco *et al.*, 2009), Israel (Steinman *et al.*, 2002), in North America since 1999 (MMWR, 2005; Ostlund *et al.*, 2001; Snook *et al.*, 2001; Trock *et al.*, 2001), and in France (Murgue *et al.*, 2001). During the high-rainfall summer of 2011, a neurovirulent mutation of the mild WNV lineage 1 Kunjin strain (Scherret *et al.*, 2001), called $WNV_{NSW2011}$, was responsible for an outbreak of disease in numerous horses in New South Wales, southeastern Australia (Frost *et al.*, 2012). The first WNV lineage 1 infection recorded in South Africa was in 2010 from a pregnant mare which died with severe neurological signs a day after she aborted: the brain of the foetus was also positive (Venter *et al.*, 2011).

Neurovirulent strains of WNV lineage 2 most likely existed in South Africa for decades but have only been brought to attention since neurological cases in experimental mice, humans and horses were investigated from 2002 until the present (Beasley *et al.*, 2002; Burt *et al.*, 2002; Venter *et al.*, 2005). Neurovirulent lineage 2 strains have been

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described since 2008 in cases of severe and fatal neurological WNV disease in South African horses (Venter *et al.*, 2009b). A zoonotic infection was also reported in a veterinary student who handled a WNV-infected brain during necropsy (Venter *et al.*, 2010). Of 206 serum or CSF specimens tested from humans hospitalised with neurological disease during summer of 2008, 40 were positive for WNV neutralising antibodies. Fewer cases of acute infection were detected either by RT-PCR or Immunoglobulin(Ig)M and IgG in cerebro-spinal fluid (CSF), suggesting WNV may have been overlooked as a cause of neurological disease in South Africa (Zaayman & Venter, 2012). In South Africa, humans with neurological disease have not routinely been tested for WNV.

In the current study the pathology of a South African neurovirulent human field isolate of WNV is investigated for the first time in laboratory mice, and compared with that of a neurovirulent lineage 1 strain. The pathology of neurovirulent lineage 2 WNV in horses has been little investigated. We previously described lineage 2 pathology in 3 South African horses (Venter *et al.*, 2009b), one of which had atypical CNS lesions. Neuropathology in 4 Hungarian horses infected with lineage 2 WNV was briefly described (Kutasi *et al.*, 2011). As part of the current study the pathology of WNV in 2 previously reported cases, including the lineage 1 case (Venter *et al.*, 2011), as well as 5 other lineage 2 naturally-infected horses which died or had to be euthanised due to severe neurological signs was investigated.

1.2 WNV VIROLOGY

1.2.1 Phylogenetic placement

WNV is a single-stranded positive sense RNA virus of the family *Flaviviridae*, genus *Flavivirus* (Hayes *et al.*, 2005), belonging to the Japanese Encephalitis (JE) virus serocomplex. Other members include several viruses associated with human encephalitis, namely, Japanese Encephalitis virus (JEV), St Louis encephalitis (SLE) virus, and Murray Valley encephalitis (MVE) virus (Petersen & Roehrig, 2001). Other JE serocomplex viruses are Alfuy and Koutango viruses (Beasley *et al.*, 2002), Cacipacore, Usutu and Yaounde viruses (Brinton, 2002). All flaviviruses are closely related

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serologically, accounting for the serological cross-reactions. To distinguish serologically between members of the JE complex, virus neutralisation assays are often required, and acute and convalescent-phase serum specimens from WNV patients are required to assess recent antibody response.

1.2.2 Genetic variation and pathogenicity

WNV isolates have been grouped into 2 main genetic lineages based on signiature amino acid substitutions or deletions in their envelope (E) proteins (Brinton, 2002) and 5, possibly 6 lineages are known (Bakonyi *et al*., 2005; Bondre *et al*., 2007; Lvov *et al*., 2000; Vazquez *et al*., 2010). The original WNV isolate from Uganda in 1937 (Smithburn *et al*., 1940) was of lineage 2 (Hayes & Gubler, 2006). Lineage 1 WNV is the most widespread in north Africa, Europe, Asia, Russia, North and South America, the Caribbean and in Australia, where it is called Kunjin (WNV_{KUN}) (Scherret *et al*., 2001). The Australian equine neurovirulent mutant Kunjin strain is called $WNV_{NSW2011}$ (Frost *et al*., 2012).

Lineage 2 had previously been considered to be less pathogenic in humans (Jupp, 2001), nonpathogenic in horses (Guthrie *et al*., 2003), and was found until 2004 only in Southern Africa and Madagascar (Burt *et al*., 2002; Jupp, 2001) apart from a mild strain isolated from a bird in Cyprus in 1968 (Beasley *et al*., 2002). Prior to 2002, only rare single fatalities were ascribed to lineage 2 WNV in humans and animals: a Botswanan dog in 1977, an ostrich chick in 1994 from Prince Albert in the Western Cape, a 6-month old Thoroughbred foal with neurological signs in 1996 from Somerset West in the Western Cape, and a human from northern Pretoria in 1989 with severe necrotic hepatitis (Burt *et al*., 2002). One person with WNV-associated renal failure (1989) and one with fever, rash, myalgia, arthralgia and encephalitis, recovered (Jupp, 2001). Jupp (2001) also mentions a further two South Africans who recovered from renal failure and 2 who recovered from meningoencephalitis (Jupp, 2001). The Special Pathogens Unit of the National Institute of Communicable Diseases (NICD) in Johannesburg had diagnosed 5 – 15 cases of WNV a year in humans, however, apart from those mentioned in Jupp (2001) and Burt *et al*, (2002), these cases were not published.

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Lineage 2 WNV has been recorded as a cause of severe and often fatal neurological disease in horses in South Africa when neurological cases were specifically investigated (Venter *et al.*, 2009b). Lineage 1 WNV was diagnosed for the first time in South Africa in the brains of a mare which died of severe neurological disease, as well as her aborted foal (Venter *et al.*, 2011). This strain differed from lineage 2 strains by 23.6-23.9% (Venter *et al.*, 2011). Lineage 1 strains have been further divided into 4 clades: Kunjin, Indian, A and B (which includes the Indian isolate) that are also referred to as lineage 5 (Hayes *et al.*, 2005). The isolates of clade B which includes strains from the USA, are all virulent in mice, with lineage 2 and other clades of lineage 1 having both virulent and attenuated strains (Beasley *et al.*, 2004; Beasley *et al.*, 2002).

The phenotype of neurovirulent WNV lineage 1 and 2 strains was found to correlate with genotype not lineage (Beasley *et al.*, 2002; Venter *et al.*, 2005) and some strains of both lineages 1 and 2 were found to be equally neurovirulent in mice (Beasley *et al.*, 2002; Venter *et al.*, 2005). All strains of South African neurovirulent WNV were found to possess the envelope-protein glycosylation site previously postulated to be associated with virulence, however major deletions were found in the 3' noncoding region of 2 lineage 2 strains previously shown to be less or non-neuroinvasive (Botha *et al.*, 2008).

Isolates classified as lineages 3 and 4 were reported from central and Eastern Europe (Lvov *et al.*, 2000), lineage 5 from India (Bondre *et al.*, 2007), and a possible new lineage from Spain (lineage 6) (Vazquez *et al.*, 2010).

1.3 WNV DIAGNOSIS

Confirmation of WNV infection is possible directly by identification of the virus or nucleic acids, or indirectly by testing for antibodies in clinical specimens; suitable specimens include necropsy tissue specimens, CSF, whole blood or serum (Castillo-Olivares & Wood, 2004). It is generally accepted that WNV diagnosis can be confirmed either by finding specific IgM antibodies or viral nucleic acid in appropriately-timed samples (Rizzo *et al.*, 2011). Negative virus detection test results alone should never be

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regarded as evidence of absence of WNV (Castillo-Olivares & Wood, 2004). Different flaviviruses have antigenic cross-reactivity, therefore techniques like plaque reduction neutralisation assay (PRNT) or sequence analysis of RT-PCR products are necessary for unequivocal diagnosis of WNV as causative agent of disease (Brinton, 2002; Lanciotti & Kerst, 2001).

Confirmatory laboratory diagnostic criteria for WNV from human patients with clinical neurological disease include isolation of virus, or detection of viral antigen or nucleic acid in tissue, blood or cerebro-spinal fluid (CSF) (Beasley, 2005). Detection of virus-specific IgM and IgG in CSF or serum are also used but require careful interpretation (Beasley, 2005) due to wide cross-reactivity between flaviviruses, differences between species of occurrence of antibodies, and timing of appearance and disappearance of WNV-specific neutralising, IgG and IgM antibodies (Ledermann *et al.*, 2011; Theiler & Casals, 1958).

1.3.1 RT-PCR

Real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) is able to detect approximately 50 viral RNA copies, making this approximately 1000-fold more sensitive than viral culture or *in situ* hybridisation for WNV detection.(Hayes *et al.*, 2005) Blood specimens collected early during infection may have nucleic acids that can be detected by RT-PCR. RT-PCR in 28 human patients with serologically confirmed WNV neuroinvasive disease was positive in 57% and 14% of patients in CSF and serum samples respectively (Hayes *et al.*, 2005). A combination of PCR and serology would therefore give the most decisive results.

In experimental WNV infection of 6- to 9-month old serologically-naive horses, rRT-PCR detected nucleic acid in 68.8% (11/16) of serum samples for up to 6 days postinfection, with levels detected corresponding to 1 to 100 plaque-forming unit (PFU) equivalents per ml of serum (Ledermann *et al.*, 2011). This test is important for diagnosis of early infection in live patients especially where viruses, like WNV, cause minimal viraemia

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and where, according to Ledermann *et al.,* (2011), less than 10% of infected horses develop clinical disease (Ledermann *et al*., 2011).

Kleiboeker *et al.,* (2004) in a retrospective diagnostic study found that WNV RNA was detected by RT-PCR and RT-nPCR (nested RT-PCR) in neural tissue of 46 of 64 cases. In 5 horses where myocardium, renal tissue or lungs were submitted, all extraneural tissues were devoid of WNV RNA (Kleiboeker *et al*., 2004). This has similarly been found in South African horses where extraneural tissues tested by RT-PCR or viral isolation in lineage 2 WNV CNS-positive cases were negative (Venter *et al*., 2009b).

1.3.2 Virus isolation

Isolation of WN virus is the "gold standard" for virus detection and confirmatory diagnosis in patients with WNV neurological disease, however it requires biosafety level 3 (BSL3) facilities, it takes more time (Beasley, 2005), is expensive since it still needs to be controlled by RT-PCR or immunofluorescent assay (IFA) with specific monocloncal antibodies (Hayes *et al*., 2005), and requires specialised training (Ledermann *et al*., 2011). WNV is best isolated in cell culture using Vero, RK-13 cells or mosquito cell lines (Castillo-Olivares & Wood, 2004), or in suckling mice (Hayes *et al*., 2005). WNV is rarely isolated from blood of infected patients because viraemia is typically low and waning at the time when clinical signs of neurological disease develop (Beasley, 2005; Hayes *et al*., 2005).

1.3.3 IgG and IgM serology, PNRT and flavivirus antibody cross-reactivity

Serological antibody assays, especially enzyme-linked immunosorbent assay (ELISA) testing, are the simplest, most commonly-used and most cost-effective means of flavivirus, including WNV, diagnosis in live subjects, and require few specialised apparatus or facilities since no live virus is used (Ledermann *et al*., 2011). Commercial WNV tests are however very expensive (personal communication, M Venter). Detection of virus-specific IgM in serum or CSF by IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) is strong evidence of recent WNV infection (Beasley, 2005; Hayes *et al*., 2005). WNV ELISA positive samples have to be

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confirmed by the more specific PRNT in endemic countries and where other flaviviruses occur (Beasley, 2005; Papa *et al*., 2011b) since WNV antibodies cross react with other flaviviruses. Production of cross-protective antibodies between closely related flaviviruses has been shown experimentally (Gould & Fikrig, 2004; Parks *et al*., 1958; Theiler & Casals, 1958). Convalescent sera of experimentally and naturally WNV-infected animals and humans were shown to contain neutralising antibodies against Japanese B, St Louis and Murray Valley flaviviruses, which are closely related to WNV; antibodies to less closely related Dengue and Russian spring-summer flaviviruses were also shown (Parks *et al*., 1958). This effect was reported in two human Australian laboratory infections with Kunjin virus (Allan *et al*., 1966).

Plaque reduction neutralisation testing (PRNT) is traditionally considered the "gold standard" for serological diagnosis and confirmation of viral infection, however it requires containment facilities, well-trained technical staff, is time consuming, expensive and uses live virus; it may also provide the most conservative interpretation of aetiology, however cross-reactivity between different flavivirus seropgroups may occur (Ledermann *et al*., 2011).

Detection of virus-specific IgM in serum confirmed by detection of IgG antibody in the same or a subsequent sample by PRNT are used for WNV diagnosis but require careful interpretation. Four-fold or greater increase in virus-specific serum antibody in paired samples confirms a recent infection (Beasley, 2005). Following an outbreak of lineage 2 WNV in Greece in summer-autumn of 2010, in 225 human patients IgG antibodies were found in serum after a mean of 8.1 +-4.9 days of onset of neurological disease and 12.6 +- 11.3 days of onset of non-neurological disease (Papa *et al*., 2011c). During this outbreak, which was linked to lineage 2 WNV after isolation of lineage 2 from mosquitoes (Papa *et al*., 2011a) and a sentinel chicken (Chaskopoulou *et al*., 2011), 262 people had WNV clinical disease and 35 died (Papa *et al*., 2011c). Serum IgM levels after the same outbreak persisted in 12% of infected individuals for up to 181-270 days post-infection with IgG levels remaining positive at all stages of follow-up in all

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patients, signalling caution in interpretion of WNV IgM positivity in serum and CSF of humans in endemic areas as due to recent infection (Papa *et al*., 2011b).

IgM antibody does not readily cross the blood-brain barrier so CSF IgM indicates CNS infection: more than 90% of human patients with meningo-encephalitis have IgM antibody in CSF within 8 days of symptom onset (Gould & Fikrig, 2004). IgM similarly does not cross the placenta, therefore the presence in serum and/or CSF of a human infant would indicate infection by WNV(Alpert *et al*., 2003).

Anti-WNV IgM response has been used as a diagnostic tool for detecting WNV infection in horses (Durand *et al*., 2002; Kleiboeker *et al*., 2004; Matter *et al*., 2005; Ostlund *et al*., 2001; Snook *et al*., 2001; Trock *et al*., 2001). Experimental infection of horses with WNV lineage 1 or lineage 2 (a 1978 Madagascan bird isolate) via the intradermal/intramuscular route resulted in WNV-specific antibodies by PRNT and MAC-ELISA by day 8PI. PRNT levels remained high until the end of the experiment (D27PI) while IgM peaked at days 8-12PI and decreased progressively until day 27PI. Lineage 1 and 2 produced similar antibody responses and both sera neutralised the lineage 1 strain used in the PRNT (Castillo-Olivares *et al*., 2011). Since IgM is short-lived in serum of horses, the presence of IgM in serum from a neurological horse is confirmatory of recent infection. A four-fold increase in PRNT titre in paired blood samples 14 days apart is needed to confirm recent WNV infection (Castillo-Olivares *et al*., 2011).

1.3.4. Immunohistochemistry and *in situ* hybridisation

Immunohistochemistry (IHC/IMH) and *in situ* hybridisation (ISH) are performed on formalin-fixed wax-embedded tissues and may be used to detect WNV with variable reliability in different species, and with the possibility of flavivirus cross reactivity depending on the specific antibody used.

IHC staining of formalin-fixed WNV infected human tissues using specific monoclonal and polyclonal antibodies was found to be more sensitive than viral culture, showing

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WNV antigen in up to 50% of fatal WNV neuroinvasive disease cases, and was particularly useful in patients dying during the first week of illness when virus concentrations in CNS were high (Hayes *et al.*, 2005). Antigen was usually found within neurons and their processes, especially in the brain stem and anterior horn of the spinal cord, however antigen was generally focal and sparse except in immunosuppressed patients where it is seen extensively throughout the CNS (Hayes *et al.*, 2005).

IHC of CNS in lineage 1 WNV positive horses has proved often disappointing and unreliable when compared with other diagnostic techniques, due to scant detectable antigen found with either monocloncal or polyclonal antibodies (Buckweitz *et al.*, 2003; Cantile *et al.*, 2001; Castillo-Olivares & Wood, 2004; Kleiboeker *et al.*, 2004), when compared with the amount of antigen in various organs and brain of other species like birds (Steele *et al.*, 2000) and fatal immunocompromised human cases (Hayes *et al.*, 2005). Horses with WNV neurological disease are frequently negative with IHC testing in the presence of positive PCR results (Buckweitz *et al.*, 2003), and the amount of virus does not correlate with the degree of inflammation, with distribution being very spatial. Cantile *et al.,* (2001) found scant antigen in the cytoplasm of few neurons, fibres, glial cells and macrophages despite inflammatory changes, suggesting that the immunopathological component of equine WNV infection was more prominent than in other species (Cantile *et al.*, 2001; Kleiboeker *et al.*, 2004). Hamsters, by comparison, show the opposite trend with much antigen, neuronal apoptosis likely caused by the virus, and little inflammation (Castillo-Olivares & Wood, 2004; Kleiboeker *et al.*, 2004).

In a recent publication (Pennick *et al.*, 2012), in formalin-fixed paraffin-embedded brain tissue sections with microscopic lesions from 16 horses diagnosed positive by RT-PCR for WNV, 0/16 were found positive by IHC and only 2/16 were positive with ISH. It was concluded that IHC and ISH are not effective for the diagnosis of equine neurological cases of WNV. Avian WNV-infected tissues had to be used in that study as the WNV positive control due to difficulty in identifying suitable positive equine brain tissue. RT-PCR for WNV was used as the gold standard to confirm infection (Pennick *et al.*, 2012).

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1.3.5 Electron microscopy

Visualisation of particles of WNV in tissues by electron microscopy (EM) is rare, and when found they are seen within endoplasmic reticulum of neurons (Hayes *et al*., 2005).

Transmission electron microscopy (TEM) on fixed WNV-infected bird tissues from the New York 1999 outbreak (Steele *et al*., 2000), showed 35-45nm diameter flavivirus-like particles with typical morphology (dense core surrounded by a thin diffuse outer layer) frequently in cerebrum and cerebellum of a Chilean flamingo but infequently in heart of this bird. Particles were usually in cytoplasmic vacuoles in the perikaryon and in neuritic processes, and less frequently in dilated endoplasmic reticulum (rER) of the perikaryon and extracellular spaces in the neuropil. Some virions were in vacuoles of stromal cells in connective tissue septae of the heart. There were also membranous vesicles of about 100nm diameter in dilated rER and vacuoles; these being identical to smooth membrane structures described by others in the rER cisternae of flavivirus-infected cells. Nonstructural proteins of Kunjin virus had also been associated with smooth membrane structures. In the perikaryon of a few neurons and occasionally in oligodendrocytes of a pheasant, aggregates of electron-dense granules found associated with dense membrane vesicle structures and convoluted membranes were consistent in morphology with flavivirus-induced structures described with JEV-infected neurons and Kunjin virus-infected Vero cells. Cells containing flavivirus-like particles or flavivirus-induced structures also had disorganisation of rER and Golgi apparatus and marked vesiculation and vacuolation of the cytoplasm. Some brain sections of the Chilean flamingo showed vacuolar breakdown of myelin sheaths. Other bird specimens examined did not contain flavivirus-like particles or intracellular inclusion bodies. Post embedment immunoelectron microscopy of cerebrum and cerebellum from the Chilean flamingo showed diffuse gold-sphere labelling of amorphous electron-dense material and associated membranes in the perikaryon and neuronal processes, but not associated with distinct structures. Labelling was, however, considered WNV-specific. Virions were much less intensely labelled than the presumed flaviviral-induced dense membrane vesicle structures and convoluted membranes and these findings were

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consistent with immunoelectron microscopy of WNV-infected Vero cells which had sparse labelling of virions (Steele *et al*., 2000).

Several studies on ultrastructural changes during flavivirus replication generally concluded that these viruses mature in close association with the endoplasmic reticulum. The rare phenomenon of plasma membrane budding was seen in lineage 2 Sarafend strain WNV infected Vero cells by TEM (Ng *et al*., 1994), and in C6/36 mosquito tissue culture cells infected with Dengue-2 virus (PR-159 strain) (Hase *et al*., 1987).

1.3.6. Histopathology

There are no pathognomonic lesions in WNV infection (Hayes *et al*., 2005), however patients with WNV neuroinvasive disease generally have nonsuppurative encephalo-myelitis and variable meningitis. Histopathological findings are nonspecific (Kelley *et al*., 2003) and similar in several viral encephalitides and so causal differentiation between them has to rely on specific serologic or CSF testing (Kelley *et al*., 2003), demonstration of WNV antigen in the tissues by antigen labelling with specific antibodies using IHC or immunofluorescence, by virus isolation or by demonstration of viral RNA by means of RT-PCR or *in situ* hybridization.

1.3.7 Differential diagnoses in horses

Clinical signs of meningo-encephalomyelitis may be caused by most neurotropic viruses including rabies virus; neurotropic arboviruses including the flaviviruses WNV and Wesselsbron (Venter *et al*., 2009b), Old World alphaviruses in Africa including Sindbis and Middelburg (unpublished M.Venter, S. Human, S.Van Niekerk *et al.,*), the orbiviruses causing equine encephalosis and African horsesickness (latter unpublished J Williams, M Venter *et al.,*), and the orthobunyavirus Shuni (Van Eeden *et al*., 2012); equine herpes virus -1 and - 4; Borna disease which has not yet been reported in South Africa, and the New World alphaviral encephalitides reported only in the Americas (Eastern-, Western- and Venezuelen Equine Encephalitis) (Castillo-Olivares & Wood, 2004). Bacterial meningo-encephalitis and botulism occasionally affect horses in South

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Africa. The protozoans *Sarcocystis neurona* and *Neospora hughesii* which cause equine protozoal myeloencephalitis (Wobeser *et al*., 2009) have not yet been diagnosed in South Africa. Non-infectious diseases or conditions include CNS trauma, CNS neoplasia such as pituitary *pars intermedia* adenoma, CNS granulomatous lesions such as cholesteatoma, and metabolic disturbances which include hypocalcaemia, secondary renal and hepatic encephalopathies and intestinal hyperammonaemia. Toxicities leading to neurological signs include lead ingestion, and *Fusarium verticillioides* fungal ingestion with fumonisin B1-induced leucoencephalomalacia ("mouldy corn poisoning"). The neurotropic nematode *Halicephalobus gingivalis* has recently been diagnosed in a horse which had been moved from the western Cape to Namibia, and was suspected of having WNV infection (unpublished J. Williams, A. Hartmann, V. Schwann). Diseases in other body systems causing clinical signs which may be confused with primary neurological signs include laminitis, myopathies, musculo-skeletal trauma or congenital defects, primary ocular disease resulting in blindness, and weakness, collapse or seizures due to dehydration, anaemia, respiratory or cardiovascular disease.

1.4 EPIDEMIOLOGY

1.4.1 Natural life-cycle of WNV

1.4.1.1 Vectors

Culex spp, breeding in fixed standing water bodies, are the most preferred vectors of WNV (Jupp, 2001; McLean *et al*., 2002) but *Aedes* spp, breeding in seasonal temporary flood waters, are also involved in some regions (MMWR, 2005; Rodhain *et al*., 1985). WNV has also been isolated from *Anopheles, Minomyia* and *Mansonia* mosquitoes in Africa, Asia and the USA (Brinton, 2002) as well as from ixodid and argasid ticks in the Asian republics of Russia, from *Ornithodorus* sp collected from nests of herring gulls on islands on the Caspian sea where mosquitoes are absent (Malik Peiris & Amerasinghe, 1994), and from a *Haemophysalis leachi* tick found on a dog in South Africa (Blackburn *et al*., 1989). Ticks were thought to be of less likely importance in the natural life-cycle than mosquitoes (Malik Peiris & Amerasinghe, 1994). *Argas persicus* ticks were used to successfully transmit WNV to chickens 20 days after feeding on a virus-serum mix,

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suggesting, however, that ticks could play a biological role in transmission (Abbassy *et al.*, 1993).

1.4.1.2 Birds

In 1999 WNV appeared in the Western Hemisphere for the first time, killing many birds, especially corvids, as well as several horses and humans in New York, from where it spread annually across North America and southward into Latin America and the Caribbean (Hayes & Gubler, 2006; Matter *et al.*, 2005). This spread was due most likely to local dispersal movements of house sparrows as well as of the mosquito vectors (Rappole & Hubalek, 2003).

The WNV New York 1999 strain that arrived by uncertain means in the USA was most closely homologous to an isolate from a dead goose in Israel in 1998 (Lanciotti *et al.*, 1999). Transmission cycles of WNV in Europe and North America are typically maintained in passerine birds, in particular house sparrows (*Passer domesticus*), which is the only New World avian host with a prolonged (5-6days) high titre viraemia (Komar, 2000; Komar *et al.*, 2003).

During the initial outbreak of WNV in New York in 1999 (Ludwig *et al.*, 2002), morbidity and mortality occurred in the Bronx Zoo/Wildlife Conservation Park's bird collection. None of the mammals died but 8% had WNV antibody. 83% (14 of 17) of bird deaths occurred in New World bird species versus 5% (3 of 57) Old World species, suggesting that co-evolution of WNV with these birds may have led to genetic resistance/stability where infection rates are high but disease and mortality low. Birds native to the New World have not had sufficient time to acquire or exploit genetic resistance to the virus. As with other observations, *Corvidae* had the highest rate of disease, but also affected were owls, penguins and ibis families (Ludwig *et al.*, 2002).

Live or dead bird surveillance has been extremely useful in predicting outbreaks of WNV lineage 1 in humans or horses, since they are the first to show viraemias and or illness or death (Eidson *et al.*, 2001; McLean *et al.*, 2002), with chickens being a useful

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live sentinel species since they are relatively resistant to WNV disease but produce antibodies, with the exception of young birds (Jupp, 2001; Langevin *et al*., 2001; Senne *et al*., 2000; Styler *et al*., 2007).

Southern African bird species were found or assumed in early research to be clinically unaffected by natural or experimental infection with local lineage 2 strains (Jupp, 2001; McIntosh *et al*., 1969; McIntosh *et al*., 1976; McIntosh *et al*., 1968). Lineage 2 WNV research in indigenous birds in South Africa showed 27 wild bird spp of 14 families to have antibodies to WNV (Jupp, 2001; McIntosh *et al*., 1968). Raptors and corvids were not tested. Adult wild birds of 13 spp trapped between 1962 and 1965 at Olifantsvlei near Johannesburg (McIntosh *et al*., 1969) were inoculated intramuscularly with WNV strain H442, which had been isolated from a human with mild febrile illness at Ndumu in Natal in 1958 (Burt *et al*., 2002). Only one sacred ibis failed to develop viraemia and red bishop, masked weaver, laughing doves, and cattle egrets, all had titres of >6 $\log_{10}LD_{50}$/mL. Viraemias lasted for 3 days. McIntosh *et al.,* (1969) state: "While light mortality occurred among birds during the period of viraemia and also on later days, this was probably caused by handling and confinement and it seemed that no observable ill-effects could be attributed to either virus". Diagnostic necropsies were not mentioned for the dead birds. Sindbis, an Alphavirus, which has the same life-cycle as WNV, had also been inoculated in different birds (McIntosh *et al*., 1969).

1.4.1.3 WNN transmission in other species

Several vertebrates other than birds have been found to be susceptible to WNV infection but are incidental or dead-end hosts due to viraemias insufficient to transmit virus via vectors (McLean *et al*., 2002; MMWR, 2005). Other species, such as domestic pigs after experimental infection (Teehee *et al*., 2005), develop antibodies without apparent signs of illness.

Alligators (Klenk *et al*., 2004), Madagascan lemurs (Rodhain *et al*., 1985), cats (Austgen *et al*., 2004) and dogs (Blackburn *et al*., 1989) have, however, been shown experimentally to develop viraemias sufficient to actually, as in lemurs with *Aedes*

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aegyptii, or theoretically, permit vector infection and further transmission, thus not being dead-end hosts. Madagascan lemurs showed inapparent infection with virus amplification (Rodhain *et al*., 1985). Rhesus monkeys (Pogodina *et al*., 1983) and hamsters (Tonry *et al*., 2005) show post-infection prolonged viral persistence.

1.4.2 South Africa

An epidemic of West Nile fever occurred in the arid Karoo region affecting thousands of people in the summer of 1973-1974 (McIntosh *et al*., 1976), and another large human outbreak together with Sindbis virus occurred on the highveld in summer of 1984 (Jupp, 2001; Jupp *et al*., 1986). Lineage 2 strains in general caused only sporadic cases of mild flu-like symptoms including fever, myalgia, arthralgia and a maculopapular skin rash in humans (Jupp, 2001). Prior to 2002, Burt *et al.,* (2002) listed, as mentioned previously, only single fatalities ascribed to WNV (Burt *et al*., 2002). Occasional cases of WNV-associated renal failure, fever, rash, myalgia, arthralgia and encephalitis recovered (Jupp, 2001) and two South Africans recovered from meningo-encephalitis.

An early (1956) small survey of 17 horses at Isis Farm near Johannesburg (Kokernot *et al*., 1956) found 1/17 horses had protective antibodies to Sindbis virus, and 8/17 to WNV. A 1962 survey (McIntosh *et al*., 1962) of blood from 18 horses and mules from Vaalhartz and 5 from Lake Chrissie on the highveld plateau of South Africa, showed the Lake Chrissie horses to be HI positive for flavivirus antibodies, and 4 of those sera were also neutralisation positive for WNV, whereas 5 of the Vaalhartz sera were HI positive with only one of those also having neutralising antibodies.

Serum tested of Thoroughbred mares and foals from 2000-2001 in South Africa showed 75% of mares to have WNV neutralising antibodies, and seroconversion of 11% of foals occurred by the time of the annual yearling sales, without record of prior illness (Guthrie *et al*., 2003). An infection study of 2 seronegative horses reported in the same publication using a local WNV lineage 2 isolate did not result in clinical signs or viraemia. Subsequent mouse studies using northern hemisphere lineage 1 and South

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African lineage 2 isolates showed that both lineages have similarly neuroinvasive and mild strains (Beasley *et al*., 2002; Venter *et al*., 2005).

Later investigation beginning in 2007 showed that lineage 2 WNV is a cause of severe and often fatal neuroinvasive diseases in horses in South Africa (Venter *et al*., 2009b). The first case of lineage 1 WNV fatal neurological infection, was subsequently reported in a Thoroughbred mare and her aborted foal from the western Cape (Venter *et al*., 2011). The lineage 1 strain sequenced from brain of both mare and foal was closest genetically to a Tunisian strain isolated from a person who died in 1997 of neurological disease (Venter *et al*., 2011), with the E-protein being similar to Tunisian and Russian strains.

1.5 IMMUNOLOGY OF WNV

1.5.1 Humans

Humans with compromised immune systems such as transplant patients and patients on anti-T cell therapies (Klein & Diamond, 2008), of advanced age over 50 years (Sampathkumar, 2003), and with underlying conditions like diabetis mellitus, are at increased risk for manifesting neurological signs with WNV infection (Lim *et al*., 2011). WNV can be transmitted to humans, which are incidental hosts, via infected mosquito bite, accidental aerosol inhalation (Nir *et al*., 1965; Nir, 1959), handling of infected equine brain (most likely aerosol inhalation) (Venter *et al*., 2010), intrauterine transmission (Alpert *et al*., 2003), infected blood or blood product transfusion (Montgomery *et al*., 2006; Sampathkumar, 2003), breast-feeding, transplacentally, and by occupational exposure in laboratory workers (Sampathkumar, 2003; Venter *et al*., 2009a). Peripheral immune responses can prevent encephalitis, however up to 40% of immunocompetent animals infected with virulent WNV strains develop lethal neuroinvasive disease, suggesting the immune system as playing a role in the associated pathology (Lim *et al*., 2011). In older people the thymic output of naive T cells diminishes responses to novel antigens such as WNV, while clonal expansion has been found to lead to defects in the T cell repertoire and blunted responses of memory T cells to conserved epitopes of the influenza virus (Lim *et al*., 2011). People on anti-T-

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cell therapies have a high incidence of WNV neuroinvasive disease (Klein & Diamond, 2008). Macrophages from elderly people cannot down regulate toll-like receptor 3 (TLR3) in infected cells, with the hypothesis being that these cells then produce high levels of pro-inflammatory and vasculogenic cytokines and resultant neuropathology (Lim *et al.*, 2011).

Rash, occurring in 50% of patients and typically maculopapular of skin of chest, back and arms and lasting usually less than 1 week, has been reported as a prognostic indicator of severe WNV disease and death. Acute signs last for 3 to 10 days but full recovery may take up to 60 days (Rizzo *et al.*, 2011). Neurological disease occurs in approximately 1% of infections, manifesting as meningitis, encephalitis or acute flaccid paralysis. The proportion of West Nile Neurological Disease (WNND) over total WN virus disease (WNVD) is approximately 30% for children and young adults, and 50% in adults over age 50 years. Meningitis is more commonly described in children and has a more favourable prognosis than encephalitis, despite some longterm sequelae. Acute WNV-associated flaccid paralysis is asymmetrical and may occur without CNS involvement (Rizzo *et al.*, 2011).

1.5.2 Mice

Most nonfatal WNV infections appear to be cleared by several host immune responses (Diamond *et al.*, 2003b; Hayes *et al.*, 2005; Wang & Fikrig, 2004).

A 2008 review by Klein and Diamond (2008) states that there is a high incidence of WNV neuroinvasive disease in patients on anti-T-cell therapies and in T cell deficient mice; both T and B lymphocytes protect against disseminated infection, shown in severe combined immunodeficiency (SCID) mice which uniformly succumb to WNV infection. Humoral immune responses control viral load peripheral to the CNS and prevent dissemination, whereas T-cell responses are required for the clearance of WNV within the CNS. Recent work addressing the extent that the immune responses are pathological, indicate that the presence of virus-specific cytolytic T cells in the CNS

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during infection by virulent strains of WNV, appear to diminish immunopathology (Klein & Diamond, 2008).

Various experiments in mice have shown that B cells with IgM and IgG production play a crucial role in preventing dissemination of WNV infection to the CNS by terminating viraemia (Diamond *et al.*, 2003a; Diamond *et al.*, 2003c), while CD8+ T cells have a very important role in clearing infection from tissues, especially the CNS, and preventing viral persistence (Shrestha & Diamond, 2004; Shrestha *et al.*, 2006a; Wang *et al.*, 2006; Wang *et al.*, 2003). Sitati and Diamond (2006) showed in CD4+ T cell deficient mice that CD4+ T-cells are necessary to prevent protracted high-level WNV CNS infection: WNV-specific CD8+ T cells were not affected by the absence of CD4+ cells at day 9 (d9) post infection (PI) but were markedly compromised by d15. They concluded that the dominant protective role of CD4+ T cells during WNV infection is to help antibody responses and sustain WNV-specific CD8+ T cell responses in the CNS enabling viral clearance (Sitati & Diamond, 2006). CD4+ T cells were, however, not found by immunohistochemistry in the brains of mice in one study (Wang *et al.*, 2003).

High doses of lineage 2 Sarafend strain WNV in C57B/61 mice resulted in CNS infection within 24h and minimal neuronal destruction with average survival time only 6 days; low doses elicited more prominent CNS immunopathology and longer mean survival times (Wang *et al.*, 2003). Despite a normal quantitaive antibody response, WNV can, however, still enter the brain (Lim *et al.*, 2011).

The speed and way in which B cells respond to WNV infection are key factors influencing viral neuroinvasiveness and the outcome of disease (Papa *et al.*, 2011c). Early polyclonal B-cell activation requires sustained signalling through the type 1 interferon (IFN)-alpha/beta receptor, and innate immunity and adaptive immune regulation are critical for the control of WNV infection by regulating the level, the IgG class switching, and neutralising capacity of antibodies (Papa *et al.*, 2011c).

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The increase in blood brain barrier (BBB) permeability in rodents (Morrey *et al*., 2008a) was found to be lacking or inconsistent in WNV infections in different mouse strains or rodent species. The need for CD4+ and CD8+ T cells to enter and clear WNV from the CNS was emphasized with the supposition that increased BBB permeability may not be required for T cells to access the CNS. It was suggested that a first wave of low-efficiency migration of T lymphocytes may cross into the CSF from blood via the choroid plexus where the BBB in not completely intact, or from subarachnoid blood via meningeal vessels or from blood to parenchymal perivascular spaces. Different model systems have also shown that activated lymphocytes can enter the CNS of normal individuals (Morrey *et al*., 2008a). The apoptotic effect on T lymphocytes by glucocorticoids (Herold *et al*., 2006) therefore make glucocorticoids a controversial treatment in viral meningo-encephalitides.

Several non-structural proteins of arboviruses from different familes have the properties of causing apoptosis, inhibiting host-protein synthesis and blocking type 1 interferon, suggesting that these abilities to induce death and to subvert the innate immune system are critical for their maintenance in nature (Hollidge *et al*., 2010).

Neutrophils appear to play a paradoxical role in WNV pathogenesis, and were found to be the most abundant cell type in the peritoneal cavity as early as 12 hours after WNV inoculation – they seem to be the predominant immune cells initially and rapidly recruited to sites of WNV infection (Bai *et al*., 2010). Mice depleted of neutrophils had significantly lower WNV levels in blood 2 to 3 days PI and increased survival rates. If mice were infected before depletion of neutrophils 1 to 2 days after infection, they showed higher viral loads and reduced survival rates. It was concluded that WNV may replicate in neutrophils and increase viral load in blood early during infection, but later they contribute to control of infection (Bai *et al*., 2010). It is interesting to speculate that neutrophils might play a critical role in WNV replication and dissemination *in vivo*, especially in humans (Lim *et al*., 2011).

1.5.3 Horses

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1.5.3.1 Immunology

Anti-WNV IgM response has been used as a diagnostic tool for detecting equine WNV infection (Durand *et al*., 2002; Kleiboeker *et al*., 2004; Matter *et al*., 2005; Ostlund *et al*., 2001; Snook *et al*., 2001; Trock *et al*., 2001).

Experimental infection of horses with WNV lineage 1 (035EDV0301 from the spinal cord of a horse affected during the 1999 North American outbreak) or lineage 2 (a 1978 Madagascan bird isolate) via the intradermal/intramuscular route resulted in WNV-specific antibodies by PRNT and IgM antibody-capture ELISA (MAC-ELISA) by day 8PI. Only 2 ponies infected with lineage 1 WNV developed mild muscle fasciculations. PRNT levels remained high until the end of the experiment (D27PI) while IgM peaked at days 8-12PI and decreased progressively until day 27PI. Lineage 1 and 2 produced similar antibody responses and both sera neutralised the lineage 1 strain used in the PRNT (Castillo-Olivares *et al*., 2011). Since IgM is short-lived in serum of horses, the presence of IgM in serum from a neurological horse is confirmatory of recent infection. A four-fold increase in PRNT titre in paired blood samples 14 days apart is needed to confirm recent WNV infection (Castillo-Olivares *et al*., 2011).

In WNV serologically-naive young horses infected with WNV lineage 1, 21 days after either a single or two successive inoculations of SLEV (2 cohorts of 6 horses each), WNV IgM ELISA, IgG ELISA and blocking ELISA were generally negative until days 9 to 12 PI, with a single animal having detectable antibody at day 3PI (Ledermann *et al*., 2011). Some horses never generated detectable ELISA antibodies to either virus but did have neutralising antibodies to SLEV at days 21 and 30PI. Two horses positive for IgG, flavivirus- and WNV-specific blocking ELISA, never demonstrated an IgM-specific response. One horse developed WNV IgM, IgG and blocking ELISA antibodies but not neutralising antibodies; it is likely that the IgM generated in early infection in this animal was not neutralising, as has been shown in humans. The remaining horses developed IgM responses which appeared to be cross-reactive, with SLEV antibodies only occurring after WNV infection and mirroring those of WNV. In one horse WNV IgM persisted for only 7 days. IgG was produced by all 6 of the SLEV-WNV group between

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days 6 and 15 after WNV injection and persisted until the end of the experiment (day 21PI). The WNV-specific blocking ELISA was very specific and never generated a false positive even after repeated flavivirus exposure, thus being an excellent option for WNV diagnosis in horses with a history of, or possible exposure to previous flavivirus infection. The equine-adapted IgG ELISA used unexpectedly generated results similar to these suggesting it is more specific for WNV than the human assay (Ledermann *et al*., 2011).

Results of a DNA plasmid vaccine study in horses (Chiang *et al*., 2005), showed no association between detection of IgM response and detection of viraemia after experimental challenge**.**

1.5.3.2 Equine WNV vaccines

Fort Dodge, taken over in 2010 by Pfizer Animal Health, now renamed Zoetis, produced two vaccines for use in horses: a formalin-inactivated product (Tesh *et al*., 2002) called Duvaxyn-WNV, and a DNA plasmid vaccine, WN Innovator DNA (Chiang *et al*., 2005; Nalca *et al*., 2003). The former was licensed for use in the USA since 2003 and since 2010 in Europe and the United Kingdom. Duvaxyn-WNV is the lineage 1 vaccine tested in the cross-lineage mouse vaccine study from which most of the control non-vaccinated, infected mice were sourced for this MSc pathology study (Venter *et al*., 2013). The Fort Dodge killed vaccine was tested in hamsters and found to be protective against lineage 1 challenge, but 2 of the 9 animals had detectable viraemia suggesting that the immune response was not sufficient to completely inhibit replication of the challenge virus (Nalca *et al*., 2003).

Other WNV vaccines were constructed and tested in laboratory animals and horses, including a WNV recombinant plasmid expressing WNV prM an E proteins which afforded protection in horses and mice (Davis *et al*., 2001), a live attenuated chimeric virus vaccine (Tesh *et al*., 2002), and a recombinant canarypox vaccine which carries the prM/E WNV genes and is marketed for equine use in the USA and Europe by Merial (Minke *et al*., 2011; Minke *et al*., 2004) and has recently been registered for use in

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horses in South Africa (personal communication 2013 Drs Hein Hesse and Lauren Gerarty, Merial, South Africa).

Horses in the USA and Europe have been largely protected against massive outbreaks of WNV disease since the introduction of equine WNV vaccines in the early 2000s in the USA and 2010 in Europe, and this protection continues in areas of high WNV risk where vaccination is correctly implemented (Schuler *et al.*, 2004). During the summer of 2002 1,698 cases of equine WNV were reported in Texas, USA, of which 68.9% of cases had not been vaccinated during the prior 12 months. Vaccines were found to reduce the risk of death by 44% even if they had not been given early enough in advance of WNV infection (Ward *et al.*, 2006).

Since the recognition that WNV of both lineages 1 and 2 is a cause of severe fatal neurological equine disease in South Africa (Venter *et al.*, 2011; Venter *et al.*, 2009b) Pfizer/Zoetis Animal Health is in the process of registration of Duvaxyn for equine use in this country. The mouse cross lineage vaccine experiment from which control non-vaccinated infected mice were sourced for this thesis was a first step towards this process (Venter *et al.*, 2013).

1.6. PATHOGENESIS OF WNV INFECTION

1.6.1 The use of mice and hamsters for WNV studies.

It has been stated that all classical laboratory strains of mice develop encephalitis with 100% mortality by intracerebral and intraperitoneal routes of WNV infection (Nalca *et al.*, 2003). Eldadah *et al.,* (1967) used the EG101 lineage 1 strain of WNV isolated from a human and concluded that suckling mice are highly susceptible to all infections leading to fatal disease, with no sublethal infections. Recent studies showed that older mice show less susceptibility to some mild lineage 1 and lineage 2 strains of WNV (Beasley *et al.*, 2002; Venter *et al.*, 2005). Mice are the cheapest and preferred animal models for WNV experiments, over monkeys (Pogodina *et al.*, 1983) and hamsters (Steele *et al.*, 2000), which latter two species are also variably susceptible and the

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brains of which become persistently infected post inoculation. The hamster model more closely represents human disease than the mouse model (Nalca *et al*., 2003).

1.6.1.1 Pathogenesis of WNV infection in experimental mice and hamsters

The original West Nile virus isolate from a febrile Ugandan woman in 1937 (Smithburn *et al*., 1940) was a lineage 2 strain (Hayes & Gubler, 2006), and was found to be neurovirulent in mice when injected by various routes (Smithburn *et al*., 1940). Inoculated mice became hyperactive with roughened coats, and later hypoactive, weak, hunched and at times with paralysed hindquarters. Coma preceded death, which occurred within 12 to 72 hours after the first clinical signs. After a few passages of virus, death of mice usually occurred on the 4th or 5th day but at times occurred by the 3rd day after intracerebral inoculation. With the minimal infective dose, the incubation period was 4 to 5 days and survival time 5 to 10 days. Intracerebral, intranasal and intraperitoneal routes of infection resulted in disease but subcutaneous infection did not. After intraperitoneal injection, virus reached the brain within 24 hours, and multiplied there reaching high titres by the 3rd day.

Since 1940 numerous pathogenesis studies have been performed in experimental mice of different ages and strains using various field and laboratory strains of lineages 1 and 2 WNV, and also in several hamster experiments (Eldadah & Nathanson, 1967; Eldadah *et al*., 1967; Garcia-Tapia *et al*., 2007; Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Monath *et al*., 1983; Morrey *et al*., 2010; Morrey *et al*., 2008b; Shrestha *et al*., 2003; Wang *et al*., 2011). The immunity and pathogenesis of WNV has been recently reviewed by Lim *et al.,* (2011) wherein the authors state that most of our knowledge of the immune response and pathogensis of WNV infection has been derived from mouse studies (Lim *et al*., 2011).

After a WNV-infected mosquito bite (Lim *et al*., 2011), WNV is believed to infect keratinocytes and Langerhans cells which then migrate to regional lymph nodes for initial replication before spreading systemically to visceral organs including kidney and spleen (Brown *et al*., 2007; Shrestha *et al*., 2006b), where replication occurs again,

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presumably in epithelial cells and macrophages. Dependent on the level of viraemia, WNV may cross the BBB causing meningo-encephalitis, with differences in the protein at position 67 on the viral E glycoprotein domain III (the receptor binding domain) being implicated in flaviviruses being either neuroinvasiveness or having the tendency to cause haemorrhagic disease (Lim *et al*., 2011).

Entry of WNV into the CNS is proposed by several mechanisms (Lim *et al*., 2011). These include passive transport through vascular endothelium (Brown *et al*., 2007; Wang *et al*., 2004) or choroid plexus epithelial cells (Hunsperger & Roehrig, 2006; Morrey *et al*., 2008a); olfactory neuron infection with spread to the olfactory bulb (Brown *et al*., 2007; Eldadah & Nathanson, 1967; Hunsperger & Roehrig, 2006; Monath *et al*., 1983; Nir *et al*., 1965; Wang *et al*., 2004); by infected “Trojan horse” trafficking via infected immune cells passing into the CNS (Garcia-Tapia *et al*., 2006); by direct axonal retrograde transport from peripheral neurons (Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Morrey *et al*., 2008b; Samuel *et al*., 2007; Wang *et al*., 2009); by centrifugal spread to peripheral nerves after intracerebral inoculation (Eldadah & Nathanson, 1967); or by multiple point entry into CNS (Brown *et al*., 2007).

Eldadah *et al.,* (1967) found that lineage 1 EG101 strain WNV multiples in suckling mice primarily in the cytoplasm and major dendrites of neurons, where it is found within 12 hours of inoculation, rising to high titres at day 2, peaking at days 3 and 4 and dropping on day 5, preceding death. Adult mice followed a similar pattern but one day later, with peak titres approximately tenfold lower, and animals survived an extra day. WNV has been found in suckling mice to be pantropic, producing marked infection of muscle, cartilage and a number of other tissues, but with little, if any, widespread infection in resistant animals after intracerebral inoculation. Spinal cord lesions were infrequent and always mild, suggesting that the severe clinical paralysis was probably of cerebral origin. Virus was present in all the many regions of brain examined and in late stages in peripheral nerves probably via centrifugal spread. In sensory ganglia it was found around the nuclei, suggesting spread via axons (Eldadah & Nathanson, 1967).

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Focal infection of the grey matter of the anterior horn of the spinal cord has been proposed as the pathogenesis for acute flaccid paralysis in WNV-infected human patients showing asymmetric weakness, areflexia, and yet no sensory abnormalities; with histopathology supporting a poliomyelitis-like syndrome (Morrey *et al.*, 2008b). Experimental inoculation of WNV into peripheral spinal cord neurons of hamsters resulted in limb paralysis that was directly associated with infection and injury of anterior/ventral horn motor neurons in the lumbar spinal cord (Morrey *et al.*, 2008b). The authors also injected WNV into the sciatic nerve or spinal cord, mapped the lesion to the ventral motor horn of the grey matter, and found neuronal apoptosis and diminished cell function by specialised staining (TUNEL and choline acetyltransferase staining) due to paucity of light microscopically visible lesions (Morrey *et al.*, 2008b). Similar multifocal poliomyelitis with presence of WNV antigen in ventral and lateral horns of the spinal cord grey matter are found in naturally infected horses (Cantile *et al.*, 2001) and birds (Steele *et al.*, 2000).

More recently, scientists also using hamsters (Morrey *et al.*, 2010; Wang *et al.*, 2011) sought to find neurological explanations for the respiratory, gastrointestinal, bladder and cardiac dysfunction found in some humans with WNV infection. WNV-infected hamsters show gastric and intestinal distension at day 9 after viral challenge, and electromyographs (EMG) of intestinal muscles were significantly reduced. Myenteric neurons innervating the intestine as well as brain stem neurons which control autonomic function were infected with WNV, suggesting that these were the cause of the gastro-intestinal (GI) dysfunction (Wang *et al.*, 2011). Autonomic function and heart rate variability were suppressed in WNV infected hamsters leading to reduced HRV and reduced EMG amplitudes of the GI tract (Wang *et al.*, 2011). EMGs of diaphragms of subcutaneously WNV-infected hamsters showed suppression at day 3 which continued to day 17PI; WNV-immunoreactive neurons were, however, only found in the brain stem and some cervical spinal cords in these animals (Morrey *et al.*, 2010). When WNV was injected directly into the ventrolateral medulla containing the respiratory functions and into spinal cord at C4, EMG suppression of diaphragms occurred within 4 days, with WNV detection in brain stem neurons at day 3, suggesting effects via the vagal

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afferents acting on respiratory control neurons in the brain stem. Brain auditory evoked response deficiency only occurred after day 11, suggesting multiple phases of WNV-induced neurological disease with WNV infection (Morrey *et al*., 2010).

A recent study (Kimura *et al*., 2010) using footpad lineage 1 WNV inoculated 6-week-old C3H mice resulted in encephalitis affecting the grey matter of the CNS from day 9 post infection (Kimura *et al*., 2010). Viral antigen was found by IHC initially in a few neurons of the ipselateral ventral horn of the lumbar spinal cord on day 7PI, suggesting transneural spread via the sciatic nerve. Foci of antigen were found in multiple regions of the brain, always intracytoplasmically, mostly in neurons and neuronal processes, but occasionally in astrocyte-type cells as well. Only a small number of mice showed diffuse severe inflammation and antigen distribution. The Kimura study also described for the first time in mice similar gastro-intestinal distension with watery fluid as described previously in WNV-infected hamsters, and concluded that this resulted from WNV infection of myenteric ganglia, many of which showed histologic lesions and IHC positivity (Kimura *et al*., 2010). In the same study, WNV immunoreactivity was not detected in the spleen and lymph nodes of infected mice, both known major organs of viral replication (Brown *et al*., 2007; Shrestha *et al*., 2006b), possibly due to levels being too low for IHC detection (Kimura *et al*., 2010). Surviving immunocompetent mice in this study cleared WNV from all tissues within 2-3 weeks post infection. This paper stated that histological events occurring during infection especially in peripheral tissues had not been fully characterised in mice: this prompted comparable investigation of extra-CNS lesions in the mouse study of the current dissertation with both lineages 1 and 2 in BALBc mice.

Peripheral subcutaneous infection simulates mosquito inoculation as in human infection. Footpad inoculation of mice differed from intraperitoneal inoculation where younger animals were found to be more susceptible than older animals in one study (Hunsperger & Roehrig, 2006). The sensitive TaqMan testing indicated early low level (d1 PI in brainstem and d2 in spinal cord) CNS infection, before the end of viraemia (d5 PI), which then diminished and later increased from days 6-9 PI. The large diameter dorsal

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root ganglion neurons of the peripheral nervous system (PNS) correspond to discriminatory touch pressure in ascending sensory tracts and muscle proprioception (position sensory) pathways; these neurons also enervate dermal epithelium, where primary inoculation of virus occurs, suggesting retrograde transport of virus from the inoculation site. Previous work suggested that dorsal root ganglion (DRG) cells can remain persistently infected without cytopathic effect, and possibly contribute to persistent IgM expression in humans sometimes for 500 days PI. These DRG neurons send peripheral information to the somatosensory and piriform cortex for processing, via tracts that synapse onto the thalamus – the presence of viral antigen in both sites suggested introduction of virus from peripheral neurons into the brain via retrograde transport. The piriform cortex is also involved in sensory information primarily from olfactory neurons and has been implicated in seizures. Peripheral introduction of virus into the brain is likely to occur from olfactory neurons whose connections terminate in the piriform cortex (Hunsperger & Roehrig, 2006).

Garcia-Tapia *et al.,*(2007) (Garcia-Tapia *et al.*, 2007), (Table 1) in lineage 1 WNV mouse experiments, found the first clinical signs and deaths on day 9PI: mice would die within 12 hours of showing illness. Most mice at day 9 also had the most severe histological lesions, and highest CNS viral titres ($10^{6.79}$), but no virus was found in blood. These findings correlated with those of Shrestha *et al.,* 2003 (Shrestha *et al.*, 2003). On day 4 PI (Garcia-Tapia *et al.*, 2007) virus was still present in macrophages and fibroblasts of the skin, few macrophages in spleen and some epithelial tubular cells of the kidney on IHC, without signs of inflammation. Neutralizing antibodies, predominantly IgM, were found initially on day 6PI. The last deaths were on day 11 and on day 13PI a low quantity of virus was still detected in brain, and occasionally lymph nodes, kidney and spleen, but none in blood. On day 21PI no virus was present in tissues by plaque assay and 2/4 mice still had IHC positive neurons in the CNS, suggesting potential persistent infection; after day 13PI mice were considered survivors. In euthanised animals splenic and thymic lymphoid tissue was severely lymphocyte depleted (Table 1).

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Neuronal tissue culture and hamster WNV experiments (Samuel *et al.*, 2007) concluded that WNV reaches neuronal or non-neuronal target cells by viral release extracellularly at distal axon sites, and that WNV uses specific pathways to enter the CNS, resulting in distinct disease phenotypes.

1.6.1.2.Immunopathogenesis of WNV

Immunopathogenesis of WNV infection continues to be intensively researched and has recently been reviewed by Lim *et al* (2011).

The BBB comprises the vascular endothelial cell tight junctions and the *glia limitans* of astrocyte foot processes, which also includes collagen (Lim *et al.*, 2011). Most infiltrating leukocytes are retained in the perivascular space after transmigration of the vascular wall The chemokine system controls a critical aspect of migration of specific subsets of leukocytes into the brain. Chemokine CXC motif receptor 3 and C-C chemokine receptor type 5 (CCR5) knock-out mice cannot clear infection resulting in increased mortality. Expression of CXC motif chemokine 12 (CXCL-12) at the perivascular space is most likely responsible for retention of leukocytes in this space in the CNS, since neutralisation of CXCL-12 promotes leukocyte entry into the brain parenchyma. Tumour necrosis factor-alpha (TNF-α) increases the vasuclar permeability allowing penetration of leukocytes into the perivascular space (Lim *et al.*, 2011). Matrix metalloproteinases (MMP) produced by both monocytes and glial cells have, however, been shown to be involved in migration of leukocytes to the perivascular space and migration through the *glia limitans*. MMPs were significantly induced in WNV-infected human brain cortical astrocytes (HBCA) and incubation of naive human brain microvascular endothelial (HBMVE) cells with supernatant from WNV-infected HBCA cells resulted in loss of tight junctions, providing evidence that astrocytes represent a source of MMP in the brain (Verma *et al.*, 2010). When the BBB is compromised, immune cells may enter the brain parenchyma contributing to WNV clearance as well as immune-mediated damage.

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Gamma interferon was found in mice to play a dominant protective role against WNV infection via antiviral properties, by occurring in peripheral lymphnodes and preventing viral dissemination to the CNS (Bai *et al*., 2009; Shrestha *et al*., 2006b).

Garcia-Tapia *et al.,* (2007) (Garcia-Tapia *et al*., 2007) in their mouse WNV experiment measured various chemokines and found early (day 6PI) up-regulation of monocyte chemoattractant protein-5 (MCP-5; CCL12), interferon gamma inducible protein (IP-10; CXCL10), and monokine induced by gamma interferon (MIG; CXCL9) and hypothesized that IP-10 and MCP-5 initiate recruitment of leukocytes into perivascular spaces where antigen presentation occurs, leading to activation of circulating leukocytes that produce IFN-gamma, IL-1α, and TNF-α. Upregulation of TNF-α and IFN-gamma occurred on day 9PI: these molecules increase the permeability of brain capillaries and facilitate entry of inflammatory cells into the brain parenchyma, helping eliminate the virus in the CNS. They also possibly facilitate entry of infected monocytes which increase viral burdens and neuronal infection. IP-10 was surmised to have an important role in induction of strong inflammation in the brain, more than in clearing of virus, thus playing a role in immunopathology of encephalitis caused by WNV. MCP-5 has also been suggested to play an important role in autoimmune inflammation in the brain after cortical injury in a murine model of autoimmune encephalomyelitis, and the WNV experiment supported this possibility of MCP-5 initiating inflammation (Garcia-Tapia *et al*., 2007).

Research using wild type and genetically Toll-like receptor 3 (Tlr-3) deficient mice (Wang *et al*., 2004) found that a major pathogenetic mechanism of rapid early entry into the brain, despite route of inoculation of WNV, is by induction of Tlr-3 from the cytoplasm of marginal zone splenocytes near blood vessels. This leads to production of inflammatory cytokines including interleukin 6 and TNF-α and these compromise the BBB, allowing CNS viral entry with subsequent neuronal replication, injury and inflammation. The BBB is primarily penetrated via the olfactory bulbs, coinciding with the early research mentioned previously, and also via the cerebellar vascular route. Immune-response-induced apoptosis additionally contributes to neuron injury after WNV infection. Tlr-3 is similarly produced in microglia in the brain, causing further local BBB

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breakdown. The host inflammatory response is primarily CD8+ T lymphocytes as well as microglial and macrophage-associated inflammation which may induce further neuropathology. CD45 labels leukocytes and activated microglia and most CD45+ cells, many of them monocytes, were found in the olfactory bulbs in the initial stages after intraperitoneal inoculation of mice with WNV. These were associated with WNV positive cells and cell injury and by day 6PI dysmorphic neurones were visible. WNV is believed also to directly induce neuronal injury (Wang *et al*., 2004).

As mentioned previously in Lim *et al.,* (2011), Tlr3 is down regulated by macrophages of young individuals but not in the elderly, and this failure possibly contributes to production of high levels of pro-inflammatory and vasculogenic cytokines, and by inference increased susceptibility to WNV (Lim *et al*., 2011).

Bai *et al.,* (2009) (Bai *et al*., 2009) found that interferon (IFN) and IFN-gamma provide immediate defence against WNV replication and dissemination. CD4+ and CD8+ T cells also help hosts to recover from WNV infection. Interleukin 10 (IL-10) is needed to stop immunopathology by suppressing cytokine production, which does not occur in autoimmune disease, but if there is deficient IL-10, an increase in T helper 1 cells occurs which are necessary for viral eradication. Genetically IL-10 deficient mice show much diminished WNV infection, and blockage of IL-10 signalling by neutralising antibodies increases survival of WNV infected mice. Wang *et al.,* (2006) (Wang *et al*., 2006) found that CD8+ T cell-mediated immune response in lineage 2 Sarafend-strain West Nile virus infection in C57BL/36 mice is the dominant subpopulation of lymphocytes in the brain and that a deficiency in IFN-gamma did not affect mortality to two strains of WNV in mice (Sarafend and Kunjin strains), or significantly affect brain virus titres (Wang *et al*., 2006).

In perforin-deficient mice, using a lineage 1 New York isolate, higher viral burden in the CNS and increased mortality after WNV infection were found, with viral persistence in the CNS for several weeks in the mice that survived initial challenge (Shrestha *et al*.,

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2006a). They found that CD8+ T cells required perforin to control WNV infection in the CNS.

Lim *et al.,* (2011) also concluded in their review that WNV-infected neurons may undergo apoptosis via caspase -3 and -9 and neurons respond to WNV infection by up-regulating pro-inflammatory cytokines including IL-1β, -6, -8 and TNF-α, also contributing to neuron damage (Lim *et al.*, 2011). Astrocytic glial cell infection leads to upregulation of CXCL10, IL-1β and indolamine-2',3'-deoxygenase (IDO), which together with MMP expression result in loss of tight junctions at the *glia limitans* and increased BBB permeability (Lim *et al.*, 2011). They finally speculated that WNV likely enters the brain of many more patients than show neurological disease, but in a small percentage, host factors such as an overactive inflammatory response lead to an increased BBB permeability and excessive neuronal death, thus overruling the protection of the immune system.

Several WNV encoded proteins directly affect WNV-mediated CNS disease including the NS3 which induces apoptosis via the extrinsic, death receptor-linked caspase-8 pathway, and NS1, NS2A and NS4B proteins which are interferon antagonists (Hollidge *et al.*, 2010). The non-structural flavivirus proteins have virus replication and assembly functions: NS1 and NS4A participate in virus replication, NS2A in assembly and virion release, NS3 and NS2B have proteolytic activities and NS5 acts as an RNA-dependent RNA polymerase and methyltransferase participating in the methylation of the 5'-cap structure (Castillo-Olivares & Wood, 2004). Flavivirus proteins E, prM, NS1, NS3 and NS5 have also been identified as antigens, and specifically the prM and NS3 proteins of WNV can be recognised by WNV-specific mouse and horse antisera, and human patient sera consistently recognise WNV NS5 (Castillo-Olivares & Wood, 2004). Several non-structural proteins of arboviruses from different families have the properties of causing apoptosis, inhibiting host-protein synthesis and blocking type 1 interferon, suggesting that these abilities to induce death and to subvert the innate immune system are critical for their maintenance in nature. These characteristics may lead to

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development of broadly applicable treatment modalities for arboviral infections (Hollidge *et al*., 2010).

1.6.2 Horses

1.6.2.1 Experimental infection and WNV pathogenesis in horses

Some studies using small numbers of horses have shown that few animals develop viraemia or clinical signs following experimental WNV inoculation by the cutaneous route (Guthrie *et al*., 2003; Schmidt & El Mansoury, 1963)

.

Bunning *et al.,* (2002) infected 12 seronegative horses of different breeds and ages via bites of *Aedes albipictus*. 6 were infected with a horse brain isolate (BC787) and the other 6 with a crow brain isolate (NY99-6625). Post-infection, uninfected female *Aedes albipictus* fed on 8 of the infected horses 3 - 5 days after inoculation. Only 1 of 12 horses developed encephalomyelitis and levels of viraemia were very low and transient (1 day). Neutralising IgM (MAC-ELISA) antibodies occurred in all horses from 7 to 11 days PI, continued to rise up to day 13, and ranged from 1:10 to 1:320. The highest viraemia measured was 460 Vero cell PFU/mL in CNS tissues of the mare which developed encephalomyelitis, but blood levels were low. All negative mosquitoes feeding on horses remained negative. Seven infected horses developed detectable levels of viraemia, range 10^1PFU/mL to 10^3PFU/mL, and virus-positive samples were obtained during days 1 to 6. Mosquitoes fed on the single horse which developed fever and neurological signs on days 8 and 9, were incubated for 10 days and found to be negative for virus. The affected mare became ill on day 8 after infection and progressed rapidly until she had to be euthanised 24 hours later. She had severe encephalomyelitis and virus titres of 10^4 to $10^{6.8}$ PFU/mL in several areas of the brain and spinal cord (Bunning *et al*., 2002). It appeared that horses were not amplifying hosts for the virus, they would be unlikely to transmit WNV via mosquitoes, and only a low percentage of infected horses would develop neurological disease, which corroborates the findings in natural infections (Beasley, 2005; Hayes & Gubler, 2006).

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Guthrie *et al.,* (2003), after finding WNV neutralising antibodies in a large percentage of Thoroughbreds tested in South Africa without prior history of illness, infected 2 seronegative horses with a South African human-origin lineage 2 strain of WNV. This strain was later found to be of low virulence in mice (Venter *et al*., 2005). Neither clinical signs nor viraemia occurred in the horses (Guthrie *et al*., 2003). They concluded that WNV lineage 2 was a common equine infection in horses in South Africa but not associated with neurological disease (non-pathogenic) in horses. In retrospect, when comparing these findings to the Bunning *et al.,* (2002) study, it appears that results from a study sample of only 2 horses would be insufficient to draw this conclusion. Studies in the USA have shown that most horses that seroconvert do not have clinical signs, even when infected with NY99 lineage 1 strains (Castillo-Olivares *et al*., 2011). Later investigation of horses with neurological signs in South Africa showed several to have severe and often fatal neurological lineage 2 WNV infections (Venter *et al*., 2009b; Venter *et al*., 2010) and also discovered the first lineage 1 WNV natural infection in a horse which died after severe neurological signs and abortion (Venter *et al*., 2011).

Garcia-Tapia *et al.,* (2006) (Garcia-Tapia *et al*., 2006) created a successful *in vitro* cell model of primary equine monocytes, CD4+, CD8+ and B lymphocytes from heparinised blood drawn from 9 healthy horses, only 1 of which was immunologically naive to WNV. They showed a positive/protective immunomodulatory effect of cellular cytokine responses to *Escherichia coli* 0128-B12 lipopolysaccharide (LPS) on WNV infection and replication in peripheral monoclear cells (Garcia-Tapia *et al*., 2006).

Immunohistochemistry of CNS in lineage 1 WNV positive horses has elicited scant antigen in CNS cells of naturally-infected animals (Cantile *et al*., 2001; Kleiboeker *et al*., 2004), despite inflammatory changes, when compared with the amount of antigen in various organs and brain of other species and fatal human cases. This suggested that the immunopathological component of equine WNV infection was more prominent than in other species like hamsters, where the opposite trend occurred with much antigen, neuronal apoptosis likely caused by the virus, and little inflammation (Castillo-Olivares & Wood, 2004).

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Kleiboeker *et al.,* (2004) in a retrospective diagnostic US study found that WNV RNA was detected by RT-PCR and nested RT-PCR (RT-nPCR) in neural tissue of 46 of 64 cases, but in 5 horses where myocardium, renal tissue or lungs were submitted, all extra neural tissues were devoid of WNV RNA (Kleiboeker *et al*., 2004). This was confirmed in the first South African study of 7 lineage 2 WNV infected neurological horses (Venter *et al*., 2009b)

1.7 PATHOLOGY OF WNV INFECTION

1.7.1 Humans

WNV lesions in up to 20% of patients presenting with benign fever included lymphadenopathy, hepatosplenomegaly and maculopapular or roseolar rash (Burt *et al*., 2002; Hayes & Gubler, 2006). Approximately 1% of humans contract severe disease manifesting as meningitis, encephalitis, or acute flaccid paralysis (Morrey *et al*., 2008a; Rizzo *et al*., 2011). Most fatalities occur in people over 50 years of age with the elderly and immune-compromised individuals being most at risk of severe neurological disease and fatality (Sampathkumar, 2003).

Neuropathology of WNV meningo-encephalitis in 2 fatal elderly human cases (Kelley *et al*., 2003), included mononuclear perivascular and leptomeningeal chronic inflammation, microglial nodules and neuronophagia especially involving the temporal lobes, brainstem and lumbar spinal cord of the patient with polio-like paralysis. Inflammatory cells were mostly CD3 positive T lymphocytes (CD8+ and CD4+) with CD68+ macrophages and rare CD20+ lymphocytes (Kelley *et al*., 2003). Lesions occurred predominantly in the brainstem and anterior horns of the spinal cord in patients with cord-related symptoms; this syndrome mimicked the extremity paralysis of poliomyelitis (Kelley *et al*., 2003). Patients with WNV clinical signs of encephalitis or rhombo-encephalitis have pathology predominantly in the brainstem and cerebrum. Van Marle *et al.,* (2007) showed in CNS taken from human WNV encephalomyelitis autopsies, that WNV infected both neurons and glial cells (van Marle *et al*., 2007).

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Rarely, WNV-associated pancreatitis (Perelman & Stern, 1974), myocarditis (Pergam *et al.*, 2006; Smith *et al.*, 2004), lymphocytic orchitis (Armah *et al.*, 2007; Smith *et al.*, 2004), myositis (Smith *et al.*, 2004), rhabdomyolysis (Gupta *et al.*, 2008; Li *et al.*, 2003; Medarov *et al.*, 2005; Montgomery *et al.*, 2005; Saad *et al.*, 2005), fulminant fatal haemorrhagic fever (Paddock *et al.*, 2006) or necrotic hepatitis (Burt *et al.*, 2002) have been reported in humans. A 20-year-old African-American woman with WNV-associated flaccid parapalegia after flu-like symptoms during her second trimester of pregnancy, gave birth to a daughter with congenital chorioretinal scarring and severe CNS malformations, with infant serum and CSF both positive for WNV-specific IgM and neutralising antibodies (Alpert *et al.*, 2003).

1.7.2 Mice (see Table1.7.2)

The macroscopic necropsy findings in mice inoculated with the original WNV isolate from a febrile Ugandan woman in 1937 (Smithburn *et al.*, 1940) were of marked cerebral hyperaemia and oedema. The only extra-CNS lesion mentioned was occasional nuclear degenerative changes in the adrenal cortex even in mice inoculated intraperitoneally. Histopathological studies confirmed that the virus was neurotropic and neuropathogenic. Brain lesions were widely scattered and varied considerably in extent, locality and in different animals. See Table 1.7.2 for details of histopathology.

CNS pathology was subsequently recorded in several other mouse experiments spanning many decades, using various ages, sexes and strains of mice of variable susceptibility, inoculation with different WNV lineages and strains of varying virulence, as well as differing viral doses and routes of infection. Several of these experiments were selected and their pathology findings are summarised in Table 1.7.2 (Eldadah & Nathanson, 1967; Eldadah *et al.*, 1967; Garcia-Tapia *et al.*, 2007; Kimura *et al.*, 2010; Nir *et al.*, 1965; Odelola & Oduye, 1977; Shrestha *et al.*, 2003; Wang *et al.*, 2004; Wang *et al.*, 2003).

In most of these experiments, neuropathology, especially nonsuppurative meningo-encephalitis with associated neuronal and glial changes, was described in detail, but

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extra-neural lesions were either not mentioned (Eldadah *et al.*, 1967; Shrestha *et al.*, 2003; Wang *et al.*, 2003), briefly mentioned (Garcia-Tapia *et al.*, 2007; Odelola & Oduye, 1977; Smithburn *et al.*, 1940; Wang *et al.*, 2004), or stated as absent (Nir *et al.*, 1965). Kimura *et al* (2010) described for the first time in lineage 1 WNV-infected mice fluid-dilation of the upper small intestine and sometimes stomach (Kimura *et al.*, 2010). Odelola *et al* (Odelola & Oduye, 1977) however found no significant histopathology of small intestines. In the Kimura study it was stated that only a small number of mice showed diffuse severe CNS inflammation and antigen distribution (Kimura *et al.*, 2010).

The mouse-virulent laboratory Sarafend lineage 2 WNV strain was used in WNV experiments by several researchers (Ng *et al.*, 1994; Wang *et al.*, 2004; Wang *et al.*, 2006; Wang *et al.*, 2003), the pathology of 2 of which are recorded in Table 1.7.2 (Wang *et al.*, 2004; Wang *et al.*, 2003).

Table 1.7.2

Macroscopic and microscopic CNS and extra-CNS lesions recorded in experimental mice infected with WNV.

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Table1.7.1. Review of gross and microscopic CNS and extra-CNS lesions recorded in experimental mice infected with WNV

Study	**WNV strain**	**Mice & route**	**CNS gross and microscopic lesions**	**Other lesions**
Smithburn *et al/,* 1940	Original Lineage 2 Uganda	Mice not specified I/cerebral, i/nasal, i/peritoneal Various concen-trations	Gross -marked hyperaemia & oedema brain. Micro -variable widely scattered. PVC lcts, some macrophages, occ granulocytes; severe neurone damage cerebrum; vasc congestion & dilatation; pia mater, cerebral nuclei or cortex, large pyramidal cells – chromatin disarray or margination, vesicular nuclei. Nuclear pycnosis Ammon's horn & cerebellar granular layer. Purkinje cell acidophilic degeneration, dissolution , disappearance; cell shrinkage.	No gross lesions in visceral organs. Occasional adrenal cortex degenerative change.
Nir *et al.,* 1965	Not specified	3 wk-old Swiss albino mice I/nasal, i/tracheal 2.4×10^4 – 1.6×10^5 mouse intracerebral (MIC) LD50	No lesions olf epith. 84hPI some infl cells betw mitral and glomerular olf bulb layers; 96hPI infl cells in pia mater, olf bulbs & ant piriform area; PVC & some polymorphs; 120hPI mitral cells olf bulb degeneration; 144hPI marked cell degen & PVC. Hippocampus PVC degen & infiltr lcts & polymorphs scattered foci. Dilated congested bl vs, PVC & some Purk degen cerebellum 144hPI.	Multiplication in lung macrophages; no lung, liver, spleen or kidney pathology.
Eldadah & Nathanson 1967	E101 (lin 1)	I.C.R Mice 1-3days old. I/cerebral 100 host specific LD_{50} per 0.02ml inoculum	Micro – neuronal necrosis, neuronophagia, neuronal outfall, meningitis, PV focal & diffuse infl changes. Cerebral cortex & hippocampus most severe neuronal destruction esp suckling mice. Severe lesions only on day preceding death. Spinal cord lesions (cervical & lumbar) infrequent & always mild.	WNV pantropic in suckling mice – muscle, cartilage, many other tissues. Lesions not mentioned.
Odelola & Oduye 1977	Nigerian strain	Adult Swiss albino mice Oral suspensions 0.1ml of 10^3-$10^4 LD_{50}$	Brain – massive lymphocyte PVC around dilated vs in cerebellum & meninges.	No significant histopath in SI. Pulm oedema & congestion. Numerous foci of degenerated hepatocytes with fragmenting nuclei.
Shrestha *et al.,* 2003	NY 2000 (strain 3000.0259)	C57BL/6J (H-2^b) 8-12 week old inbred wild-type mice 10^2 PFU into footpad	Inf. non-paralysed mice- sp cd mild leucocyte infiltr, near N neurones. Inf par mice 10x incr CD45+ WCs in CNS; sp cd many pycnotic neurons, altered morph or frank degen – esp ventral horns. Brains similar in par & non-par. IMH- patchy distribution in brain stem and cerebral cortex esp par mice; some par had much neuronal inf in cerebellar granular &	Not mentioned.

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			Purkinje neurons with neuron & neur.architecture loss. Sp cd – approx 35% neurons IMH + in par mice with incr neuron granularity, altered morphology, incr no CD45+ cells in region of inf neurons.	
Wang *et al.*, 2003	Lin 2 Sarafend	C57B/6 I/v tail vein. High dose [10^7-10^8PFU] Low dose [10^3PFU]	High dose – minimal CNS lesions – vascular congestion cerebral cortex, scatt small PV infected foci & WCs, oedema, glial proliferation & necrosis. Low dose – cortical infl WCs in parenchyma & leptomeninges; PV oedema & dilated bl vs granulocytes & mononuclear cell margination & PV accum; PV foci of infection; dilated ventricles; neuronal degen & marked PVC. IMH – CD8+ T cells in CNS PV & parench foci.	Low dose 1-10PFU no clinical signs, all seroconverted 12 d – no virus brain. D4PI virus in hypogastric ln & spleen (gone d7PI). No virus other extraneural tissues, no viraemia.
Wang *et al.*, 2004	Lin 2 Sarafend	Wild-type & genetically Tlr-3 deficient mice I/peritoneal $1x10^3$ PFU	Primary penetration at olf bulbs, also cerebellar vasc route. Immune response induced apoptosis. Host response mainly CD8+ T lcts & microglial & macrophage-assoc infl. CD45+ cells in olf bulbs soon after inoc- assoc with WNV pos cells & cell injury- by d6PI dysmorphic neurones visible.	Not mentioned apart from marginal zone splenocytes inducing Toll-like receptor -3.
Garcia-Tapia *et al.*, 2007	WNV lin 1 from American kestrel died Missouri 2002	SPF C57BL/6 female 7-8 wk mice. S/cut footpad. 1000PFU	Glial - microglial nodules (from d6PI); mononuclear PVC with scant neutrophils, neuronal necrosis, neuronophagia, satellitosis all at d9PI and decr by d13PI, and some lesions remaining except nods & neuronophagia at d21PI. No lesions days 1,2 or 4PI. Day 9PI most virus & worst lesions and day of 1st clinical signs. IMH from d6PI pos brain & sp cord esp ventral horns, neurons hippoc, cerebral cortex, cerebellar gran cells & a few stellate neurons in cytoplasm, axons, dendrites, neurons & astrocytes. Day 11 last day of death. Dd21PI no viral RNA but 2/4 mice IMH pos -possible persistent infection.	Spleen, liver, kidney, ln, skin, thymus, adr glands, muscles, lung, heart for histopath & IMH. IMH pos in macrophages, fibrocytes of skin; few macrophages spleen & some renal tub epith cells; no inflammation. Euth sick mice – spleen & thymus severe lymphoid depleted – lcts with karyorrhexis or pycnotic nuclei.
Kimura *et al.*, 2010	NY99-6922	C3H mice 6 week old. Footpad 1000PFU	Neurone necrosis, infiltrating reactive microglia, neuronophagia, PV macroph/microglia & lcts –in CNS GM fr d9PI. Brain stem, cerebral cortex, caudate putamen, thalamus & SC; often hippoc & thalamus, with mild meningitis & small hem foci. IHC+ a few neurones ipselateral VH lumbar SC d7pi. IHC+ brainstem, cer cortex, caud put, cerv SC (i/cytoplasmic) in neurons, neur	From d7PI dilation upper SI & sometimes stomach- greenish watery content. Microscopic - villi markedly shortened; deg, necrosis IMH+ upper SI myenteric gangl, but also gangl other areas. Thymic atrophy common in sick mice. No IMH pos in spleen and lnn. Survivors

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			processes, occ astrocyte-types. Neuronophagia and glial proliferation in heavily inf foci; sometimes vasc endoth cells +; PVC macroph/ microglial cells - virus in affected foci. Only few mice with diffuse severe infl & antigen distribution.	cleared WNV from all tissues in 2-3 wks.

Abbreviations: occ, occasional; lcts, lymphocytes; vasc, vascular, olf, olfactory, epith, epithelial; infl, inflammation; betw, between; ant, anterior; PVC, perivascular cuffing; degen or deg, degeneratiobn; infiltr, infiltration; bl vs, blood vessel; Purk, Purkinje; esp, especially; SI, small intestine; pulm, pulmonary; histopath, histopathology; inf, infection/ed; sp cd or SC, spinal cord; N, normal; incr, increased; par, paralysed; non-par, non-paralysed; neur, neuronal; approx., approximately; scatt, scattered; accum, accumulation/ed; PV, perivascular; parench, parenchymal; In, lymph node; d, day; inoc, inoculated; hippoc, hippocampus; gran, granular; macroph, macrophage; GM, grey matter; fr, from; VH, ventral horn; caud, caudal; put, putamen; cerv, cervical; cer, cerebral; endoth, endothelial; ag, antigen; gangl, ganglia; Inn, lymph nodes.

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1.7.3 Horses

Gross necropsy lesions with lineage 1 WNV infection were either absent in CNS and/or internal organs (Antorino *et al*., 2002; Bunning *et al*., 2004; Cantile *et al*., 2000; Snook *et al*., 2001) or included pulmonary oedema and congestion (Tber Abdelhaq, 1996), visceral congestion and occasional haemorrhages (Schmidt & El Mansoury, 1963), occasional haemorrhages especially of the heart (Steinman *et al*., 2002), mild pericardial effusion (Steinman *et al*., 2002), meningeal congestion, oedema and/or haemorrhages (Schmidt & El Mansoury, 1963; Tber Abdelhaq, 1996), lumbosacral spinal cord haemorrhage in a single equine post mortem (Steinman *et al*., 2002), spinal cord congestion with lumbar and thoracic segment haemorrhage (Cantile *et al*., 2000), occasional cases with CNS visible perivascular haemorrhages (Cantile *et al*., 2001) and a single case of meningeal hyperaemia with subdural exudate and fibrin tags (Bunning *et al*., 2004).

Horses with neurological signs due to WNV have polio-encephalomyelitis, particularly evident in the lower brain stem and ventral horns of the spinal cord, and less commonly in cerebral cortex and cerebellum (Castillo-Olivares & Wood, 2004). The lesions are inflammatory in nature with scant viral staining, and include perivascular cuffs of lymphocytes and macrophages with frequent haemorrhages; scattered foci of microgliosis; and in severe cases neuronal degeneration with cytoplasmic swelling and chromatolysis. Extra-neural tissue lesions are rarely described in horses; virus was not detected in vascular endothelium in horses (Castillo-Olivares & Wood, 2004).

Descriptions of histopathology of the central nervous system (CNS) of lineage 1-associated natural or experimental WNV equine cases in north Africa, Europe and North America have been published (Antorino *et al*., 2002; Bunning *et al*., 2004; Bunning *et al*., 2002; Cantile *et al*., 2001; Cantile *et al*., 2000; Guillon *et al*., 1968; Schmidt & El Mansoury, 1963; Snook *et al*., 2001; Steinman *et al*., 2002)..

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A study in Italy of horses infected with WNV showed mild rhombencephalitis and more prominent thoraco-lumbar spinal cord nonsuppurative poliomyelitis with constant involvement of the ventral horns of thoracic and lumbar cord regions (Cantile *et al*., 2000). The spinal lesions in the grey matter were described as being bilateral and symmetrical especially of ventral and lateral grey matter horns, and only occasionally was there unilateral ventral horn involvement. The lumbar spinal cord occasionally had more ring and petechial haemorrhages with scanty inflammation. These horses had no significant cerebral or cerebellar cortex lesions, only occasional neuronal degeneration or neuronophagia, and haemorrhage was surmised to be related to acute infections. Moderate inflammation and rare neuronophagia were found in the pons and medulla oblongata, with milder lesions in the basal nuclei, thalamus and midbrain. Mild inflammation and oedema were rarely found in the meninges of brain and spinal cord. In the 2008 outbreak of WNV in horses in Italy, 794 cases occurred of which 33 (Monaco *et al*., 2009) were neurological and 5 died (Calistri *et al*., 2010b). This was caused by a lineage 1 strain closest to a 1998 Tuscany strain (Calistri *et al*., 2010b); no necropsy or histopathological findings were recorded.

In contrast with the 6 horses in Italy, which had more prominent spinal cord and mild brain lesions, a small comparative study found that 4 horses from northeastern USA showed more severe rhombencephalitis, which extended from the basal nuclei through the brain stem and to the sacral spinal cord; 50% of these horses had perivascular petechiae (Cantile *et al*., 2001). Snook *et al.,* (2001) necropsied 3 American horses that were euthanised and found severe perivascular lymphocytic rhombencephalitis with perivascular haemorrhage, neutrophils and multifocal gliosis - spinal cord examined in a single case had similar lesions (Snook *et al*., 2001). Antorino *et al.,* (2002) mention in 6 horses in Italy, specifically mild-to-moderate nonsuppurative polio-encephalomyelitis with consistent ventral horn involvement of thoracic and lumbar spinal cord and lower brain stem, with perivascular lymphoplasmacytic and histiocytic cuffs, occasional neutrophils, scattered small glial nodules and focal gliosis in the grey matter. Some cases also had ring and petechial haemorrhages and neuronal degeneration in the lumbar cord (Antorino *et al*., 2002). Other researchers lump the lesions into the general

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term nonsuppurative encephalomyelitis (Bunning *et al*., 2004; Steinman *et al*., 2002), or viral encephalitis (Kleiboeker *et al*., 2004) without further detail.

The gross and microscopic lesions of 3 of 7 cases of lineage 2 WNV infection in horses in South Africa (Venter *et al*., 2009b) and of the lineage 1 case (Venter *et al*., 2011) were briefly described. From August to October of 2010, 17 horses contracted WNV in Hungary (Kutasi *et al*., 2011); 12 survived the neurological signs and of the 5 which died or were euthenised, 4 were histologically examined and 2 tested for WNV in CNS brainstem and lumbar spinal regions by RT-PCR. Both were positive for WNV which sequenced as lineage 2. Gross pathology was described as “negative” in the horses and histological lesions were briefly described as lymphoplasmacytic perivascular infiltration, gliosis with some focal neutrophil infiltration, and neuronal degeneration. Lesions were found mostly in brain stem, medulla and grey matter of the spinal cord, with the most severe lesions being found in the cervico-thoracic and lumbar regions (Kutasi *et al*., 2011).

1.8. AIMS AND HYPOTHESES

1.8.1 Hypotheses

1.8.1.1. The pathology caused by South African virulent lineage 2 WNV strains in horses and laboratory mice is similar to that recorded by virulent lineage 1 WNV strains in the northern hemispere in horses and in mice.

1.8.1.2. CNS and extra-CNS pathology in male 3-4 month-old BALBc mice caused by neurovirulent lineage 1 and 2 WNV strains are similar.

1.8.2 Aims

1.8.2.1 Investigation and description of the pathology of lineage 2 WNV (SPU93/10) in experimental mice and comparison with that of lineage 1 WNV (NY99/385).

1.8.2.2 Investigation and description of the pathology of natural WNV lineage 2 cases in horses in SA, and comparison with that of the single SA equine lineage 1 case.

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CHAPTER 2

Comparative Pathology of Neurovirulent Lineage 1 (NY99/385) and Lineage 2 (SPU93/01) West Nile Virus Infections in BALBc mice

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2.1 INTRODUCTION

West Nile virus (WNV) is a widespread, single-stranded, positive sense RNA virus of the genus *Flavivirus*, family *Flaviviridae*. WNV strains primarily fall into 2 lineages, 1 and 2, with neurovirulent lineage 1 strains mainly found in the northern hemisphere (Hayes & Gubler, 2006; MacKenzie *et al*., 2004; Petersen & Roehrig, 2001), and lineage 2 being endemic to southern Africa and Madagascar (Burt *et al*., 2002). Neurovirulent lineage 2 strains have caused severe and fatal disease in horses (Burt *et al*., 2002; Venter *et al*., 2009b) and humans (Burt *et al*., 2002; Zaayman & Venter, 2012) in South Africa, and since 2004 in Europe in birds (Bakonyi *et al*., 2006; Erdelyi *et al*., 2007; Savini *et al*., 2012), horses (Kutasi *et al*., 2011), and humans (Danis *et al*., 2010; Papa *et al*., 2011a; Papa *et al*., 2010a; Platonov *et al*., 2008; Sirbu *et al*., 2011). In 2010 a single fatal equine case was identified as lineage 1 in South Africa (Venter *et al*., 2011).

The original 1937 WNV isolate from a febrile woman in Uganda was of lineage 2 (Hayes & Gubler, 2006) and neurovirulent in mice, causing illness and death from 6 to 8 days post inoculation, and with viral-induced lesions essentially limited to the CNS (Smithburn *et al*., 1940). Different lines of experimental mice have since been infected with WNV of various lineages and strains, with detailed descriptions having been recorded mainly of CNS lesions (Eldadah & Nathanson, 1967; Garcia-Tapia *et al*., 2007; Kimura *et al*., 2010; Nir *et al*., 1965; Odelola & Oduye, 1977; Shrestha *et al*., 2003; Smithburn *et al*., 1940; Wang *et al*., 2004; Wang *et al*., 2003). Extra-CNS lesions during the same experiments were either not recorded or were absent (Eldadah & Nathanson, 1967; Shrestha *et al*., 2003; Wang *et al*., 2004; Wang *et al*., 2003), scant (Nir *et al*.,

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1965; Smithburn *et al*., 1940), or varied (Garcia-Tapia *et al*., 2007; Kimura *et al*., 2010; Odelola & Oduye, 1977). Kimura *et al.,* in 2010, using 6-week-old C3H mice infected with a lineage 1 (NY99-6922) strain, reported for the first time in mice distinctive macroscopic gastric and upper small intestinal dilation and filling with watery green content from day 7 post-infection (PI). Microscopically there were shortened villi, and degeneration, necrosis and immunohistochemical positivity especially of upper small intestinal myenteric ganglia but also of ganglia of other areas (Kimura *et al*., 2010). Odelola and Oduye in 1977 reported no significant histological lesions in small intestine of adult Swiss albino mice infected with a Nigerian WNV strain; they did, however report numerous foci of degenerated to necrotic hepatocytes (Odelola & Oduye, 1977). Brown *et al.,* in 2007 cited duodenum and pancreas as novel sites of WNV replication in C3H and C57BL/6 mice after footpad WNV inoculation, but did not mention lesions (Brown *et al*., 2007).

Thymic atrophy, and thymic and splenic lymphoid depletion due to lymphocyte necrosis were also recorded in sick C3H mice as well as in 8-week-old female C57BL/6 mice subcutaneously footpad-inoculated with 1000 and 100 plaque-forming units of lineage 1 WNV USA strains in the WNV pathogenesis experiments of Kimura *et al.,* (2010) and Garcia-Tapia *et al.,* (2007) respectively (Garcia-Tapia *et al*., 2007; Kimura *et al*., 2010). Kimura *et al.,* (2010) commented that pathological events occurring in peripheral tissues during WNV infection of mice have been incompletely characterised (Kimura *et al*., 2010).

The pathology in mice caused by a neurovirulent South African field strain of lineage 2 WNV has not previously been reported nor been compared with that caused by a known neurovirulent lineage 1 WNV strain, and WNV-associated extra-CNS lesions in mice have only partially been investigated. The present study describes, illustrates and compares gross and microscopic CNS and extra-CNS pathology in male 3-4 month-old BALBc mice after intraperitoneal infection with either neurovirulent New York lineage 1 WNV strain NY385/99 or the South African neurovirulent lineage 2 WNV strain SPU93/01. The presence of both strains was tested for by nested real-time RT-PCR

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and immunohistochemistry (IHC). Electron microscopy was done of the control positive lineage 1 WNV-infected avian tissue sourced from Canada and brain of one of the RT-PCR and IHC lineage 2 positive mice (case No. 18) in an attempt to demonstrate the presence of WNV in the IHC positive areas of cell cytoplasm.

2.2 MATERIALS AND METHODS

2.2.1 Case and virus source

Specific pathogen free (SPF) WNV-infected mice and non-infected control mice mock-infected with phosphate buffered saline (PBS), all of which had been previously mock-vaccinated with PBS, were sourced from two registered, WNV vaccination and infection/challenge experiments performed in biosafety level 3 facilities at Deltamune (Pty.) Ltd. (Roodeplaat, Gauteng, SA) and the National Institute of Communicable Diseases (NICD) (Sandringham, Johannesburg, Gauteng, SA) respectively, after approval of the protocols by both the National Health Laboratory Services (NHLS) Animal Ethics and the University of Pretoria Animal Use and Care Committees (project number H019-10). In the experiment in which challenge was with both lineages 1 and 2 WNV, there were 10 mice per group. One each of the mock-vaccinated infected groups was sacrificed on day 0 of infection for blood studies. From the other experiment conducted under identical circumstances, 5 mock-vaccinated lineage 2-infected mice were sourced for the current study. The lineage 2 WNV strain, SPU93/01, was isolated in 2001 from a 21-year-old South African woman hospitalised with meningo-encephalitis, who recovered (Burt *et al.*, 2002), and was later reported as a neuroinvasive accidental laboratory-infection in a 29-year old woman (Venter *et al.*, 2009a). It was obtained from Professor Janusz Paweska, Centre for Emerging Diseases and Zoonoses, NICD. The lineage 1 infection strain, NY385/99, was isolated from a bird which died in 1999 in New York and obtained from the late Dr. R.E. Shope and Dr. D.W.C. Beasley of the University of Texas Medical Branch, Galveston. Both strains were known to be highly neuroinavsive (Botha *et al.*, 2008; Venter *et al.*, 2009a; Venter *et al.*, 2005).

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2.2.2 Infection of Mice

Healthy PBS mock-vaccinated susceptible male BALBc mice of 3-4 months of age were infected on day 0 of a 21-day vaccine – challenge/infection trial with WNV lineage 2 strain SPU93/01 (Burt *et al*., 2002), (n=13: case Nos. 6-9, 11-13, 16-19, 23 and 24), or the lineage 1 strain, NY385/99 (n=8: case Nos. 10, 14, 15, 20-22, 25 and 26) (Table 2.3.1) (Venter *et al*., 2013). As non-infected controls, one healthy mouse was euthanised on day 0 of the vaccine trial, 49 days prior to day 0 of infection/challenge (case No. 1), and four PBS mock-vaccinated, mock-infected healthy mice, were euthanised either on day 0 of the infection/challenge trial (case Nos. 2 and 3), day 7 (case No. 4) or day 21 post-infection (case No.5) (Table 2.3.1).

The viruses were titrated in Vero cells to determine the dilution to be used. A dose of 1×10^4 $TCID_{50}$/ml of both strains, shown in previous mouse studies to be 100% fatal (Beasley *et al*., 2002; Venter *et al*., 2005), was used for intraperitoneal inoculation to ensure successful infection of all mice for the vaccine studies (Beasley *et al*., 2002).

Mice were observed for signs of disease for 21 days following infection. They were evaluated as paralysed when they remained stationary with variable intermittent body tremors, rocking, or were unable to move their limbs on stimulation or on being lifted from the cage (Shrestha *et al*., 2003). Mice seen unable to move or too disorientated to reach food or water were euthanised.

Euthanasia was either by intraperitoneal injection of sodium pentobarbitone 200mg/ml (Euthanaze, Bayer Animal Health (AH), Isando, SA), or by intramuscular xylazine hydrochloride 20mg/ml (Rompun 2%, Bayer AH, Isando, SA) at 5mg/kg body mass combined with ketamine hydrochloride 100mg/ml (Anaket-V, Bayer AH, Isando, SA) at 35mg/kg body mass, with exsanguination from the heart as soon as deep anaesthesia had occurred. Two surviving mice from each lineage infection group were euthanised on day 21 PI by carbon dioxide inhalation.

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Digital images were taken with Panasonic Lumix FZ10 and FZ45 cameras (Panasonic Corporation, Ariake Tokyo, Japan). Images of histological lesions of all cases were taken using an Olympus BC50 microscope (Olympus, Hamburg, Germany) with associated CC12 soft imaging system, and analySIS software (Olympus, Hamburg, Germany).

2.2.4 Necropsies and Collection of Tissue Specimens

After blood and aseptic fresh tissue collection from euthanised mice and aseptic tissue collection from mice which died, the abdominal and thoracic cavities were opened ventrally and left laterally after reflection of the diaphragm, to allow the 10% neutral buffered formaldehyde to penetrate. The dorsal cranium was removed and the skin along the dorsum of the neck and trunk opened and dissected away from the subcutis and spine. After a minimum of 48 hours and in most cases at least 7 days, full necropsies were performed. Tissue slices of 1-2mm width were cut from approximately 30 tissues from each mouse and placed in a single cassette for routine alcohol dehydration, wax-embedding, 5µm thick microtome sectioning, HE staining and light microscopic examination.

Tissues sampled for histopathology included haired skin from trunk or left hind leg, left quadriceps muscle, bladder, liver, spleen, left and right kidneys, both lungs, serial horizontal sections of heart, serial vertical transverse sections of brain and cerebellum, tongue, ethmoid region, transsected lumbar or thoracic vertebrae incorporating vertebral body bone marrow and occasionally spinal cord, pyloric region of stomach, pancreas, duodenum, jejunum, ileum, caecum, descending colon, mesenteric lymph node, testis, mesenteric fat, footpads and digits of left hind foot, and occasionally adrenal gland, eye, oesophagus, and dissected-out lumbar spinal cord. Regions of the brain examined included olfactory lobe, cerebral cortex, hippocampus/thalamus, pons/medulla oblongata and cerebellum. The thin thread-like spinal cord or bits thereof, and occasionally spinal nerves, in most cases of lumbar origin and either in trans- or longitudinal section, were examined in case Nos. 2-5, 8, 10 –13, 16-19, 23- 26: in the remaining cases it was likely situated in a deeper plane in the wax blocks or was cut

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away during section preparation.

2.2.5 Immunohistochemistry

Immunohistochemical labelling of all tissues was performed using Thermo Scientific (Pierce Biotechnology, Illinois, USA) rabbit polyclonal WNV E antibody at dilution 1:50 according to the avidin-biotin complex technique (Haines & Chelack, 1991). Trial labelling of known RT-PCR positive avian heart infected with lineage 1 WNV (Dr Josepha DeLay, University of Guelph, Ontario, Canada) as well as a known RT-PCR positive mouse brain from this study (case No. 18, day 9 PI with WNV SPU93/01) was done to develop a suitable protocol. Mouse case No.1 sacrificed on day -49 was used as negative control. The Novolink Polymer Detection System (Novocastra Laboratories Ltd., Newcastle upon Tyne, NE12 8EW, United Kingdom) was used. Novored Substrate (Vector Laboratories, Burlingame, CA) was applied, whereafter differentiation was done manually, with haematoxylin counterstaining and mounting in Entellan (Merck KGaA, 64271 Darmstadt, Germany) mounting medium. WNV positive mouse brain and avian heart controls, the negative control mouse, as well as known positive rabies tissue sections were also labelled immunohistochemically with polyclonal rabies antibody (prepared by the late Dr Ken Charlton, Animal Diseases Research Institute, Ontario, Canada) at dilution 1:500 as irrelevant antibody.

2.2.6 WNV RNA detection

Nested real-time RT-PCR with WNV specific primers was carried out on the fresh tissues of all cases. This was performed with a nested real-time RT-PCR amplification method as previously described (Zaayman *et al*., 2008).

2.2.7 Electron microscopy

IHC-positively labelled cells from the Canadian avian lineage 1 WNV control positive heart as well as thalamic neurons of mouse case No.15 were prepared for electron microscopy (EM) by the pop-off method for re-embedding in resin (Hayat, 2000). Briefly, the relevant areas on the light microscope slides are flooded with a xylene and epoxy resin mixture before inverting the slide onto a resin-filled pre-shaped polyethylene

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mould with hinged lid (BEEM® capsule, Agar Aids, 66A Cambridge Road, Stansted, Essex CM 24 8DA) for polymerization. This technique enables examination of specific cells identified by light microscopy, in this case avian cardiomyocytes and mouse neurons that were positively labelled by IHC, to investigate which structures were being labelled. Ultra-thin resin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips CM10 (Philips Electron Optical Division, Eindhoven, The Netherlands) transmission electron microscope operated at 80 kV, and digital images captured with a Megaview III side-mounted digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and iTEM software (Olympus Soft Imaging Solutions GmbH, Munster, Germany) to adjust brightness and contrast.

2.3 RESULTS

One mouse of each infection group died unexpectedly on day 1PI of unrelated causes, leaving 8 in the lineage 1 group and 13 in the lineage 2 WNV infection group for this pathology study. Two lineage 2-infected mice were euthanised on day 5PI prior to showing clinical signs, and 2 each of lineage 1 and 2 infections were euthanised on day 21PI after showing clinical recovery (case Nos.23 - 26). All other WNV-infected mice died (case Nos. 9, 11, 12, 14-18, and 20-22) or were euthanised while sick (case Nos. 8, 10 and 19). The procedure, diagnostic test results and outcome of the experiment as well as main gross and histological findings are summarised in Table 2.3.1.

Intraperitoneal infection of 3-4 month-old male BALBc mice with neurovirulent lineage 1 NY99/385 or South African lineage 2 SPU93/01 WNV strains resulted in similar neurological signs in all infected mice and death in the majority of animals from days 7-11PI, with 2 mice infected with each lineage surviving infection. Gross and microscopic pathology were indistinguishable between the lineages and the study confirmed the pathogenicity and neurovirulence in mice of SPU93/01 isolated from a South African female with naturally-acquired WNV neurological disease (Burt *et al.*, 2002). Detailed results are further described under specific sub-headings in the text.

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Table 2.3.1

Results of real-time RT-PCR, IHC, gross and microscopic pathology in control non-infected and WNV lineage 1 (NY99/385) and 2 (SPU93/01) infected 3-4-month-old male BALBc mice.

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Table 2.3.1. Results of real-time RT-PCR, IHC, gross and microscopic pathology in control non-infected and WNV lineage 1 (NY99/385) and 2 (SPU93/01) infected 3-4-month-old male BALBc mice.

Day PI	Specimen number	Case No.	WNV lin1 lin2	Euthanised, sick, died	rRT-PCR WNV	IHC on all tissues	Gross PM	Microscopic lesions					
								SI	LI	Spleen	Lymph nodes	CNS	Other
-49 0 7 21	4845-10 4286-11D, 1286-11E 1364-11C 1567-11C	1*^ 2,* 3* 4* 5*	NI	Not sick E E E E E	1-5 neg	1-5 neg	-	villi stunted(4)	-	neg; NE(3)	hyperplasia(1) NE(3)	+PVhem(1)	hepatosis (3 and 5)
Day 5	4957-105A	6	lin2	E not sick	neg	neg	-	+	+	+	NE	+	-
	4957-105B	7	lin2	E not sick	neg	neg	-	-	+	+-	NE	-	high protein lung oedema, hepatosis
Day 7	1364-11A	8	lin2	E sick	+	neg	st SI dist	+++	+	+++necr lcts DMs marginal	++ lct necrosis	+	interstitial pneumonia
	4962-10	9	lin2	D	+	neg	st SI dist	+++	+	+++	++ lct depl	+	ethmoid lct necrosis
	1364-11B	10	lin1	E sick	+	+ neur deep brain stem	st SI dist	+++	-	+++	++	+	hepatosis
Day 8	1389-11A	11	lin2	D	+	+ cecal myenteric ganglion	st SI dist	+++	mucus	++marginal lct depl	++ SH lct depl	+	lipid hepatosis
	1389-11B	12	lin2	D	+	neg	st SI dist	+aut	-	NE	+++ lct depl	aut	lipid

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	4963-10	13	lin2	E sick	+	neg	st SI dist	+++ Villus fusion	+	+++marginal lct necrosis	++ lct necrosis	+	hepatosis pleuritis, diaphragm vasculitis, peritonitis, interst pneum, hepatosis
	1389-11C	14	lin1	D	neg	neg	st SI dist	+++ neuts	mucus	+++	++depl SH	+	hepatosis
	1389-11D	15	lin1	D	neg	neg	st, SI dist	+++	-	++lct depl	++depl SH	+	lipid hepatosis
Day 9	1497-11A	16	lin2	D	+	++cortex	st SI dist, melena	+++	-	++lct depl	++lct depl	+	lipid hepatosis
	1497-11B1	17	lin2	D	neg	neg	st SI dist	+++	+	+lct depl	+SH	+	hepatosis
	1497-11B2	18	lin2	D	+	+cortex, thalamus	st SI dist, melena	+++	+	+	+lct depl	+	lipid hepatosis
	4969-10	19	lin2	E sick	+	+cortex, brain stem	st SI dist, yellow-brown	+++	mucus	+ lct depl	++lct depl SH	+	-
	1497-11	20	lin1	D	+	+brain stem	st SI dist, melena	+++	neuts	+	+	+	hepatosis, purulent mesenteritis
	1497-11C2	21	lin1	D	+	neg	st SI dist	+++	-	NE	+	+	hepatosis
Day 11	1498-11	22	lin1	sick 2dys D	+	+cerebellar peduncle, cortex, deep brain stem	st SI dist, melena. meningeal hem	+++	mucus	+++	NE	+	FB pleuritis, mesenteric. vasculitis, lipid hepatosis
Day 21	1566-11A	23	lin2	E	+	neg	spleno-megaly	neuts	-	-	NE	+	hepatosis
	1566-11B	24	lin2	E	neg	neg	-	villi stunted	-	-	-	PVhem	hepatosis

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1566-11C1	25	lin1	E	neg	neg	-	neuts	-	-	-	focal neur calc	hepatosis, distended bladder
1566-11C2	26	lin1	E	neg	neg	-	neuts	-	-	-	PVhem	hepatosis

Lesions as listed in the text: +, mild lesions; ++, moderate lesions; +++, marked lesions; *, control, non-infected mice from infection trial; ^, control mouse from day 0 of vaccine trial; lin 1, lineage 1; lin 2, lineage 2; PI, post infection; d, day; D, died; E, euthanised; NI, not infected; PM, post mortem; -, neg, negative; st, stomach; dist, distension; SI, small intestine; LI, large intestine; NE, not examined; PV, perivascular; hem, hemorrhage; neuts, neutrophils; pycn, pycnotic; neur, neurons; lct, lymphocyte; depl, depleted; aut, autolysed; necr, necrotic; DMs, dendritic macrophages; SH, sinus histiocytosis; interst, interstitial; pneum, pneumonia; calc, calcification

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2.3.1 Clinical Signs

Clinical signs shown by both lineage 1 and 2 WNV infected mice were similar and included depression, hunched body posture, ruffled fur, dehydration as tested by lifting a skin fold, body tremors or lateral rocking while stationary, circling, inability to drink or eat from the overhead water spout and food cage, very slow crawling movement when touched, paresis, paralysis, and abdominal distension.

Table 2.3.1 shows daily progress of illness, death or euthanasia of mice infected with both lineages. Ten mice were found dead after showing mild or no clinical signs during the twice-daily observations: time from onset of clinical signs to death was generally less than 12 hours except for case No. 22 (lineage1) which was hunched and showed slow movement and rocking for 2 days prior to death. Mice started dying on day 7 in both lineage 1 and 2 groups.

2.3.2 Gross Necropsy Findings

All control mice, infected mice euthanised prior to manifestation of clinical signs (case Nos. 6 and 7 inoculated with lineage 2), and mice surviving infection with both lineages showed no significant gross lesions apart from visceral congestion and pulmonary haemorrhages (Table 2.3.1).

WNV-affected mice were generally dehydrated, had distended abdomens and ruffled fur. Small intestine and stomach were markedly fluid- and sometimes gas-distended, and serosae were congested (Table 2.3.1) (Fig. 2.3.1). After formalin fixation when necropsies were continued, the stomachs deflated on removal, and the distended proximal small intestines were fragile and occasionally broke releasing watery content. Comparative gastro-intestinal distension in various mice is illustrated (Figs. 2.3.2, 2.3.3 and 2.3.4). Gastrointestinal contents were variably green-grey fluid, watery to granular dark grey-brown, pasty brown-yellow or dark red-brown to red-black granular fluid (Fig. 2.3.4) (Table 2.3.1). Melaenic contents were present in 3 mice dying at day 9PI (case Nos. 16 and 18 with lineage 2 and case No.20 with lineage 1 infection) and case No. 22, the lineage 1 infected day 11PI mouse (Table 2.3.1).

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In all fatalities or sick euthanised mice, distal jejunum, ileum, caecum and descending colon appeared atrophic with very little content; faecal pellets if present were generally few, dry, small and firm. Control mice, lineage 2-infected case Nos. 6 and 7, and the 4 survivors (case Nos. 23 and 24 of lineage 2, and case Nos 25 and 26 of lineage 1) had by contrast small stomachs filled with granular food content, narrow, relatively empty small intestines, caecae filled with pasty grey-green faeces and several large elongated faecal pellets in their colons.

Mice euthanised on day 5PI (lineage 2-infected case Nos. 6 and 7) had ample abdominal fat, no intestinal distension, and their stomachs contained only a small amount of granular commercial food. Abdominal fat was generally mildly to markedly reduced in amount in clinically-affected mice when compared with that of healthy control mice, case Nos. 6 and 7, and surviving mice.

Meninges, brain, cerebellum and lumbar spinal cord were congested, and case No.22 (lineage 1) had visible meningeal haemorrhage.

Lungs generally appeared consolidated due to pulmonary haemorrhage and congestion.

Livers in some uninfected controls, sick mice and the survivors were pale yellow-tan in colour (Table 2.3.1) and slightly friable.

One surviving mouse had mild splenomegaly (case No. 23, lineage 2) and bladder size varied with some being empty and that of case No. 25 (lineage 1 survivor) moderately distended.

2.3.3 Histopathology

CNS lesions were present but scant and subtle in all mice showing neurological signs infected with both lineages 1 and 2 WNV. All affected mice showed moderate to

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marked diffuse meningeal, cerebral, cerebellar and brainstem vascular congestion and the most common lesion was small multifocal perivascular petechiae, seen randomly in meninges (case Nos. 6, 8 -10, and 12 – 22), and in various affected mice in all parenchymal regions studied including cortical grey and/or white matter, hippocampus/thalamus, occasionally medulla/pons, cerebellar white matter, and/or choroid plexus. Congestion and perivascular haemorrhages were less prominent in survivors and lineage 2-infected case No. 7 euthanised prior to clinical signs. Diffuse or regional mild gliosis was found in case No. 9 (lineage 2) of cerebellar white matter and medulla/pons, case No. 20 (lineage 1) of brainstem, and case Nos.10, 14 (lineage 1), 11 and 13 (lineage 2) of cortex. Single or very few lymphocytes and rare neutrophils were seen perivascularly affecting very few vessels of meninges (case Nos. 6 and 16 (lineage 2), and 10, 13, 15 and 20 (lineage 1)), cortex (case Nos.14, (lineage 1) and 23 (lineage 2 survivor)), hippocampus/thalamus (case Nos. 11, 16, 17, 20 and 22 (lineage 2), and 15 (lineage 1)), periventricularly (case Nos. 11, 20 and 21 (lineage 1)), medulla/pons (case Nos.17 and 22 (lineages 2 and 1)), brainstem (case No. 14, lineage 1) and choroid plexus (case No.11, lineage 2). Foci of cortical oedema (case No. 22, lineage 1), occasional large neuron necrosis in brainstem (case No. 13, lineage 2), and intermittent cerebellar Purkinje cell necrosis (case Nos. 13 and 19, lineage 2) were also seen. By comparison, in the 5 non-infected control mice, the only lesion found was occasional perivascular petechiae in the medulla/pons of case No. 1.

Lumbar or distal thoracic spinal cord of control case Nos. 2 - 5, lineage 2 WNV-affected case Nos. 9, 12, 13, 16-19 and survivor case No. 24, and lineage 1 infected case No.10 and survivors case Nos. 25 and 26 showed no significant lesions. Grey matter gliosis was seen in case Nos. 8 (lineage 2 day 7PI) and 11 (lineage 2 day 8PI). No significant lesions were found in other neural tissues examined in both lineage 1 and 2 infected mice, including several spinal ganglia, mesenteric ganglia, spinal nerves, peripheral nerves of skin, foot and tongue, ethmoid/nasal epithelium, eyes, and optic nerves.

Case Nos. 6 and 7 (lineage 2) euthanised on day 5PI prior to clinical signs had no macroscopic lesions, however case No. 6 had mild early intestinal histological lesions

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similar to those in mice showing macroscopic lesions from day 7PI. These included scattered neutrophils in the small intestinal lamina propria, mild crypt single cell necrosis with sloughing, some Paneth cell degranulation, mild serum exudation into intestinal glandular lumens, occasional splenic white pulp lymphocyte necrosis, and occasional perivascular round cell presence in the meninges.

The most striking histological lesions in the clinically-affected WNV-infected mice of either lineage (case Nos. 8 - 22) occurred in the distended proximal small intestine and included those lesions seen in case No. 6 but were severe in comparison (Fig. 2.3.5). Also seen were mild to moderate lamina propria neutrophil influx, with some neutrophils, lymphocytes and plasma cells undergoing necrosis, occasional villus tip haemorrhages, and variable sloughing of villus tip enterocytes along with necrotic leukocytes and serum exudation into the intestinal lumen. Villus fusion was found in one small intestinal segment of case No. 13 (lineage 2) and shortened villi in control case No. 4 and lineage 2 survivor case No. 24 (Table 2.3.1).

Excessive caecal luminal mucus, mild occasional colonic crypt and/or luminal mucosal cell necrosis with sloughing (Fig. 2.3.6), and occasional neutrophils in the colonic mucosa were also seen in clinically-affected mice with both lineages (Table 2). Bacteria, generally mixed and in varying numbers, were present in the lumen of both small and large intestines of most cases.

Myenteric ganglia of control mice and case Nos. 6 and 7 (lineage 2 infected and euthanised on day 5PI prior to clinical signs) were easily visualised as small clusters of normal neurons, however in the WNV-affected cases which died, marked intestinal distension resulted in ganglia being difficult to identify. In the large intestine of some of the mice showing mucosal lesions, some ganglia appeared more cellular than normal, with neutrophils occasionally identified. In one colon section of case No. 17 (lineage 2) neutrophilic vascular leucostasis and exocytosis into the muscle layers was found and lymphatics draining necrotic cell debris and neutrophils made visualisation of myenteric

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ganglia difficult. Neuronal vacuolation seen in some ganglia of some mice which had died was ascribed to autolysis (Wohlsein *et al.*, 2013).

Phagocytosis of splenic follicular marginal zone lymphocytes by dendritic macrophages was prominent in all mice infected with both lineages 1 and 2 which succumbed or were euthanised on day 7PI and continued on day 8 (Fig. 7), with follicular lymphocytes becoming progressively depleted from days 8 to 9 except in case Nos. 18 (lineage 2, day 9PI) and 20 (lineage 1, day 9PI) (Table 2.3.1). Splenic extramedullary haemopoiesis diminished in red pulp of affected mice and haemosiderophages increased in number when compared with spleens of healthy control and recovered animals.

Scattered macrophage phagocytosis of lymphocytes, follicular lymphocyte depletion or lymph node sinus histiocytosis were found in mesenteric lymph nodes and intestinal Peyer's patches from days 7 to 9PI with both lineages (Table 2.3.1).

In survivor case Nos. 23 (lineage 2), 25 and 26 (lineage 1) neutrophils remained scattered in small intestinal villus tips and case No. 24 (lineage 2) had slightly shortened villi. Splenic and lymph node follicles of all survivors had recovered to normal lymphocyte density and splenic red pulp once again showed extramedullary haemopoiesis with very few haemosiderophages. CNS in these cases showed minimal lesions including occasional cortical (lineage 2 case No. 24) or hippocampal/thalamic (case No. 26, lineage 1) perivascular petechiae, with occasional cortical perivascular lymphocytes in the only survivor with brain still RT-PCR positive for lineage 2 WNV (case No. 23). A deep thalamic small cluster of mineralising neurons was found in lineage 1 survivor case No. 25 (Table 2.3.1).

Lesions found in individual mice included submucosal ethmoid lymphocyte necrosis (case No. 9, lineage 2); necrotic vasculitis, purulent peritonitis and pleuritis affecting both surfaces of the diaphragm (case No. 13, lineage 2); and necropurulent to haemorrhagic foreign body mediastinitis, pleuritis, peri- and epicarditis affecting the

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caudo-dorsal thoracic cavity, associated with mixed bacteria, foreign material, interstitial pneumonia and bacterial colonies in the spleen in case No. 22 (lineage 1) (Table 2.3.1).

Many mice, including healthy controls and infected animals, had mild microscopic to macroscopically visible hepatosis which was either hydropic vacuolar cytoplasmic change, diffuse mitochondrial swelling (case Nos.7 and 10), or fatty vacuolation, varying from periportal to pericentral or bridging in distribution (Table 2.3.1).

Lungs lesions were present in most mice including controls and included congestion, areas of atelectasis, haemorrhage, diffuse mild to moderate vascular neutrophilic leucostasis, and case No.7 also had high protein alveolar oedema.

Testicles from all mice were undergoing spermatogenesis. Vertebral body bone marrow of control and infected mice in general showed a predominance of neutrophil granulopoiesis. Thymus was not examined, being too small to visualise macroscopically for intentional sectioning. Hyperkeratosis of lingual epithelium and oesophagus occurred in most anorexic, paralysed sick mice. Mast cells with prominent granules plentiful in the soft tissues of the tongue of healthy controls and surviving mice, were in general variably degranulated in sick mice.

2.3.4 Immunohistochemistry

IHC on all tissues of all mice identified WNV brain positivity in 6 mice on days 7, 9 and 11PI; 3 of lineage 1 on days 7, 9 and 11PI and 3 of lineage 2 on day 9PI. Positivity was intracytoplasmic, granular and intense (Figs. 2.3.8 and 2.3.9) in few small or large single scattered neurons or neurons in locally-extensive areas of cortical grey matter (case Nos. 18 and 19 of lineage 2 and case No. 22 of lineage 1) (Fig. 2.3.8), thalamus (lineage 2 case No. 18), cerebellar peduncle white matter (case No. 22) and/or deep brain stem (lineage 1 case Nos.10, 20 and 22, and lineage 2 case No. 19) (Fig. 2.3.9) (Table 2.3.1). Specific nuclei were not identifiable due to sections often being cut at angles and to the scattered nature of most of the single positively-labelled neurons. In case No. 11 (day 8PI, lineage 2) neurons in a single caecal myenteric ganglion were

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also IHC positive (Table 2.3.1). IHC positive neurons showed neither light microscopically visible pathology nor associated glial or inflammatory leukocyte infiltration. The negative control and rabies-positive sections showed no antigen on WNV-IHC staining and none of the sections stained positively for rabies antigen except the positive rabies control.

Plate 1 (Figs 2.3.1 -2.3.9)

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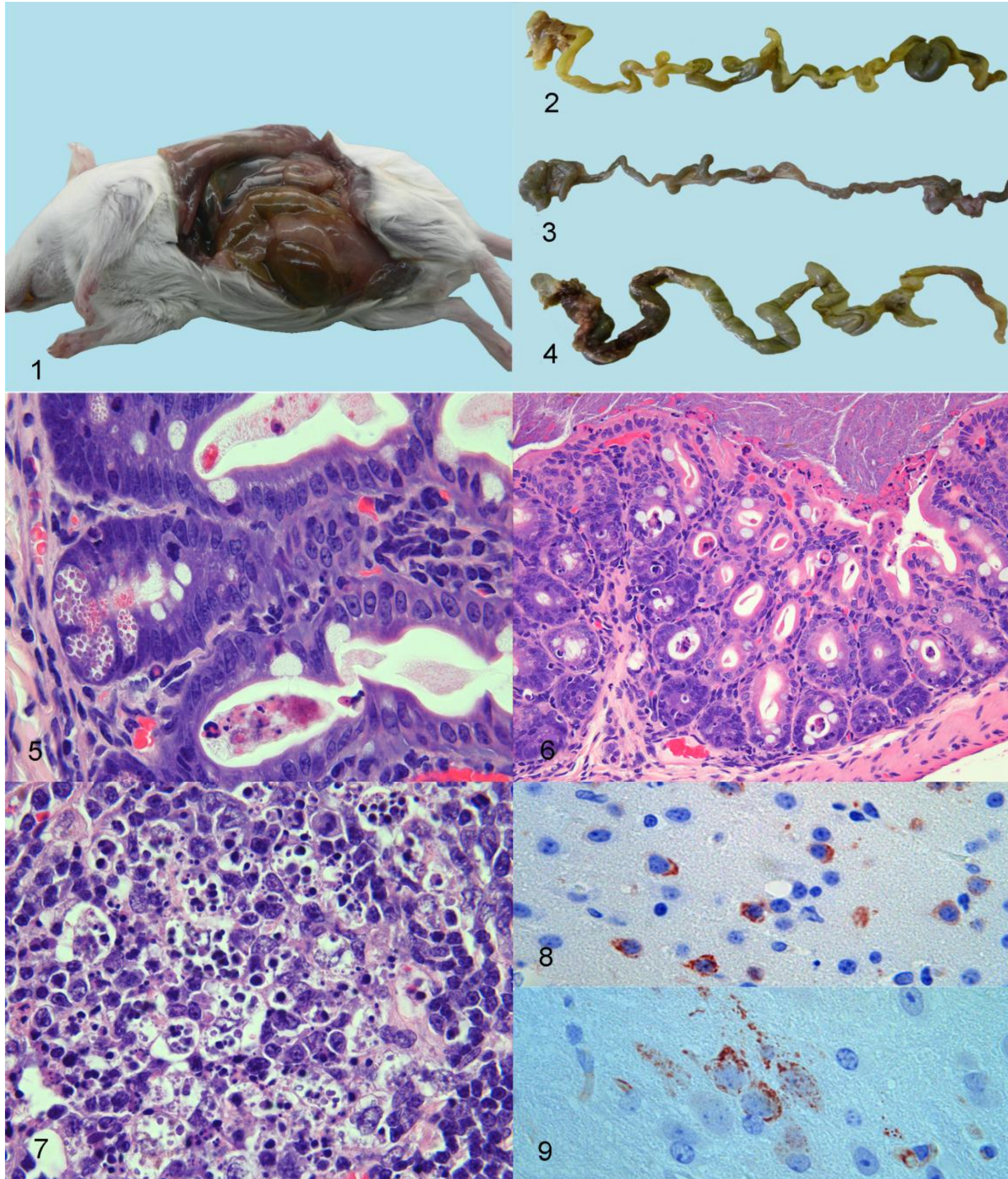

Figure 2.3.1. Opened abdomen; case No. 10, lineage 1 WNV, euthanised sick day 7PI. Marked visceral congestion, gastric and small intestinal distension. **Figure 2.3.2.** GI tract; case No. 4, euthanised day 7. Normal control; stomach to the left of image. **Figure 2.3.3.** GI tract; case No. 6, lineage 2 WNV, euthanised day 5PI. Apparently normal; stomach on left of image. **Figure 2.3.4.** GI tract; case No. 18, lineage 2, died day 9PI. Deflated stomach on left of image; marked proximal small intestinal distension with melaenic contents; empty caecum and colon. **Figure 2.3.5.** Small intestine; case No.8, lineage 2, euthanised sick day 7PI. Crypt enterocyte necrosis and sloughi ng, Paneth cell degranulation and protein exudation. HE. **Figure 2.3.6.** Colon; case No. 8, lineage 2 WNV, day 7PI. Crypt and luminal enterocyte necrosis and sloughing. HE. **Figure 2.3.7.** Spleen; case No. 8, lineage 2 WNV, day 7PI. Follicular marginal zone dendritic macrophage phagocytosis of lymphocytes and lymphocyte necrosis. HE. **Figure 2.3.8.** Cerebral cortex; case No. 19, lineage 2 WNV, euthanised sick day 9PI. WNV E protein present in scattered neurones as cytoplasmic granular staining without visible pathology. Immunoperoxidase with Novored as chromogen and haematoxylin counterstain. **Figure 2.3.9.** Deep brain stem; case No. 10,. WNV E protein positive cytoplasmic staining of some neurones without visible lesions. Immunoperoxidase with Novored as chromoge n and haematoxylin counterstain.

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2.3.5 WNV RNA detection

Viral RNA could be detected in brain of 12/15 mice which died or were euthanised sick and infected with both lineages 1 and 2 WNV, from days 7 to 11PI (Table 2.3.1). Tissues of infected case Nos. 6 and 7 (lineage 2) euthanised on day 5PI prior to development of clinical signs as well as fatal lineage 1 case Nos. 14 and 15 (day 8PI) and 17 (day 9PI) were WNV RT-PCR and IHC negative. Of the 4 surviving infected mice euthanised at day 21, only one lineage 2 infected mouse (case No. 23) was RT-PCR positive in brain tissue (Table 2.3.1).

2.3.6 Ultrastructure

Distinct WNV particles were absent in the ultra-thin sections prepared from the IHC, RT-PCR positive avian cardiac fibres, however roughly spherical, faintly paracrystalline or amorphous structures, sometimes associated with endoplasmic reticulum, were present (Fig. 2.3.10). Occasional vesiculation or vacuolation related to these structures was evident. Pleomorphic amorphous structures were seen adjacent to dilated endoplasmic reticulum and vesicles in the lineage 2 WNV antigen-positive cytoplasmic areas of mouse thalamic neurons (lineage 2 infected case No.18) (Fig. 2.3.11), with a cluster of 50nm diameter WNV-like particles with central dense cores and some with pale envelopes found in an IHC non-labelled area (Fig. 2.3.12).

Plate 2 (Figs 2.3.10 – 2.3.12)

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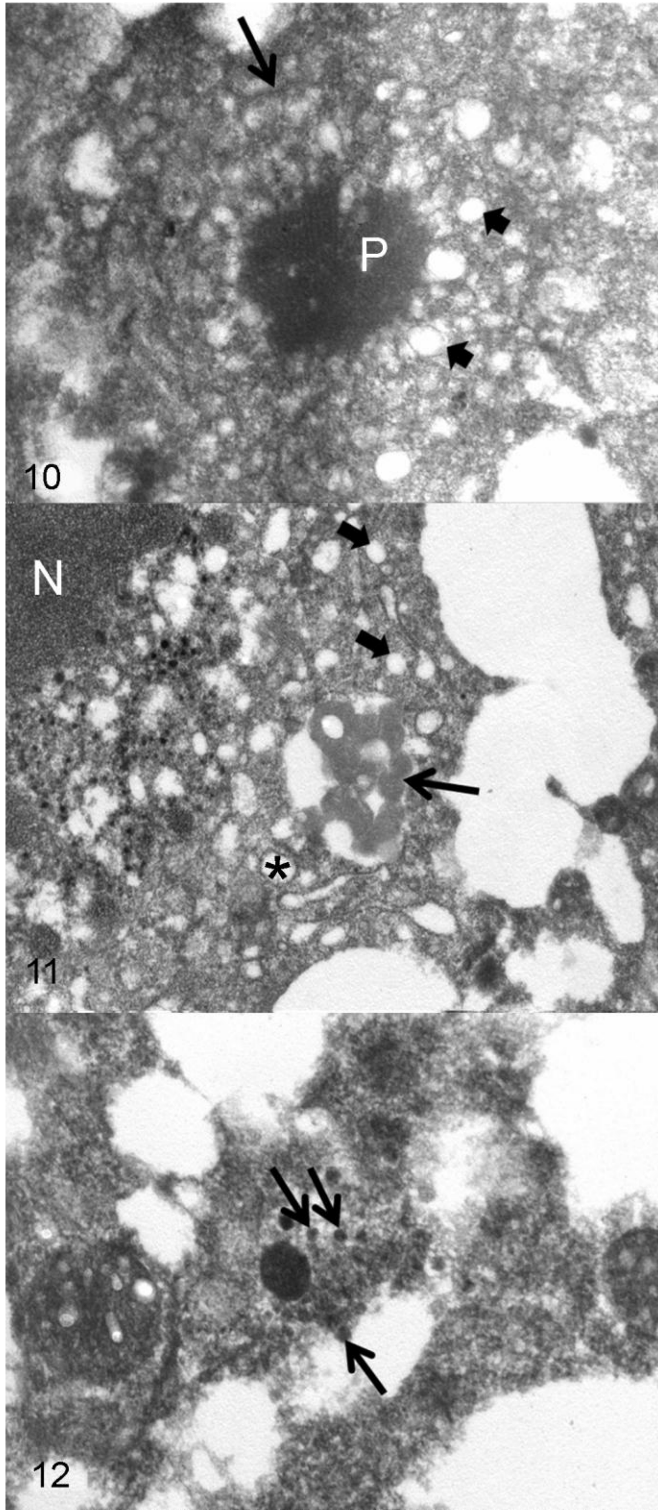

Figure 2.3.10. Avian cardiomyocyte; lineage 1 WNV, Canadian control; ultrastructure. Paracrystalline structure (P) associated with vesicular structures (short arrows) and endoplasmic reticulum (long arrow). **Figure 2.3.11.** Thalamic neuron ultrastructure; case No. 18, lineage 2 WNV, died day 9PI. Amorphous cytoplasmic inclusion (long arrow) associated with dilated endoplasmic reticulum (*) and vesicles (short arrows) in an IHC-positive area in the mouse neurone. Nucleus (N). **Figure 2.3.12.** Thalamic neuron; ultrastructure; case No.18, lineage 2 WNV, died day 9PI. Cluster of cytoplasmic virus-like particles (arrows) measuring approximately 50nm in diameter in a non-IHC positive cytoplasmic region.

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2.4 DISCUSSION

Gross and microscopic pathology were indistinguishable between lineages 1 and 2 WNV infections in 3-4-month-old male BALBc mice and the study confirmed the pathogenicity and neurovirulence in mice of the WNV lineage 2 SPU93/01 strain isolated from a South African female with naturally-acquired WNV neurological disease (Burt *et al*., 2002).

Intraperitoneal inoculation was chosen for the vaccine studies from which the mice for this pathology study were sourced to ensure that all mice developed WNV neurological disease, as previously described (Beasley *et al*., 2002; Venter *et al*., 2005). For a strictly pathogenesis study, however, intra-dermal or subcutaneous inoculation to mimic natural infection by mosquito vectors would have been preferable (Garcia-Tapia *et al*., 2007; Hunsperger & Roehrig, 2006; Kimura *et al*., 2010). The general lack of light microscopically visible lesions and IHC positivity in the spinal cord in mice of this study was likely due to direct haematogenous spread of virus from the peritoneal cavity and visceral lymphoid sites of replication to the brain. Brain entry by WNV in mice is commonly reported from blood via olfactory neuroepithelium and olfactory bulbs (Brown *et al*., 2007; Wang *et al*., 2004), by the cerebellar (Wang *et al*., 2004) or choroid plexus (Hunsperger & Roehrig, 2006) vascular routes, or by retrograde neuronal transport via sympathetic or sensory nervous systems (Hunsperger & Roehrig, 2006). Footpad inoculation, however, results in transneural spread into the ipselateral spinal cord ventral horn via the sciatic nerve (Brown *et al*., 2007) with resultant neuronal IHC positivity (Garcia-Tapia *et al*., 2007; Kimura *et al*., 2010) and/or histological changes (Garcia-Tapia *et al*., 2007), which were absent in the current study.

Mouse numbers were limited by ethics to the minimum needed for adequate statistical analysis for vaccine-efficacy testing, which in the main study (Venter *et al*., 2013), was 10 per group. As mentioned previously, numbers of non-vaccinated lineage 1 and 2 infected mice in the main vaccine study were reduced to 8 each by day 1PI. Characterisation of the mouse-pathology caused by the South African lineage 2 SPU93/01 WNV strain was one of the primary aims of the study, hence the number of

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lineage 2 infected mice was increased by 5 sourced from the other study which had only used the lineage 2 strain. In *in vivo* animal studies, despite inbred laboratory strains of mice being relatively genetically homozygous and also specific pathogen free, there remains a degree of individual variation, manifested in the current trial as the differences in duration before individual mice showed clinical signs and in duration and severity of illness, leading to dissimilar numbers of mice dying on each of days 7 to 11, and 2 mice from each lineage infection group recovering. The highest number of mortalities occurred on day 9PI (4 of lineage 2 and 2 of lineage 1). Previous reports of lineage 1 WNV infection in experimental mice of other strains found the first clinical signs and deaths occurred on day 9PI (Garcia-Tapia *et al*., 2007; Kimura *et al*., 2010; Shrestha *et al*., 2003), with the most severe histological lesions and highest viral load in CNS on days 9 and 10PI (Garcia-Tapia *et al*., 2007; Shrestha *et al*., 2003). In these studies, as in the current study, the last deaths occurred on day 11PI, and from day 13PI, when they were considered survivors, viral load in the CNS diminished but CNS IHC labelling continued with some inflammation in half the mice in one study up to day 21PI suggesting potential persistent infection (Garcia-Tapia *et al*., 2007). Similarly, a single lineage 2 WNV survivor in the current study (case No. 23) was still rRT-PCR positive in the brain at day 21PI.

Real-time RT-PCR and IHC negativity in day 5PI euthanised mouse case Nos. 6 and 7 was expected due to low incubating virus titres prior to onset of clinical signs. Similar negativity in case Nos. 14, 15 (lineage 1 day 8PI) and 17 (lineage 2 day 9PI) might have been due to individual variation with virus levels being too low for detection; unlikely minor differences in sample handling; or due to viral fluctuations (early clearance and later re-introduction) in the brain as found in 4 – 6 week-old C57B1/6 lineage 1 (NY99) WNV infected mice by Hunsperger *et al.,* (2006) (Hunsperger & Roehrig, 2006). These mice in the current study were, however, initially infected with the same WNV dose via the same route, and all showed identical clinical signs and lesions to the other infected mice which tested positive by one or other or both tests.

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The variable locations of IHC WNV-positive neurons in this study, the paucity of light-microscopically visible inflammatory changes or light-microscopically visible pathology in virus-positive cells, and lack of virus occurring perivascularly in association with lesions, conflict with previous reports (Garcia-Tapia *et al*., 2007; Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Wang *et al*., 2004; Wang *et al*., 2003). The older mice used in this study which were WNV-infected 49 days after initial (at 7-8 weeks of age) and 21-days later booster mock-vaccinations, as well as the BALBc strain not commonly used in WNV infection experiments, may have contributed to this difference in visible pathology from other reports. In general younger mice (Garcia-Tapia *et al*., 2007; Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Nir *et al*., 1965) and especially suckling mice (Eldadah & Nathanson, 1967) are most susceptible to WNV.

WNV is known to exert its influence by coding for inflammatory cytokines (Garcia-Tapia *et al*., 2007; Lim *et al*., 2011; Wang *et al*., 2004), and these may be sources of immunopathology (Bai *et al*., 2009; Lim *et al*., 2011) as seen in species such as equines which typically have very little or no IHC positivity in CNS neurons (Cantile *et al*., 2001; Kleiboeker *et al*., 2004; Pennick *et al*., 2012) despite RT-PCR positivity and often prominent nonsuppurative encephalomyelitis (Cantile *et al*., 2001; Snook *et al*., 2001). Despite extensive areas of brain being examined, in all mice dying or euthanised sick, CNS lesions were random, subtle and without a particular pattern, unlike those in previous reports (Kimura *et al*., 2010; Odelola & Oduye, 1977).

Randomly scattered IHC positivity of neurons without light microscopical evidence of intracellular lesions or presence of localised glial or leukocyte inflammatory response, share similarity with the finding by Hunsperger *et al.,* (Hunsperger & Roehrig, 2006) in dorsal root spinal ganglion neurons of lineage 1 WNV footpad-inoculated mice which were IHC positive without cytopathic effect. Morrey *et al.,* (2008a) using hamsters as experimental model for pathogenesis studies of WNV-induced flaccid paralysis, identified neuronal apoptosis and diminished cell function in spinal cord by using terminal deoxynucleotidyl transferase-mediated BrdUTP nick end labelling (TUNEL) as well as choline acetyltransferase staining respectively where animals showed paralysis

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but no light microscopically-visible neuronal lesions or inflammatory response (Morrey *et al.*, 2008a). These labelling methods were not done but might have been enlightening in the current study.

Extra-CNS lesions found in the current study concurred in some macroscopic, but not microscopic respects with findings by Kimura *et al.,* in 2010 in lineage 1 WNV footpad-inoculated 6-week-old CH3 mice (Kimura *et al.*, 2010). Gastric and small intestinal fluid distension were found in clinically-affected mice in both studies. The shortened villi described in the Kimura study were found in only 2 mice in the current study: a healthy control mouse and a lineage 2 survivor. The widespread intestinal myenteric ganglion IHC-positivity found in the Kimura study was not evident in the current study with only one mouse showing unequivocal caecal ganglion labelling of neurons which appeared normal and without presence of leukocytes. Care was needed not to over-interpret myenteric ganglion changes especially in large intestine in the current study due to varying degrees of autolysis in all mice which died and were not euthanised. The myenteric ganglia in all survivors appeared completely normal as they were in the non-infected controls, also suggesting that changes seen in ganglia of clinically-affected mice were more likely related to autolysis or were not significant.

Recent studies (Morrey *et al.*, 2010; Wang *et al.*, 2011) seeking to find neurological explanations for the respiratory, gastrointestinal, bladder and cardiac dysfunction found in some humans with WNV infection, used WNV-infected hamsters, which, like mice (Kimura *et al.*, 2010) show gastric and intestinal distension. They found by electromyograph (EMG) electrophysiological suppression of the diaphragm either directly due to WNV-infected neurons found by immunolabelling in the brain stem and mid-cervical spinal cord, or indirectly via altered vagal afferent function (Morrey *et al.*, 2010). Autonomic function and heart rate variability (HRV) were suppressed in the WNV-infected hamsters leading to reduced HRV and EMG amplitudes of the gastrointestinal tract (Wang *et al.*, 2011). In the current study WNV IHC positivity was found in brain stem neurons in all 4 mice in which it was examined; 3 lineage 1 mice on days 7, 9 and 11, and one lineage 2 mouse on day 9PI. These findings suggested a

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similar primarily CNS-origin etiology of gastric and intestinal stasis in the BALBc mice as was found in the hamsters. The pulmonary and surrounding lesions of case No. 22 were likely associated with distal oesophageal perforation secondary to gastric distension and diaphragmatic paresis.

Intestinal lesions not previously recorded were found in mice infected with both lineages and were described and illustrated. Paneth cell degranulation was most likely in response to the presence of intraluminal bacterial overgrowth secondary to intestinal stasis. Paneth cell granules contain antimicrobial peptides and are functional in maintenance of intestinal homeostasis (Bevins & Salzman, 2011). These cells in mice specifically contain alpha-defensins as well as unique defence cryptdin-related sequences (CRS)-peptides which allow for a broad repertoire of potent microbicidal activity in the intestinal lumen (Andersson *et al*., 2012).

Similar to the findings in both lineage 1 and 2 WNV-infected mice of this study, lymphocyte or lymphoid depletion were previously recorded with lineage 1 infections of female 7-8 week-old C57BL/6 mice (Garcia-Tapia *et al*., 2007; Wang & Fikrig, 2004), and of 6-week-old C3H mice (Kimura *et al*., 2010). Lesions in the current study were first seen histologically in the follicular marginal zone in one lineage 2 infected mouse euthanised and examined at day 5PI in lymphoid tissue and also small intestine, prior to clinical signs of illness or rRT-PCR or IHC detectable virus, and progressed in both lineages from day 7PI. The marginal zone of splenic germinal follicles contains dendritic macrophages important in clearance of micro-organisms and viruses (Cesta, 2006). WNV lineage 2 Sarafend strain infection of mice via any route was found to induce Tlr-3 from the cytoplasm of marginal zone splenocytes located near blood vessels: the production of inflammatory cytokines stimulated by Tlr-3 compromised the blood brain barrier allowing WNV entry into the CNS (Wang *et al*., 2004). Similar to the Kimura report, no clear IHC positivity was found in spleen or other lymphoid tissue (Kimura *et al*., 2010).

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Hepatosis in some of the control, infected and surviving mice may have been variably stress- or disease-related or due to a change in energy metabolism since sick mice were unable to eat. Pulmonary haemorrhages were interpreted as mostly traumatic during cardiac exsanguination.

Hayes *et al.,* (2005) stated that ultrastructural visualisation of particles of WNV in WNV-infected tissues is rare, and when found they are usually within endoplasmic reticulum of neurons (Hayes *et al*., 2005). EM findings in this study concurred with immunoelectron microscopy findings in cerebrum and cerebellum from a WNV-infected Chilean flamingo studied by Steele *et al.,* (2000) (Steele *et al*., 2000). In that study, virions were much less intensely labeled than the presumed flaviviral-induced dense membrane vesicle structures and convoluted membranes and these findings were consistent with immunoelectron microscopy of WNV-infected Vero cells (Steele *et al*., 2000). IHC positive staining in the current study was therefore assumed to be of the ultrastructural pre-particulate aggregates or of crystalline structures, suspected by Ghadially in 1988 to be viral (Ghadially, 1988). The paracrystalline amorphous or vesicular structures found in the IHC-positive control avian cardiomyocytes were similar to flavivirus-induced dense membrane vesicle structures described in Japanese encephalitis virus (JEV)-infected cultured neurons (Wang *et al*., 1997). Steele also mentioned cytoplasmic vesiculation or vacuolation in association with flavivirus-induced structures (Steele *et al*., 2000). The amorphous structures found in the mouse neurons, although linked to endoplasmic reticulum and vesicles, differed from the paracrystalline structures in the avian cardiomyocytes.

2.5 CONCLUSION

Intraperitoneal inoculation of neurovirulent lineage 1 (NY99/385) or South African lineage 2 (SPU93/01) WNV strains in 3-4 month-old male BALBc mice caused similar clinical neurological disease and death with gross necropsy and histopathological lesions being indistinguishable between the lineages. CNS lesions were subtle despite severe neurological signs and may have indicated virus-associated biochemical damage with a paucity of light microscopically identifiable immunopathology in the older

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BALBc mice used. Gastro-intestinal lesions were prominent and most likely initiated by stasis which, based on IHC positivity of deep brainstem neurons, was suggestive as more likely of WNV-associated brain-stem rather than local myenteric origin, although accurate interpretation of myenteric ganglion changes was hampered by autolytic changes and small intestinal distension. Ultrastructural findings of WNV-positive IHC labelled cells concurred with previous findings in WNV-infected birds that WNV particles are rare in WNV-infected tissues and not necessarily labelled with antibody, and that other cytoplasmic structures which were labelled are most likely virus-related.

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CHAPTER 3

Pathology of Fatal Lineage 1 and 2 West Nile Virus Infections in Horses in South Africa

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3.1 INTRODUCTION

The *Flavivirus*, West Nile virus (WNV), cycles naturally between birds and ornithophilic mosquitoes, especially *Culex univittatus* in South Africa (Jupp, 2001). Lineage 1 WNV strains are known to be neuropathogenic and re-emergent in the northern hemisphere (Petersen & Roehrig, 2001), with the closely-related but less pathogenic subtype, Kunjin virus (Scherret *et al*., 2001), and its 2011 equine pathogenic variant $WNV_{NSW2011}$ (Frost *et al*., 2012), occurring in Australia. Lineage 2 WNV has been reported in South Africa since 2007-2008 as a cause of severe emerging neurological disease in horses (Venter *et al*., 2009b) and humans (Zaayman & Venter, 2012), and since 2004 in Europe as a cause of severe illness or death in birds (Bakonyi *et al*., 2006; Danis *et al*., 2010), horses (Kutasi *et al*., 2009) and humans (Danis *et al*., 2010; Papa *et al*., 2011a; Papa *et al*., 2010a; Papa *et al*., 2011c; Papa *et al*., 2010b; Papa *et al*., 2011d; Platonov *et al*., 2008). Recent outbreaks of lineage 2 WNV in Romania in 2010 (Sirbu *et al*., 2011) and lineage 1 WNV in the USA in 2012 have shown the incidence of neuroinvasive disease in humans to have risen to more than 50% of clinical cases (Chung *et al*., 2013). Horses and humans are similarly susceptible to WNV infection (Beasley, 2005), however horses have been afforded the opportunity of protection from WNV disease in the USA (Schuler *et al*., 2004) and more recently Europe since the introduction and registration of equine-specific lineage 1–derived WNV vaccines.

In the South African survey of horses with neurological signs over the summer of 2007-2008 (Venter *et al*., 2009b), seven horses were WNV lineage 2 positive by real-time RT-PCR (Zaayman *et al*., 2008) or WNV positive and analysed as lineage 2 by sequencing on blood, brain and/or spinal cord and one by viral isolation. Five died or

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had to be euthanized and gross and microscopic pathology descriptions were tabulated of 3. These had marked pulmonary, subcutaneous and/or intermuscular oedema, and hydropericardium, with microscopic lesions including those described for WNV lineage 1 (Venter *et al.*, 2009b). The microscopic pathology of one case was atypical with peripheral spinal white matter Wallerian degeneration without inflammatory leukocyte infiltration apart from phagocytosing myelinophages. One case was co-infected with African horsesickness virus (AHSV) (Venter *et al.*, 2009b).

During the autumn of 2010, of 17 horses contracting neurological lineage 2 WNV in Hungary (Kutasi *et al.*, 2011). Four of the 5 which died were examined histologically. Gross pathology was reported as "negative" and histological lesions as lymphoplasmacytic perivascular infiltration, gliosis with some focal neutrophil infiltration, and neuronal degeneration. Lesions occurred mostly in brain stem, medulla and grey matter of the spinal cord, with cervico-thoracic and lumbar region lesions being the most severe (Kutasi *et al.*, 2011).

Detailed CNS histopathology descriptions of CNS of WNV lineage 1-associated natural (Bunning *et al.*, 2002; Cantile *et al.*, 2001; Cantile *et al.*, 2000; Guillon *et al.*, 1968; Schmidt & El Mansoury, 1963; Snook *et al.*, 2001), or experimental (Bunning *et al.*, 2002) equine cases in north Africa, Europe and North America have been published. Italian cases had mild rhombencephalitis, and constant spinal cord ventral horn non-suppurative polio-encephalomyelitis mostly of thoraco-lumbar regions (Cantile *et al.*, 2001; Cantile *et al.*, 2000). A small comparative USA study showed moderate to severe multifocal perivascular lymphocytic rhombencephalitis, perivascular haemorrhages, scattered neutrophils and multifocal microgliosis; spinal cord examined in a single case had similar lesions (Cantile *et al.*, 2001; Snook *et al.*, 2001). Gross necropsy lesions were generally recorded as absent in CNS and/or internal organs except for occasional cases (Antorino *et al.*, 2002; Bunning *et al.*, 2004; Cantile *et al.*, 2000; Snook *et al.*, 2001). Visceral lesions were reported in horses by Schmidt *et al.,* (1963) in Egypt (Schmidt & El Mansoury, 1963), Tber Abdelhaq (1996) in Morroco (Tber Abdelhaq, 1996) and Steinman *et al.,* (2002) in Israel (Steinman *et al.*, 2002). These included

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pulmonary oedema and congestion (Tber Abdelhaq, 1996); visceral congestion and occasional haemorrhages (Schmidt & El Mansoury, 1963); cardiac haemorrhages with mild pericardial effusion (Steinman *et al*., 2002); meningeal congestion, oedema and/or haemorrhages (Schmidt & El Mansoury, 1963; Tber Abdelhaq, 1996); and lumbosacral spinal cord haemorrhage in one horse (Steinman *et al*., 2002). Spinal cord congestion with lumbar and thoracic segment haemorrhage were recorded in Italy (Cantile *et al*., 2000), with other authors recording occasional visible CNS petechiae in cut sections (Snook *et al*., 2001), perivascular haemorrhages (Cantile *et al*., 2001), and one described meningeal hyperaemia with subdural exudates and fibrin tags (Bunning *et al*., 2004).

Experimental infections of mice and hamsters have shown various routes of WNV CNS entry and transport after extra-cerebral inoculation, including regional motor axonal transport from footpad or sciatic nerve inoculation into the ipselateral lumbar spinal cord ventral horn grey matter (Brown *et al*., 2007; Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Morrey *et al*., 2008b; Samuel *et al*., 2007; Wang *et al*., 2009), transneuronal anterograde and retrograde intra-spinal transport (Wang *et al*., 2009), multiple point entry into the spinal cord by haematogenous or neurinvasive routes (Brown *et al*., 2007), rapid early neural transport during viraemia from the olfactory neurons into the olfactory bulbs (Brown *et al*., 2007; Hunsperger & Roehrig, 2006; Nir *et al*., 1965) as is also seen in flaviviral St Louis encephalitis infections in hamsters (Monath *et al*., 1983), with caudal progression in the brain (Nir *et al*., 1965), and circulatory CNS entry via the choroid plexus (Hunsperger & Roehrig, 2006). Dorsal sensory horn grey matter was also occasionally infected after experimental sciatic nerve (Hunsperger & Roehrig, 2006; Wang *et al*., 2009) or ventral horn infection (Wang *et al*., 2009). Neuronal tissue culture and hamster WNV experiments (Samuel *et al*., 2007) concluded that WNV reaches neuronal or non-neuronal target cells by viral release extracellularly at distal axon sites, and that WNV uses specific pathways to enter the CNS, resulting in distinct disease phenotypes.

Confirmatory laboratory diagnostic criteria for WNV in patients with clinical neurological

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disease include isolation of virus or detection of viral antigen or nucleic acid in tissue, blood or CSF (Beasley, 2005). RT-PCR and nucleic acid sequencing is approximately 1000-fold more sensitive than viral culture for WNV detection (Hayes *et al*., 2005). Immunohistochemistry (IHC) with either monoclonal or polyclonal antibodies (Buckweitz *et al*., 2003; Cantile *et al*., 2001; Castillo-Olivares & Wood, 2004; Kleiboeker *et al*., 2004; Pennick *et al*., 2012), and *in situ* hybridisation were found to be disappointing and unreliable for diagnosis in horses with WNV neurological disease (Cantile *et al*., 2001; Kleiboeker *et al*., 2004; Pennick *et al*., 2012), being often negative in the presence of positive PCR results (Buckweitz *et al*., 2003). The amount of virus did not correlate with the degree of inflammation, and virus distribution was often scant or localised.

This paper details and illustrates pathology and histopathology of 6 natural South African equine cases of WNV lineage 2 infection, including a more in-depth investigation of one of the 3 previously-described cases (HS101/08) for comparison (case 1) (Venter *et al*., 2009b), and a peracute case (case No. 5). The pathology of the recent 2010 WNV lineage1 case from the Western Cape in a pregnant mare which aborted then died (Venter *et al*., 2011), the first recorded in South Africa, is included (case No. 7) for illustration and comparison with the lineage 2 cases.

3.2 MATERIALS AND METHODS

3.2.1 Clinical Cases

Seven horses with severe neurological signs resulting in either death or euthanasia were necropsied and gross and microscopic pathology investigated (Table 3.3.1). Cases from 2008 to 2011 occurred during early (case No. 5) or late summer seasons, and were diagnosed with single WNV CNS infection (Table 3.3.2). One mare had recovered from AHS 10 days prior to developing neurological signs (case No. 6). Case histories and clinical descriptions (Table 3.3.1) were supplied by the referring veterinarians and by some owners. Case Nos. 1 - 6 were from the Gauteng highveld district of South Africa and case No. 7 from the Western Cape. Some horses had been antemortally tested for some viruses in blood (Table 3.3.2) and/or viral antibodies.

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3.2.2 Necropsy Examination

Case Nos. 1 - 6 were necropsied at the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria (UP), Onderstepoort, and case No. 7 by the referring private practitioner at Ceres in the Western Cape. Specimens from lumbar, thoracic and cervical spinal cord, as well as regions of brain variably including cerebellum, brain stem, midbrain, thalamus, hippocampus, olfactory lobe and surrounding cortex, were preserved in 10% neutral buffered formalin for a minimum of 24-48 hours. Only formalin-preserved and fresh brain were received from case No. 7. Duplicate samples of fresh CNS were placed on ice for RT-PCR (Case Nos. 2 – 7) and in case No. 1 for virus isolation with later RT-PCR of the isolate (Venter *et al.*, 2009b). Fresh brain was also submitted for rabies virus testing (case Nos. 1, 2 and 6). Samples of internal organs from case Nos. 1 - 6 and testicle of case Nos. 3 and 4 were also formalin-fixed. Fresh samples of at least spleen and lung were collected for RT-PCR for AHS, equine encephalosis virus (EEV) and equine herpes virus-1 and -4 (EHV) and for virus isolation (case Nos. 1, 3 and 6).

3.2.3 Histopathology

Selected regions of formalinised CNS (Table 3.3.1) and other tissues were prepared for routine wax-embedding, 4µm sectioning, haematoxylin and eosin (HE) staining and light microscopic examination.

3.2.4 Immunohistochemistry

IHC staining of CNS tissues in sections showing pronounced lesions from case Nos. 1 - 7 plus 1 section of testicle with orchitis from case No. 3, was performed using Thermo Scientific (Pierce Biotechnology, Illinois, USA) rabbit polyclonal WNV E antibody at dilution 1:50 according to the avidin-biotin complex technique (Haines & Chelack, 1991). Canadian origin (Dr Josepha DeLay, University of Guelph, Ontario, Canada) known RT-PCR positive lineage 1 WNV-infected avian heart and equine brain, as well as RT-PCR and IHC positive BALBc mouse brain infected with South African neurovirulent SPU93-01 lineage 2 WNV were simultaneously stained as positive controls. IHC using rabies

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polyclonal antibody (prepared by the late Dr Ken Charlton, Animal Diseases Research Institute, Ontario, Canada) at dilution 1:500, was performed on WNV positive controls as an irrelevant antibody with simultaneous staining of a rabies positive control, plus brain sections of case Nos. 3, 4, 5 and 7 to exclude rabies. IHC was similarly performed for EHV (J van der Lugt, OVI) at dilution 1:400, and the flavi-virus Wesselsbron (WESSV) (J van der Lugt, OVI) at dilution 1:500, on brainstem, spinal cord and testicle of case No.3, and for EHV and AHS (polyclonal rabbit anti-AHSV serum at dilution 1:2000, from C. Hamblin, Institute for Animal Health, Pirbright Laboratory, United Kingdom) on brain, spinal cord, spleen, heart, liver, kidney and lung of case No.4, using simultaneously-stained known positive control sections for each. IHC for equine encephalosis virus (EEV) (antiserum used at dilution 1:6000, obtained from C. Hamblin, Institute for Animal Health, Pirbright Laboratory, United Kingdom) and EHV were performed on spleen, lung and lumbar spinal cord of case No.1. (Table 3.3.2).

IHC cross-reactivity was tested on the respective control positive sections for the two flaviviruses WNV and WESSV virus, both present in South Africa, WESSV having been diagnosed previously by RT-PCR in two horses with neurological signs in South Africa, one of which was fatal (Venter *et al.*, 2008).

3.2.5 Digital images

Digital images were recorded during the majority of necropsies and preparation of formalinised specimens using Panasonic Lumex FZ10 or FZ45 cameras (Panasonic Corporation, Ariake Tokyo, Japan). Images of histological lesions of all cases were taken on an Olympus BC50 (Olympus, Hamburg, Germany) microscope with attached CC12 soft imaging system, using analySIS software (Olympus, Hamburg, Germany).

3.2.6 Viral diagnosis and viral isolation

Rabies virus fluorescent antibody testing (FAT) on fresh composite brain smears was performed at the Onderstepoort Veterinary Institute (OVI) for case Nos.1, 2 and 6.

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Fresh necropsy CNS tissues underwent RNA extraction and were tested by realtime RT-PCR for WNV by the method described by Zaayman *et al.,* (2008) (Zaayman *et al*., 2008) as well as for flavi-family.

CNS extracts of all cases were also RT-PCR tested to exclude the South African differential diagnoses of EEV, EHV and the orthobunyavirus Shunivirus (Van Eeden *et al*., 2012), with CNS of all cases being similarly tested for AHSV. Other tests done for WNV, WESSV, Sindbis (SINV) and Middelburg viruses, AHS, EEV and EHV on various tissues in various cases are recorded in Table 3.3.2 and included viral isolation using standard techniques on either Vero cells (case No. 1), or baby hamster kidney (BHK) cells; IHC; and RT-PCR on blood drawn antemortally (case Nos. 1 and 7) or directly post-mortally (case No. 5), and on organs other than CNS. The serum of case No. 1 was tested antemortally for antibodies to AHS, EEV, WNV and EHV-1 and –4 (Venter *et al*., 2009b).

RNA extracts of CNS from all cases, and blood of case No.5, were screened with an alphavirus family-specific RT-PCR as described in Sanchez-Seco *et al.,* (2001) (Sanchez-Seco *et al*., 2001).

3.2.7 Other

Due to the very early seasonal, unexpected occurrence and acute nature of case No. 5, this mare was also tested for organophosphate, organochlorine and carbamate pesticides in stomach content and for brain lead concentration at the OVI Toxicology Department. Aerobic and anaerobic bacterial cultures were performed on lung, spleen, brain and jejunal content, as well as *Salmonella* culture of the jejunum. Transmission electron microscopy (TEM) was done on testicular tissue from case No. 3 by deparaffinizing wax block material (Van den Berg Weermans & Dingemans, 1984). Ultra-thin resin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips CM10 transmission electron microscope (Philips Electron Optical Division, Eindhoven, The Netherlands) operated at 80 kV. Digital images were captured with a Megaview III side-mounted digital camera (Olympus Soft Imaging

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Solutions GmbH, Münster, Germany) and iTEM software (Olympus Soft Imaging Solutions GmbH, Munster, Germany).

3.3 RESULTS

Diagnostic testing using nested realtime RT-PCR was positive for WNV on CNS tissues in all cases, as well as with viral isolation of brain in case No. 1. All other viruses tested for by various tests were negative except for AHS which was RT-PCR positive in spleen and lung of case No.6, but did not culture positive in tissue culture. Case No. 6 had recovered from AHS 10 days prior to developing neurological signs due to WNV. All horses exhibited severe neurological clinical signs and demonstrated from mild to severe histological meningo-encephalomyelitis. The lineage 2 cases showed much variation of severity, distribution and type of CNS microscopic lesions between and within individual cases, and the lineage 1 case had mild histological lesions by comparison. Macroscopic lesions were nospecific but often resembled the oedema and haemorrhages seen with African horsesickness. Results are further described in detail and tabulated in Tables 3.3.1. and 3.3.2. Selected lesions from cases, macroscopic and microscopic, are illustrated in Plates 3 and 4.

3.3.1 Clinical signs

Signalment, clinical signs, final outcome of the case (death or euthanasia) and date of occurrence for case Nos. 1 – 7 are tabulated (Table 3.3.1).

Table 3.3.1

Signalment, clinical signs, month of disease, comparative severity and distribution of CNS histological lesions of WNV positive equine cases.

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Table 3.3.1: Signalment, clinical signs, and comparative severity and distribution of CNS histopathological lesions of WNV positive equine cases.

Case No.	Lab. Nos.	Signalment and date	Clinical signs	Olfactory region	Cerebral cortex	Thalamus	Medulla /brain stem	Cerebellum	Cervical sp. cord /br-stem	Thoracic spinal cord	Lumbar Spinal cord
Case No.1*	PM394-08 S1830-08 HS101/08	8-yr TB gelding Mid-April 2008	Recumbency, muscle twitching, loss of awareness, colic, seizures, nystagmus, died after 4days.	+	+	+	+	+	+	+	+ -++ meningeal vasculitis
Case No.2*	PM250-10 S959-10 SAE18/10	11-yr Wmbl gelding.Mid-March 2010	Sudden ataxia, depression, tongue paralysis, falling, fell dead after 5days	++	+	++	++	+	+ - ++	++	+++ Haem ++infl.
Case No.3*	PM260-10 S1024-10 SAE22/10	2-yr TB colt Early April 2010	Difficulty rising, laminitic stance, ataxia, recumbency, euthanized after 36hr	+	+	+++	+++	++	+++	+-++	+-+++ focal assym GM VH malacia
Case No.4*	PM355-10 S1606-10 SAE51/10	4-yr Arab stallion.Late April 2010	Initial fever, ataxia, hindlimbs weak, recumbent, paddling forelimbs, nystagmus, euthanised at 4 days.	+++	+GM ++WM	++	++	++ GM/WM ++ meningitis	++-+++	++	+
Case No.5*	PM740-10 S3592-10 SAE121/10	17-yr WelshX mare. Early September 2010	Progressive fever, lateral recumbency, seizures, mydriasis, loss of cranial reflexes, tachypneoa, gurgling , died after 36h.	+	+	+	Choroid plexus + PVC	-	PVhaem	PVhaem	-
Case No.6*	PM425-11 S1605-11 SAE109/11	7-yr Arab mare Late April 2011	Progressive ataxia, recumbent, quadriparetic at 3 days, assymetric forelimb paresis. Euthanasia	+	+	+ Mild meningitis	++	+	++-+++ Assym sp motor N neuritis	++-+++	++-+++

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Case No.7**	S2207-10 SAE75-10	6-yr pregnant TB mare. May 2010	Progressive ataxia, recumbent, quadriparetic at 3 days. Euthanasia.	++	+	+	+	++ Molecular layer glial flares	NE	NE	NE

Subjective light microscopic determination of the severity of inflammatory lesions in each site was made: +, mild; ++, moderate; +++, marked; -, absent; lab, laboratory; nos, numbers; PM, post mortem; S, histology specimen; HS, horse specimen; SAE, RT-PCR laboratory number; *, WNV lineage 2; **, WNV lineage 1; olf, olfactory; NE, not examined; yr, year; h, hours; TB, Thoroughbred; Wmbl, warmblood; X, crossbreed; GM, grey matter; WM, white matter; assym, assymetrical; sp, spinal; br, brain; PVC, perivascular cuffing; PV, perivascular; haem, haemorrhage; infl, inflammation; VH, ventral horn; N, nerve; AHSV, African horse sickness virus. Where lesions were unique or outspoken they are written out

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3.3.2 Viral diagnoses and genotyping

All cases were RT-PCR positive for WNV in brain (case Nos.1, 2, 4-7) and/or spinal cord (case Nos.1-4 and 6) (Table 3.3.2). Brain of case No.1 yielded virus on tissue culture, which on RT-PCR was WNV lineage 2, whereas spleen and lung from this horse were virologically negative (Venter *et al.*, 2009b). Antemortal serological testing of case No.1 by ELISA showed antibodies for AHS (1:8), EEV (1:4), and EHV-1 (1:8), with WNV and EHV-4 negative.

Case Nos. 1 - 6 were shown to be WNV lineage 2 and case No. 7 lineage 1 (Venter *et al.*, 2011) CNS positive by real-time RT-PCR. The WNV titre of case No .5 was low and positive only in the olfactory cortex and thalamic regions with all other areas of brain and spinal cord being negative.

All other tests were negative except for spleen and lung of case No. 6 which were RT-PCR positive for AHS, but from which viral isolation was negative. This mare had previously been diagnosed with AHS in blood (test and laboratory not specified), from which she had clinically recovered. Control positive sections for the rabies, WNV, WESSV, EEV, EHV and AHS antibodies all stained correctly by IHC. The WESSV and WNV antibodies did not cross-react.

In CNS sections selected from areas of marked lesions from all cases, IHC for WNV stained positively a single glial cell central to a glial nodule and 1 small cortical neurone in the olfactory cortex of case No.4, and 3 glial cells in the Canadian lineage 1 WNV equine brain control. In control positive sections WNV antigen stained in several avian cardiomyocytes and mouse brain neurons respectively.

Table 3.3.2

Viruses which tested positive and negative in CNS and other tissues by various diagnostic tests in naturally-infected WNV equine case Nos. 1-7.

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3.3.3 Other tests

In case No.5, brain lead measured 0.75 parts per million (ppm) (toxic levels range from 3.0-30ppm (Puls, 1994)); stomach content was negative for common pesticides; and aerobic and anaerobic bacterial culture from brain, lung, and spleen were negative after 72 hours' incubation, with jejunal content having normal bacterial flora and *Salmonella* species culture negative. Ultrastructure of the testicular tissue from case No. 3 did not show viral particles in the 50nm size range of WNV, nor amorphous bodies or crystalline structures which might have suggested pre-particulate aggregates of WNV (Ghadially, 1988; Steele *et al*., 2000).

3.3.4 Gross Necropsy Findings

The gross pathology common to most cases included variable external self-induced trauma especially to bony prominences; mild to severe pulmonary oedema and congestion; variable serosanguinous hydropericardium, occasional hydrothorax or ascites; subcutaneous and intermuscular oedema mostly of the neck but also occasionally of hindquarters (case No. 1) and genital region (case Nos. 1 and 3); oedema of the supraorbital fossae (case Nos. 1, 3, 5 and 6); white or blood-stained foam lining the distal trachea (case Nos. 2 and 6), filling the tracheal lumen (case Nos. 1, 3 and 4) or exuding from the nostrils (case No.3) (Fig. 3.3.1); and mild haemorrhages on serosal surfaces especially of the heart.

Case No. 5 had epicardial, subpleural, diaphragmatic, mesenteric, tracheal mucosal, adrenal cortical and bronchial lymph node petechiae, ecchymoses and suggilations, and showed moderate visceral cyanosis and dark congestion.

The tongue of case No .2 was flaccid, oedematous and pale with a single dorsal epidermal vesicle of approximately 2cm diameter. Case No. 6 had multifocal oral and lingual ulcerations of uncertain origin: she showed mild pulmonary oedema.

Macroscopic CNS lesions included ecchymotic haemorrhages of lumbar spinal grey and white matter and diffuse meningeal subarachnoid lumbar spinal haemorrhage (case No.

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2); diffuse dorsal lumbar and thoracic meningeal subarachnoid haemorrhage (case No. 6); and/or small petechiae and markedly distended vessels in spinal cord and brain grey and/or white matter, with predominance in the lower brain centres (case Nos. 2-4) (Fig. 3.3.2). CNS was otherwise variably congested.

3.3.5 Histological Findings

See Table 3 for comparison of CNS severity and distribution of lesions common to all cases and additional or severe lesions specific to certain cases.

3.3.5.1 Spinal cord

Lesions were mostly asymmetrical, not always bilateral, and included meningeal, white and grey matter perivascular petechiae, to massive lumbar cord meningeal (case Nos. 2 and 6) and intramedullary haemorrhage (case No. 2) (Fig.3.3.4), with vascular congestion especially of grey matter but also focally in white matter. Variable perivascular cuffing with lymphocytes, plasma cells, sometimes macrophages and occasional neutrophils was found (Table 3.3.1).

Other lesions included diffuse grey matter gliosis; glial nodules at times with a few neutrophils; scattered apoptotic glial cells and peri-central canal gliosis (case Nos. 1 and 2 of lumbar cord). All lesions were most prominent in ventral horn grey matter, sometimes occurred in dorsal and lateral horns, and were sporadic in white matter and meninges. Distribution and severity of lesions varied markedly between cases (Table 3.3.1). Nonsuppurative poliomyelitis was prominent in the *cauda equina* of case No. 2. Occasional segmental lumbar spinal meningeal vascular wall necrosis was seen (case No. 1) (Venter *et al.*, 2009b).

Focal unilateral lumbar cord ventral horn poliomalacia, within which were Gitter cells, other glial cells, lymphocytes, plasma cells, macrophages and some necrotic neurones, was found in case No. 3 (Fig.3.3.5). Unilateral ventral motor spinal nerve neuritis, associated with multifocal axon and myelin degeneration and mononuclear

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inflammatory cell infiltration, was found in the distal cervical cord of case No.6 (Fig. 3.3.11).

3.3.5.2 Brain and cerebellum

Lesions resembled those in the spinal cord (Figs.3.3.3 and 3.3.6), as well as occasional scattered foci of mononuclear meningitis involving cerebrum (mild in case Nos. 2, 6) and/or cerebellum (moderate in case No. 4).

Olfactory lobe and surrounding frontal cortex lesions were prominent in case No. 4 (Fig. 3.3.7), comprising multifocal grey matter glial nodules containing necrotic neutrophils, as well as moderate to severe white matter perivascular mononuclear cuffing, meningeal congestion and vascular leucostasis. This case also exhibited cerebellar molecular layer glial “flares” (Fig. 3.3.8). Occasional small vessels were found with mild perivascular mononuclear cuffing in frontal and parietal cortices (Fig. 3.3.9) and in the choroid plexus (Fig. 3.3.10) of case No. 5, with olfactory lobe and frontal cortical white matter oedema, and white matter perivascular petechiae. Case Nos. 1, 2, 5 and 7 showed moderate frontal cortex and olfactory lobe lesions, with some areas of grey matter of case No. 6 exhibiting neuronal satellitosis. Leuco-encephalitis affecting predominantly the thalamus, midbrain, pons and brainstem was relatively more pronounced in case Nos. 2 - 4 and 6. Inflammatory lesions of lineage 1 WNV (case No. 7) were by comparison mild to moderate (Fig. 3.3.12).

Plate 3 (Figs 3.3.1-3.3.6)

Plate 4 (Figs 3.3.7.-3.3.12)

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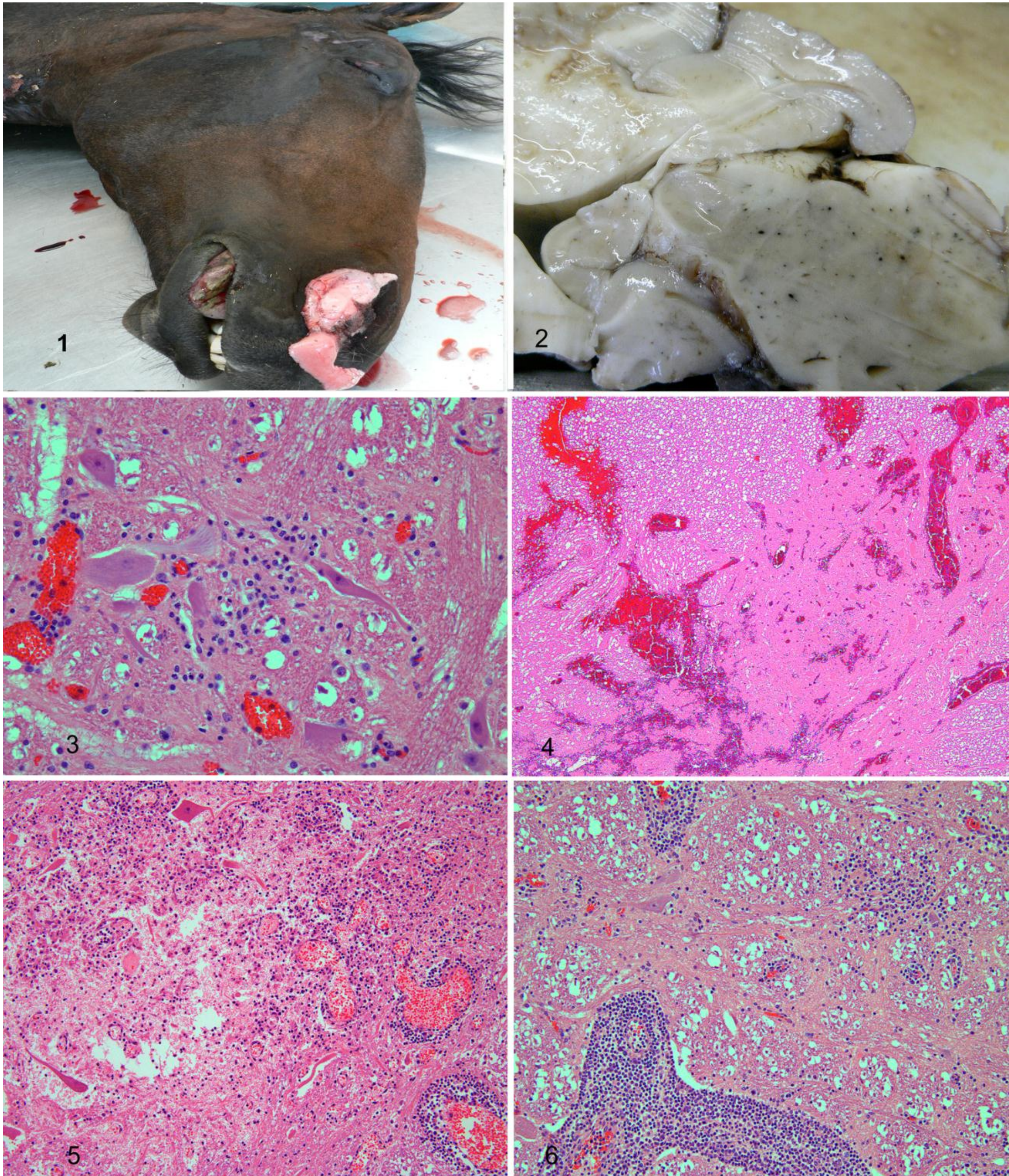

Plate 3. Figure 3.3.1. Head; case No. 3, lineage 2 WNV. Oedema of the supraorbital fossae and blood-tinged foam at nostrils. **Figure 3.3.2.** Thalamus; case No. 4, lineage 2 WNV. Macroscopic vascular distension and petechiae. HE **Figure 3.3.3.** Brain stem; case No. 1, lineage 2 WNV. Glial nodule with scattered neutrophils, mild perivascular mononuclear cuffing, and intermittent neuronal degeneration. HE. **Figure 3.3.4.** Lumbar spinal cord; case No. 2, lineage 2 WNV. Severe multifocal to coalescing haemorrhage involving white and grey matter. HE **Figure 3.3.5.** Lumbar spinal cord; case No. 3, lineage 2 WNV. Unilateral ventral horn focal poliomalacia, neurone necrosis and perivascular lymphoplasmacytic and macrophage cuffing. HE **Figure 3.3.6.** Brain stem; case No. 3, lineage 2 WNV. Severe lymphoplasmacytic and macrophage perivascular cuffing and multifocal glial nodules. HE

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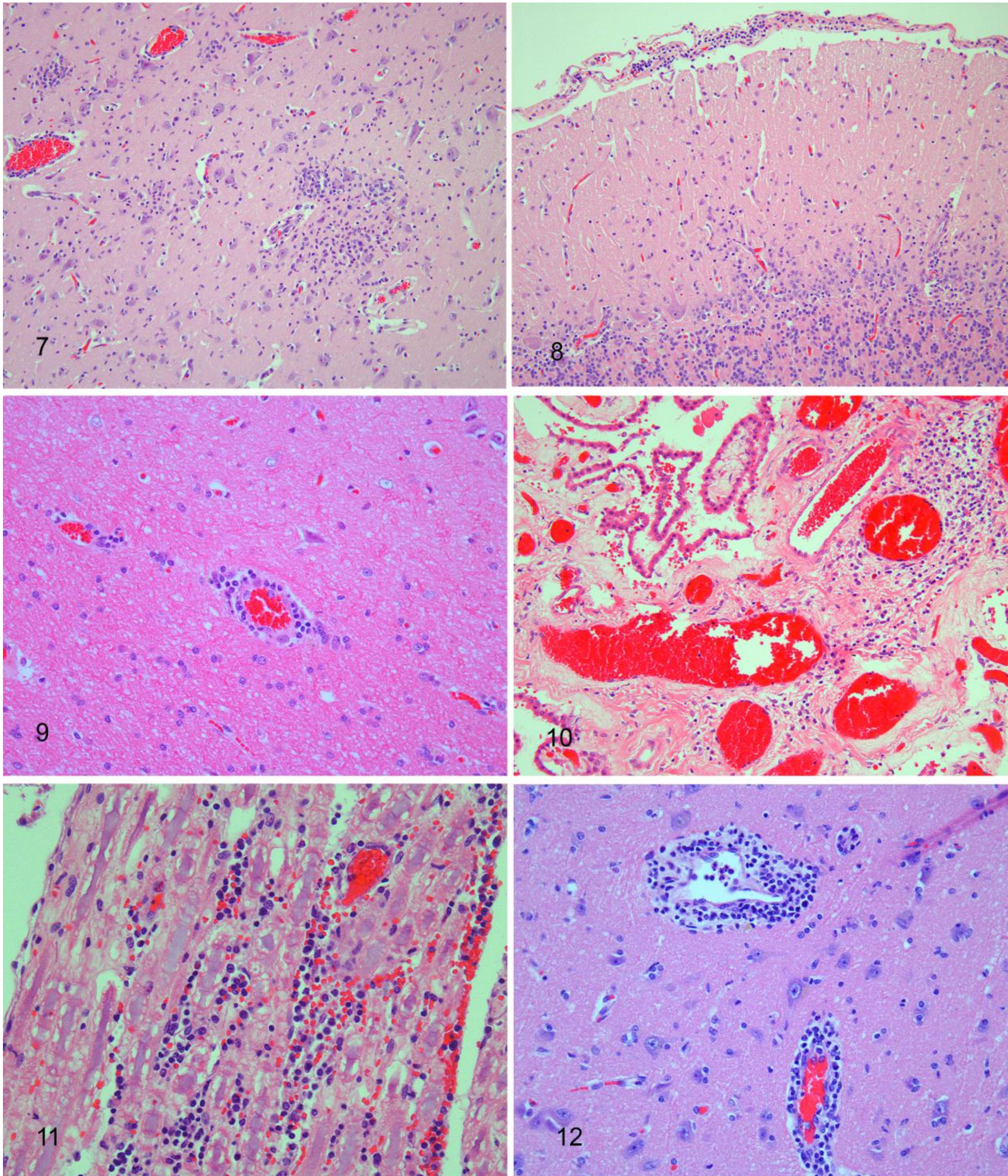

Plate 4. Figure 3.3.7. Olfactory cortex; case No. 4, lineage 2 WNV. Multifocal glial nodules, diffuse gliosis and perivascular lymphoplasmacytic and macrophage cuffing. HE **Figure 3.3.8.** Cerebellum; case No. 4, lineage 2 WNV. Molecular layer gliosis with glial flares and intermittent mild mononuclear meningitis. HE **Figure 3.3.9.** Parietal cortex white matter; case No. 5, peracute lineage 2 WNV. Mild perivascular mononuclear cuffiing. HE **Figure 3.3.10.** Choroid plexus; case No. 5, peracute lineage 2 WNV. Multifocal perivascular mononuclear inflammation and congestion. HE **Figure 3.3.11.** Distal cervical spinal cord; case No. 6, lineage 2 WNV. Locally-extensive mononuclear ventral motor spinal neuritis. HE **Figure 3.3.12.** Thalamus; case No. 7, lineage 1 WNV. Perivascular lymphoplasmacytic cuffing and multifocal glial nodules. HE.

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3.3.5.4 Other lesions

Case No. 3 showed severe genital oedema and multifocal lymphoplasmacytic degenerative orchitis. The testicles of case No. 4 by comparison were histologically normal and undergoing spermatogenesis. The lingual vesicle of case No. 2 was associated with submucosal perivascular mononuclear inflammation and underlying marked non-inflammatory inter-myofibre lingual oedema with occasional segmental myolysis. This horse also showed mild nephrosis, mild periportal hepatic hydropic vacuolisation, and occasional small foci of hepatocyte necrosis. Case No. 6 had midzonal hepatocyte hydropic change and multifocal mild necrosis with associated presence of macrophages. The lingual ulcers of case No. 6 were superficially contaminated with debris and bacteria, with an underlying necropurulent reaction zone extending deep into submucosa and muscle layers; this mare also had occasional mild interstitial lymphoplasmacytic nephritis.

3.4 DISCUSSION

This study confirmed South African field strains of WNV lineage 2 to be neuroinvasive and highly pathogenic in horses, and described and illustrated the macroscopic and microscopic lesions. The lineage 2 cases showed much variation of severity, distribution and type of CNS microscopic lesions between and within individual cases, but the majority of lesions were similar to those reported with lineage 1 WNV (Bunning *et al.*, 2002; Cantile *et al.*, 2001; Cantile *et al.*, 2000; Guillon *et al.*, 1968; Schmidt & El Mansoury, 1963; Snook *et al.*, 2001) and Hungarian lineage 2 cases (Kutasi *et al.*, 2011). Olfactory region encephalitis and asymmetrical spinal neuritis suggested varying neural routes of entry of WNV into the CNS as had been found by extracerebral inoculation of WNV in experimental mice and hamsters (Brown *et al.*, 2007; Hunsperger & Roehrig, 2006; Kimura *et al.*, 2010; Morrey *et al.*, 2008b; Samuel *et al.*, 2007; Wang *et al.*, 2009).

All positive RT-PCR products were confirmed by sequencing of the NS5 region (genome positions 9091-9191) except for case No. 5 in which viral load was too low

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indicating the value of the nested real-time PCR in distinguishing between lineages. Previous phylogenetic analysis had shown that the lineage 1 WNV identified in case No.7 was similar to a northern African Tunisian strain (PAH001) isolated in 1997 from a person who died of neurological disease, with E-protein analysis grouping it with isolates from Russia and Tunisia (Venter *et al.*, 2011).

The unilateral ventral motor spinal neuritis at the region of pectoral limb supply in case No. 6 corresponded with her clinical asymmetrical forelimb paresis. This suggested ascending motor neural transmission of virus from ipselateral cutaneous inoculation by infected mosquitoes as found after cutaneous inoculation in mice and hamsters (Brown *et al.*, 2007; Hunsperger & Roehrig, 2006; Kimura *et al.*, 2010; Morrey *et al.*, 2008a; Wang *et al.*, 2009). This pathogenesis, as well as multiple point entry into the spinal cord (Brown *et al.*, 2007), may explain clinical signs of "lameness" and/or muscle twitching seen in one or more single limbs of some horses with WNV neurological infection and also the variation in CNS microscopic lesion distribution and severity in the current series of natural cases.

Prominent olfactory lobe lesions suggested olfactory neuroepithelial entry of circulating WNV into the CNS of several of the horses. Case No. 5, which was peracute, was only real time RT-PCR positive from olfactory cortex and thalamus, with more caudal regions of CNS and spinal cord negative. Microscopic lesions in this case were similarly acute and found only in the cranial half of the cerebral cortex, with perivascular leukocyte infiltration of ventricular choroid plexus vessels suggesting also a vascular route of entry.

The 2010 late summer cases had macroscopically visible vascular lesions in the CNS and prominent microscopic lesions. The lineage 1 WNV case lesions were mild in comparison, but also included olfactory cortex lesions. All horses showed severe neurological signs despite the varying severity of histological lesions and the general lack of visualised antigen on IHC staining.

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The significance of the lymphoplasmacytic orchitis of the stabled, non-sexually active 2-year-old Thoroughbred colt, case No. 3, is uncertain since WNV IHC and TEM failed to show evidence of virus. WNV-associated lymphocytic orchitis has been reported in two immunosuppressed renal transplant humans (Armah *et al*., 2007; Smith *et al*., 2004). The testicular sections of one (Smith *et al*., 2004) were IHC negative for WNV but ultrastructurally suggestive of flavivirus particles in Sertoli cell cytoplasmic vesicles by TEM, and IHC positive in seminiferous tubular epithelia in the other (Armah *et al*., 2007). The testicles of the older stallion (case No.4) in the current study were normal and undergoing spermatogenesis. Orchitis as a cause of testicular swelling is rare in horses, especially in young stallions, and most commonly traumatic or bacterial in origin. Viral orchitis due to influenza, arteritis and equine infectious anaemia viruses is recorded in some texts (Turner, 2007: 198), however, these diseases are currently absent in South Africa. Autoimmune orchitis (Turner, 2007: 198) due to viral breaching of the blood-testis barrier is a speculative possibility. One text mentions the potential for transmission in semen of eastern and western equine encephalitis viruses, WNV, vesicular stomatitis, AHS and equine infectious anaemia viruses (Timoney, 2011). Equine viral arteritis virus is known to be transmitted in stallion semen (Timoney & McCollum, 2004).

Many of the horses in this series had gross oedema, including of the supraorbital fossae, and serosal haemorrhages mimicking lesions of horses dying of AHS (Mellor & Hamblin, 2004) and Shunivirus (Van Eeden *et al*., 2012). Case No. 6 which had recovered from AHS, was still RT-PCR AHS positive but viral culture negative from spleen and lung and showed the mildest pulmonary oedema. A recent study (Weyer *et al*., 2012) found that 16% of a group of 50 horses, vaccinated annually for AHS and living free in a high AHS infection risk area, contracted subclinical or clinical AHSV field infection, with blood samples remaining RT-PCR positive for periods of up to or more than 130 days in survivors, but with tissue culture from positive blood being negative. Erythrocyte-bound virus coated with neutralising antibody was speculated, and the duration of positivity related to the longevity of circulating erythrocytes (Weyer *et al*., 2012).

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CNS of all cases in the current study was negative by various tests for other likely locally-occurring viruses, ruling out co-infections. Successive infections, as in case No.6, or co-infections with arboviruses (Venter *et al*., 2009b) are not surprising since the mosquitoes transmitting WNV, other flavi-, bunya- and alphaviruses and *Culicoides* spp transmitting orbiviruses and some bunyaviruses, are similarly affected by temperature and rainfall and the diseases occur seasonally simultaneously (Jupp, 2001; Van Eeden *et al*., 2012; Venter *et al*., 2009b).

The mechanism/s for the gross edema and haemorrhage found in the WNV cases is unclear. In AHS, multifocal serofibrinous oedema and haemorrhages arise due to subcellular vascular injury (Erasmus, 1972), with virus mainly targeting microvascular endothelial cells and monocyte-macrophages (Clift & Penrith, 2010). AHS viral tissue tropism occurs in those tissues showing most consistent lesions (Clift & Penrith, 2010). In contrast, horses with neurological WNV infection have been found to be negative for WNV RNA in tissues other than CNS (Kleiboeker *et al*., 2004), as confirmed in the present study.

Pulmonary oedema can result from various nervous system disorders including brain malignancies, trauma with increased intracranial pressure, infections and seizures. Suggested mechanisms include increased pulmonary capillary permeability, as well as rapid and massive centrally-mediated sympathetic catecholamine-induced discharge resulting in elevated pulmonary vascular resistance (Caswell & Williams, 2007; Reuter-Rice *et al*., 2011). Barbiturate euthanasia leads to alveolar filling with proteinaceous fluid; and foam filling of the trachea and even flowing from the nose has been recorded as an incidental finding in horses and sheep dying of various causes (Caswell & Williams, 2007).

The gross necropsy findings imply that without a prior history of clinical signs, or in cases of acute death, in South Africa and likely elsewhere in sub-Saharan Africa, gross lesions of horses with WNV (Venter *et al*., 2009b) might be confused with other

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generally non-neurotropic but endotheliotropic viral infections such as AHS, EEV or EHV, or neurotropic viral infections like Shunivirus (Van Eeden *et al.*, 2012). Suitable sampling of CNS to eliminate these viruses may be overlooked, and biosafety and personal protective measures omitted, exposing prosectors to the danger of zoonotic infection. WNV was unexpectedly transmitted to a veterinary student during necropsy on a positive horse while handling brain without facial protection (Venter *et al.*, 2010).

Immunohistochemistry was confirmed in the present study to be a very poor diagnostic tool for equine WNV, as previously reported (Pennick *et al.*, 2012), and RT-PCR of CNS was used to confirm infection (Pennick *et al.*, 2012).

3.5 CONCLUSIONS

This study confirmed the neuropathogenicity of South African field strains of lineage 2 WNV in horses. Pathology was similar to that reported with northern hemisphere lineage 1 WNV strains and more severe than that of the South African lineage 1 equine case reported to date, despite all cases showing severe fatal neurological clinical signs. Based on experimental findings in mice and hamsters, CNS lesions, sites and distribution suggested various neural and/or vascular routes of WNV entry into the CNS, and explained some of the clinical signs seen. WNV IHC was confirmed in this study as a poor method for detection of viral antigen in equine CNS relative to real-time RT-PCR which could also distinguish between the lineages. Co-infections with other locally-occurring viruses including AHS, EEV and EHV-1 and 4, rabies virus and other neurotropic arboviruses of the flavi, alpha- and bunyavirus families were excluded from the CNS of all cases.

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CHAPTER 4

CONCLUDING REMARKS

In the mouse study, the hypothesis that gross and microscopic pathology caused by neurovirulent lineage 1 WNV NY385/99 would be similar to that of the neurovirulent South African strain SPU03/01 was proven: clinical signs, duration of disease, pathology and number of survivors were indistinguishable between the two lineages. Some of the gross and microscopic findings concurred with those found by Kimura *et al.,* (2010), however, several did not, possibly related to the different mouse strains and ages as well as route of inoculation used in the two experiments. The most notable differences included the subtle CNS inflammatory lesions in the 3-4month-old BALBc mice of the current study although Kimura *et al.,* (2010) did mention that in their study only a small number of mice showed diffuse severe CNS inflammation and antigen distribution (Kimura *et al.*, 2010). Microscopic intestinal lesions including necrotic to serum-exudative enteritis associated with lumenal bacterial overgrowth and crypt Paneth cell degranulation were not mentioned in the Kimura study, nor the rare caecal myenteric ganglion WNV IHC positivity but lack of staining of small intestinal ganglia without convincing evidence of neuronal injury found in the current study. In researching prior literature, the likely pathogeneses of the GI distension was extrapolated from work done in hamsters infected with WNV, where brain stem and/or cervical spinal cord central neuronal damage were linked to paresis of the diaphragm and intestines (Morrey *et al.*, 2010; Wang *et al.*, 2011). In the current study, in all 4 mice where brainstem was examined, deep brainstem neurons were IHC positive supporting the hamster findings.

The hypothesis that natural equine infections with lineage 1 WNV described in the northern hemisphere and the single lineage 1 case identified in South Africa, would result in similar pathology to natural equine infections with South African neurovirulent lineage 2 strains of WNV, was confirmed, with lesions of several lineage 2 cases being severe in comparison with the local lineage 1 case. Some specific differences were

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noticed, however, in lesion type, distribution and severity from previous reports likely due to more careful and specific sampling of different regions of CNS.

The extensive literature search prior to writing the protocol for this study was of good benefit as a background on which to design simultaneously both mouse and equine pathology investigations. Extrapolation from the experimental mouse and hamster WNV pathogenesis studies prompted the current researcher, in the equine study, to sample specific brain regions such as olfactory lobe and cortex, plus other main regions of brain and each major segment of spinal cord, for both RT-PCR and histopathology. This was done to ascertain whether equine WNV CNS infection, after natural cutaneous mosquito inoculation, might follow the same routes of entry into the brain as occurs in mice or hamsters after cutaneous inoculation. Sampling and histopathological examination of olfactory bulbs and cortex is rarely mentioned in other equine WNV pathology reports and several of the horses in this study had prominent lesions in this region. The very early olfactory route of entry as seen in mice and hamsters inoculated with WNV (Brown *et al*., 2007; Eldadah & Nathanson, 1967; Hunsperger & Roehrig, 2006; Monath *et al*., 1983; Nir *et al*., 1965; Wang *et al*., 2004) was indeed suggested in the Welsh-cross pony mare (case No. 5) who showed acute neurological signs and early death, and was rRT-PCR positive and with early encephalitic histological lesions only in the cranial regions of her brain suggesting olfactory nasal neuroepithelial entry; she also showed choroid plexus inflammation suggesting circulatory entry via the more porous BBB in that region (Hunsperger & Roehrig, 2006; Morrey *et al*., 2008a).

The clinical and histopathological findings could again be meaningfully linked and explained by extrapolation from the laboratory rodent finding of direct axonal retrograde transport of virus from peripheral neurons to the ventral spinal cord motor horns (Hunsperger & Roehrig, 2006; Kimura *et al*., 2010; Morrey *et al*., 2008b; Samuel *et al*., 2007; Wang *et al*., 2009) in equine case No. 6. This mare showed asymmetrical forelimb paresis and unilateral distal cervical (forelimb outflow region) ventral motor spinal neuritis. It is feasible that horses would be bitten in multiple sites by mosquitoes, especially in glabrous regions of skin, and be prone therefore to multiple variable,

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bilateral but nonsymmetrical routes of neuronal transport to the spinal cord, as well as to circulatory transport of virus into the CNS. This is a likely explanation for the very variable distribution, assymetry and severity of brain and spinal cord inflammatory lesions found within and between the equine cases of this study and yet also the high incidence of olfactory cortex lesions amongst them. Most researchers reporting on WNV-associated pathology in animals and man do not try to explain or correlate their findings with pathogenesis, thus under-utilising experimental findings and losing the wider purpose of experimental studies in laboratory animals. Both groups would benefit from studying the others' research, and this would surely promote more meaningful future investigations and treatment modalities substantiated by experimental and field evidence.

The poor diagnostic potential of IHC for WNV in CNS tissue of brain in the horses concurred with several prior reports (Buckweitz *et al*., 2003; Cantile *et al*., 2001; Castillo-Olivares & Wood, 2004; Kleiboeker *et al*., 2004; Pennick *et al*., 2012), and the sensitivity or real-time RT-PCR in diagnosing both lineages 1 and 2 even at very low concentrations in CNS tissue was shown (Zaayman *et al*., 2008).

Gross lesions of oedema, pericardial effusion and in some cases serosal haemorrhages found in most WNV-infected horses appeared very similar to gross necropsy findings in some other South African viral diseases, specifically African horsesickness (Mellor & Hamblin, 2004), equine encephlaosis virus, shunivirus, and equine Herpes virus, with some of these viruses also capable of causing neurological signs (Van Eeden *et al*., 2012). Of these viruses the arboviruses overlap in transmission seasons due to similar effects of weather on their insect vectors, with co-infections possible and diagnosed (Venter *et al*., 2009b). The equine cases were thus extensively tested in CNS and other tissues for possible co-infections, however only one mare, case No. 6, was still RT-PCR but not culture positive in spleen and lung for AHS, from which she had clinically recovered 10 days prior to showing the first WNV neurological signs.

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The important implications of the confusing macroscopic necropsy findings in WNV and other viral diseases include omitting to test CNS tissues for neuroinvasive viruses in the absence of a history, or omitting to take biosafety and personal protective measures during the necropsies and sampling of diagnostic specimens, thus increasing the risks of zoonotic infections (Venter *et al.*, 2010). Unexpected advantages were discovered during the recruitment of cases for the current equine study by routinely taking both fresh and formalinised CNS and organ specimens in horses showing these gross lesions and neurological signs and testing for WNV. These were the diagnosis of other neurotropic arboviruses, specifically alphaviruses (unpublished M Venter, S Van Niekerk), and the orthobunyavirus Shuni (Van Eeden *et al.*, 2012), amongst other viral or non-viral differential diagnoses, in WNV-negative cases, and of WNV or other viral infection cases with viral co-infections (Venter *et al.*, 2009b). Viral co-infections are rarely reported in horses in regions where several neurotropic arboviruses occur, such as in the USA where WNV and the alphaviral equine encephalitis viruses (Eastern- , Western- and Venezuelan equine encephalitis) share mosquito vectors, and judging from the South African experience since 2007 whilst looking for WNV, viral co-infections should be investigated not only in horses but in humans globally.

The intensive screening for other possible viruses was also done in order to convince overseas researchers as well as the South African medical fraternity reading the equine WNV pathology paper arising from this study, that the lesions and clinical signs in the horses were truly caused only by lineage 2 WNV. There is still a degree of scepticism since the Guthrie *et al.,* paper of 2003, (Guthrie *et al.*, 2003) that lineage 2 WNV is truly neuropathogenic in horses (Venter *et al.*, 2009b) and humans. Human neurovirulent WNV infections in South Africa are currently underdiagnosed and may have been either undiagnosed or underdiagnosed for many years (Burt *et al.*, 2002; Jupp, 2001; Zaayman & Venter, 2012), or virulent WNV strains have emerged as dominant strains (Venter *et al.*, 2009b; Venter *et al.*, 2010). Since the emergence and reporting of highly neurovirulent lineage 2 WNV strains in raptors in southern Hungary in 2004 (Bakonyi *et al.*, 2006) with subsequent spread, morbidity and mortality in birds (Danis *et al.*, 2010; Savini *et al.*, 2012), horses (Kutasi *et al.*, 2011) and humans (Papa *et al.*, 2011a; Papa

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et al., 2010a; Papa *et al*., 2011d), more credence is being given to the South African findings in horses, and the real hope is that the South African medical fraternity will start routinely testing humans presenting with neurological signs at least for WNV.

The mouse vaccine studies were performed with the view to registration of WNV vaccines for use in horses in this country since the veterinary and equestrian fraternities here have become increasingly aware of the pathogenicity of WNV in horses since the beginning of the project. The information arising from these studies including the equine pathology of this thesis has been broadcast by presentations at various veterinary congresses and published in local popular magazines and international scientific journals. The mouse study investigating the efficacy of the inactivated WNV lineage 1-derived Pfizer/Zoetis equine vaccine, Duvaxyn WNV, after challenge with both lineage 1 and 2 strains, and which provided most of the mouse subjects for the current study, has been published (Venter *et al*., 2013).

During preparation of the literature review underlying this study, consultation of original South African and worldwide WNV publications documenting the history of WNV discovery in 1937 and the subsequent research over time to current, was not only of great interest and benefit scientifically, but also as an exercise of appreciation of the passion, dedication, brilliance and interest of our scientific forefathers. Many recent reviews consult only recent papers, most likely considering the relatively crude methods and lack of modern precise technology used during the original WNV work as having led to imprecise findings. The author of this thesis found the original work to be elegant, thorough, logical, still relevant and remarkable under their relatively primitive circumstances. Some of the same methods are still used today and their findings are referred to by some recent researchers.

The journey of discovery during this study was enlightening, exciting, surprising, rewarding and enjoyable and has hopefully contributed information of use to future researchers.

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ANIMAL USE AND CARE COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: H019-10

18 January 2013

Prof M Venter
Department of Medical Virology
Faculty of Health Science
(marietjie.venter@up.ac.za / marietjiev@nicd.ac.za)

Dear Prof Venter

H019-10 : Pathology of West Nile virus lineages 1 and 2 in mice and horses *(JH Williams)*

The application for ethical approval, dated 18 December 2012 was approved by the Chairman of the Animal Use and Committee on 18 January 2013

Kind regards

Elmarie Mostert

AUCC Coordinator

Copy Prof R Pretorius

Dr JH Williams

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