RETROSPECTIVE ANALYSIS OF THE EPIDEMIOLOGY AND CLINICAL PRESENTATION OF WEST NILE VIRUS INFECTION IN HORSES IN SOUTH AFRICA, 2016 – 2017

by

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"Les grandes choses ne sont pas réalisées par la force, mais par la persérvérance"

- Samuel Johnson

DECLARATION

DECLARATION

I declare that the dissertation, which I hereby submit for the degree MSc Veterinary Epidemiology at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

Freude-Marié Bertram

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ABSTRACT

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Keywords: West Nile virus, horses, South Africa, epidemiology, emerging disease, encephalitis, zoonosis, neurotropic virus.

West Nile virus (WNV) has gained international attention in recent years as a globally emerging disease, particularly after large epidemics occurred in North America in the past 20 years. Although endemic to South Africa, it has only been recognised as a significant cause of neurological disease in either humans or horses since 2008. This retrospective study provides an epidemiological and clinical description of WNV disease in horses in South Africa during 2016–2017, when 54 cases, most of which occurred during 2017, were diagnosed by passive surveillance at the Centre for Viral Zoonoses (CVZ), University of Pretoria. Cases were followed up and then statistically compared to a randomly selected set of 120 WNV-negative controls from the CVZ database of the same time period, which complied with similar case descriptions. Clinical presentation of WNV cases was found to be remarkably similar to international trends, with 89% neuroinvasive disease and 39% case fatality rate, mostly displaying typical, significant neurological signs: ataxia (74%), hindleg paralysis (35%), paresis (30%), total paralysis (28%), tremors / muscle fasciculations (19%), foreleg paralysis (17%) and laminitic stance (9%). Approximately half of the

cases exhibited pyrexia. Cases that had only neurological signs were more likely to die while cases with pyrexia, with or without neurological signs, were more likely to recover.

Most of the cases were in Thoroughbred, Warmblood or Arabian horses, while local or mixed breed horses were the least represented. Cases occurred mostly in WNV-unvaccinated horses less than 5 years old, specifically in the late summer and autumn months after heavy rain in the temperate to warm Eastern parts of South Africa. Cases were located mainly in Gauteng, KwaZulu-Natal Midlands and the Northern Cape with fewer cases in the Free State and Western Cape provinces. In the multivariable logistic regression analysis, the odds of WNV infection was associated with season (higher during March-April vs. all other times), altitude (higher at 1293–1466 m vs. other categories), breed (lowest in mixed and local breeds), younger age and failure to vaccinate against WNV. Based on these findings, risk-based recommendations may be made to horse owners; in particular, vaccination against WNV, which is currently the most effective prophylactic measure available to reduce disease, severity of clinical signs and mortality.

LIST OF ABBREVIATIONS

AAEP	American Association of Equine Practitioners
Ae.	*Aedes*
AHS	African horse sickness
BSL3	Biosafety level 3
B.t.i.	*Bacillus thuringiensis israelensis*
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
CPD	Continuing professional development
CSF	Cerebrospinal fluid
CVZ	Centre for Viral Zoonoses
Cx.	*Culex*
DAFF	Department of Agriculture, Forestry and Fisheries (South Africa)
DEET	Diethyltoluamide (N,N-Diethyl-meta-toluamide)
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEV	Equine encephalosis virus
EHV	Equine herpes virus
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbent assay
ERC	Equine Research Centre
FEI	*Federation Equestre Internationale*
GIS	Geographic information system

GPS	Global positioning system
HI	Haemagglutination inhibition
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IR3535	Insect repellent 3535
KDA	Kentucky Department of Agriculture (USA)
LRT	Likelihood ratio test
MAC-ELISA	IgM antibody capture-ELISA
MIDV	Middelburg virus
NASA	National Aeronautics and Space Administration
NHRA	National Horse Racing Authority (South Africa)
NICD	National Institute for Communicable Diseases (South Africa)
OBP	Onderstepoort Biological Products
OIE	World Organisation for Animal Health
OR	Odds ratio
OVI	Onderstepoort Veterinary Institute
PRN	Plaque reduction neutralization
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
rtRT-PCR	Real-time reverse transcriptase polymerase chain reaction
RSA	Republic of South Africa
SAWS	South African Weather Service
SHUV	Shuni virus
SINV	Sindbis virus

UP	University of Pretoria
USA	United States of America
USDA	United States Department of Agriculture
VN	Virus neutralization
WHO	World Health Organization
WNV	West Nile virus
WSLV	Wesselsbron virus
ZARV	Zoonotic Arbo- and Respiratory Virus program

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CHAPTER 1: INTRODUCTION

West Nile virus (WNV) is a neurotropic, zoonotic vector-borne flavivirus, family *Flaviviridae*, endemic to South Africa (Venter et al., 2009). Mosquitoes serve as vectors of WNV, with birds as its primary host. Mosquitoes may also incidentally spread the virus to humans, horses and other species which then act as dead-end hosts (Castillo-Olivares and Wood, 2004). Approximately 20% of WNV infections in horses are symptomatic, with clinical signs ranging from fever to severe neurological signs (90%) and death (30%) (Venter, 2015). The virus was initially detected in the West Nile District of Uganda in 1937 in a febrile patient. Subsequently, periodic outbreaks have been reported in Africa, the Middle East and Europe (Castillo-Olivares and Wood, 2004). WNV lineage 1 was identified as the cause of deaths in birds, humans and later horses in New York, U.S.A., starting in August 1999, and was presumably introduced to the Western Hemisphere by the importation of infected birds or mosquitoes (Roehrig, 2013). The WNV lineage responsible for the North American outbreak was closely related to WNV isolated from a dead goose in Israel during the previous year (Lanciotti et al., 1999). Subsequently the virus spread to large parts of America, Europe and the Middle East in less than 10 years, changing the status of WNV from a minor cause of concern to being regarded as a very significant agent of neurological disease worldwide (Roehrig, 2013; OIE, 2018).

In South Africa, passive surveillance for arboviruses such as WNV (Flaviviridae) and Wesselsbron (WSLV; Flaviviridae), Sindbis (SINV; Togaviridae) and Middelburg (MIDV; Togaviridae), and Shuni virus (SHUV; Bunyaviridae) has been routinely performed since 2008 for acute febrile and neurological disease in horses and other animals by the Zoonotic Arbo- and Respiratory Virus (ZARV) programme at the Centre for Viral Zoonoses (CVZ), University of Pretoria. Multiple cases of fatal and nonfatal encephalitis in humans and other species, have been reported to be associated with lineage 2 WNV (Zaayman and Venter, 2012; Venter et al., 2017). Despite previous suggestions that endemic lineage 2 strains in South Africa were of low virulence in horses and humans (Jupp, 2001; Guthrie et al., 2003), it has been demonstrated that

highly pathogenic strains exist, causing severe neurological disease and death in humans, horses and other animals (Venter et al., 2009; Williams et al., 2014). Systematic passive surveillance by the ZARV confirmed a total of 79 clinical cases of WNV in horses in the period 2008–2015 using RT-PCR and IgM serology followed by serum neutralisation assays (Venter et al., 2017), with a 34% case fatality rate. Currently, the World Organisation for Animal Health (OIE) classifies WNV as a notifiable disease-causing agent, and regards it as a disease of importance in international trade (OIE, 2018).

During 2017, compared to previous years in South Africa, a substantially larger number of cases of WNV in horses and other animals were detected by the ZARV, most of which displayed severe neurological signs. A total of 48 clinical WNV-positive cases was diagnosed in horses in 2017 alone, compared to only 6 clinical cases in the previous year, which in combination formed 9% of the equine sample submissions to the CVZ during 2016–2017. In the preceding 8 years, 79 WNV-positive cases were diagnosed out of 1,069 equine sample submissions (Venter et al., 2017). As a vector-borne disease, its relation to the increased rainfall during the 2017 summer season, following the period of severe drought in 2016, had been an area of interest to be investigated. Annual reports of the cases detected by the CVZ have been submitted to the OIE and Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, and necessitated further investigation.

The aim of this study was to investigate and describe the epidemiology and clinical case presentation of WNV in horses, during the 2017 outbreak, in South Africa. However, equine cases detected by the ZARV program in 2016 were also included and compared. Investigations included measuring the association of severe neurological disease with certain predictor variables, including age, breed, sex and vaccination status as well as geographical location and weather variables such as average rainfall and temperature. Social considerations regarding public opinion about West Nile vaccination are discussed and whether the influence of increased public awareness of West Nile fever due to social media, WNV presentations to veterinarians and owners, and pharmaceutical promotions, may have caused increased vaccination use during 2016–2018 in South Africa.

CHAPTER 2: LITERATURE REVIEW

2.1. AETIOLOGY

West Nile fever is caused by the West Nile virus (WNV), a member of the Japanese Encephalitis virus serocomplex, and in Australia a closely related but apparently less pathogenic sublineage (lineage 1b) is known as the Kunjin virus (Siger et al., 2006; Williams et al., 2014). It is a zoonotic, mosquito-borne, positive-sense single-stranded RNA enveloped virus in the genus *Flavivirus* in the family *Flaviviridae* (Petersen and Roehrig, 2001; Castillo-Olivares and Wood, 2004; Beasley et al., 2013), and is maintained in nature by cyclic activity in numerous avian and mosquito species. Although certain avian species such as blue jays (*Cyanocitta cristata*), crows (*Corvus* spp.) and raptors, mostly red-tailed hawks (*Buteo jamaicensis*) and great horned owls (*Bubo virginianus*) (Saito et al., 2007) in the USA display fatal infections, most avian species thought to be reservoir hosts in Africa display no apparent signs of infection (Jupp, 2001; OIE, 2018; Sule et al., 2018).

Isolates of WNV fall mainly into two lineages: WNV lineage 1, which is the most widely distributed worldwide, is found in North America (lineage 1a), North Africa (lineage 1a), Europe (lineage 1a), Australia (lineage 1b/Kunjin virus) and India (lineage 1c) (Ciota and Kramer, 2013). WNV lineage 2 tends to dominate in Southern Africa and Madagascar (Venter and Swanepoel, 2010; Ciota and Kramer, 2013), although since 2004 WNV lineage 2 also caused severe illness and death in birds, horses and humans in Europe (Chaskopoulou et al., 2016). Minor lineages have recently been identified in Central and Eastern Europe (lineage 3 and 4) as well as India (lineage 5) (Venter and Swanepoel, 2010; Ciota and Kramer, 2013).

Nucleotide sequences of 25 South African lineage 2 strains were examined and the sequence identity was found to be 86.3–100%, indicating an exceptional constancy in the Southern African strains of the virus and strengthening the suspicion that local circulating foci of WNV are being maintained in certain areas, rather than migratory birds being responsible for epidemic outbreaks (Jupp, 2001; Burt et al., 2002).

Both internationally and locally, studies have shown that there is a distinct variation in neurovirulence in different strains of both lineage 1 and 2 WNV (Beasley and Barrett, 2002; Venter et al., 2005; Samuel and Diamond, 2006), but it is not yet clear exactly why. Different receptor binding sites in the brain membranes in mice and humans (Beasley et al., 2001), major deletions in the 3' noncoding regions of WNV (Botha et al., 2008; Venter and Swanepoel, 2010), variation in the nonstructural genes, particularly those of nonstructural protein 5 (Botha et al., 2008) and the envelope-protein glycosylation site of the virus have all been postulated to have an effect on potential neuropathogenicity (Beasley et al., 2005). Increased expression of certain genes in the brain, liver and spleen of mice was seen after experimental infection with most neuroinvasive WNV strains, relative to the less neurovirulent strains (Venter et al., 2005). Increased expression of particular acute proteins, CNS specific proteins and T-cell hepatitis associated proteins may potentially also be involved in the increased virulence of certain WNV strains (Venter et al., 2005). Other studies implicate tumour necrosis factor alpha facilitated changes in permeability of endothelial cells and the lack of interferon alpha/beta/gamma or interferon receptors in potential neuroinvasion (Samuel and Diamond, 2006).

In humans it is known that persons with certain genetic defects in the genes which modulate host response to exogenous viral RNA, are more likely to have anti-WNV antibodies than persons without (Petersen et al., 2013). Conversely, mice and humans with chemokine receptor CCR5 deficiencies (CCR5 acts at the level of leukocyte trafficking to the brain) have displayed enhanced susceptibility to development of symptomatic WNV-infection (Glass et al., 2006). Other factors which may also play a role in the risk of developing neuroinvasive WNV infection in humans may be advanced age, immunocompromised patients, a history of cancer, diabetes, hypertension, renal disease, alcohol abuse and certain chemokine receptor deficiencies, feasibly implicating the immune response in the potential for WNV syndrome development post infection (Samuel and Diamond, 2006; Petersen et al., 2013).

2.2. HISTORY

West Nile virus was first isolated in a female human patient with mild febrile illness in Uganda in 1937 (Smithburn et al., 1940), but until the early 1990s was considered relatively unimportant, causing sporadic outbreaks of pyrexia and encephalitis, mainly in Africa, Southern Europe, Middle East and Central Asia, in equines and predominantly elderly people (Castillo-Olivares and Wood, 2004; Beasley et al., 2013). In the 1950's large scale epidemiological research was done in Egypt describing WNV infection as a self-limiting, non-fatal, febrile childhood disease with seroprevalence of up to 61% in the Nile Delta and 40% in Southern Sudan. This study also first postulated the avian – mosquito cycle as well as first describing the seasonal fluctuations of the disease and suspecting the possible infection of horses (Taylor et al., 1956).

The first cases of neurological WNV infection in horses were diagnosed in Egypt and France in the 1960's (Schmidt and El Mansoury, 1963; Joubert et al., 1970). In Egypt neutralizing antibodies were found in 67% of horses, 47% of donkeys and 44% of mules sampled in the Nile Delta and upper Egypt. Most of the infections seemed to occur in the first 5 years of life. WNV was isolated in 1959 from a 12-year-old horse in Upper Egypt, that displayed colic, haematuria and distinct neurological signs such as urinary retention, ataxia and progressive hindlimb paralysis, recumbency and eventual death (Schmidt and El Mansoury, 1963). In Camargue, France, in the 1962–1963 WNV epidemic, at least 80 horses showed neurological signs such as ataxia with a 26–30% case mortality rate (Castillo-Olivares and Wood, 2004).

The first report of equine WNV encephalomyelitis occurring in Italy, was during an epidemic affecting 14 horses in Tuscany in 1998 (Cantile et al., 2000). Other substantial epidemics of WNV have also occurred in the Mediterranean and Eastern Europe, North Africa and Asia (Petersen and Roehrig, 2001). Since 2000 more than 27,000 horses have been diagnosed with WNV neuroinvasive disease in the USA, with a case fatality rate of 30–40%. It is now considered endemic in USA, Canada, Mexico and the Caribbean, with an average of 300 equine cases annually just in the USA (Weese, 2017). The increase in neurological WNV-positive cases, as was seen globally since the early 1990's in both horses and humans, may be due to a suspected increase in neurovirulence and emerging distribution of neuroinvasive strains of WNV

in recent years (Petersen et al., 2013; Williams et al., 2014; Chaskopoulou et al., 2016). This global increase in diagnosed WNV cases of a severe neurological nature was very likely also influenced by advances in molecular technology and improved immunological tests, resulting in increased diagnostic sensitivity. Increased technological advances in communication such as cellular phone technology and the internet, had likely caused both greater national and international awareness of the disease, resulting in a greater amount of correctly diagnosed cases. Increased international human and freight travel by aeroplane, may also have assisted the global dissemination and emergence of WNV strains in recent years.

Presently WNV is regarded as the most widely geographically distributed arbovirus, causing the most cases of arboviral encephalitis globally (Ciota, 2017), and is classified as a globally re-emerging pathogen with an increased proportion and severity of neurological disease cases in humans and horses as well as high mortality rates in birds in the Western Hemisphere (Castillo-Olivares and Wood, 2004). Typically, humans show mild symptoms which include headaches, weakness, myalgia, arthralgia, morbilliform or maculopapular rash and fever (often low grade or absent), of which less than 1% may progress to more severe disease such as meningoencephalitis, encephalitis, hepatitis, flaccid paralysis, with a 10% fatality rate for neurological cases (Petersen and Marfin, 2002; Venter et al., 2005; Zaayman and Venter, 2012; Petersen et al., 2013).

Historical reports indicated that neuroinvasive WNV infection had been very rarely observed in horses in the Republic of South Africa (RSA) prior to 2003 (Jupp, 2001; Burt et al., 2002). Lineage 2 WNV was postulated not only to be of low virulence but the only widely endemic WNV lineage present in horses in South Africa (Guthrie et al., 2003). However, as of 2007 both lineage 1 and lineage 2 WNV have been identified in horses in South Africa as being associated with severe neurological disease, with a 35% case fatality rate. Nevertheless, only 2 cases were of lineage 1, while the vast majority of severe cases were lineage 2 WNV (Venter and Swanepoel, 2010; Williams et al., 2014; Venter et al., 2017). Table 1 shows a summary of clinical WNV detected in horses during 2008–2015 by the ZARV program at the CVZ.

Table 1: WNV infection, co-infection, disease and death in horses by year, South Africa 2008–2015.

	No. (%) horses								
Category	2008	2009	2010	2011	2012	2013	2014	2015	Total
Total specimens	71	76	150	164	89	138	193	188	1,069
Confirmed WNV positive†	9 (12.7)	6 (7.9)	18 (12.0)	12 (7.3)	3 (3.4)	4 (2.9)	23 (11.9)	4 (2.1)	79 (7.4)
WNV PCR positive†	5 (7.0)	3 (3.9)	8 (5.3)	2 (1.2)	0 (0)	1 (0.7)	4 (2.1)	1 (0.5)	24 (2.2)
WNV IgM positive†	5 (7.0)	3 (3.9)	12 (8.0)	10 (6.1)	3 (3.4)	3 (2.2)	20 (10.4)	3 (1.6)	59 (5.5)
Deaths‡	5 (55.6)	3 (50.0)	8 (44.4)	3 (25.0)	1 (33.3)	1 (25.0)	5 (21.7)	1 (25.0)	27 (34.2)
Any neurologic signs‡	8 (88.9)	6 (100.0)	16 (88.9)	11 (91.7)	2 (66.7)	4 (100.0)	21 (91.3)	4 (100.0)	72 (91.1)
Fever‡	2 (22.2)	2 (33.3)	3 (16.7)	6 (50.0)	1 (33.3)	1 (25.0)	10 (43.5)	3 (75.0)	28 (35.4)
Co-infections‡ and co-infecting viruses	2 (22.2), 2 AHSV	2 (33.3), 2 SINV	1 (5.6), 1 SHUV	2 (16.7), 2 MIDV	2 (66.7), 1 AHSV, 1 SINV	0	4 (17.4), 2 MIDV, 1 SHUV, 1 EEV	1 (25.0), 1 SHUV	14 (17.7), 3 AHSV, 3 SINV, 3 SHUV, 4 MIDV, 1 EEV

*AHSV, African horse sickness virus; EEV, equine encephalitis virus; MIDV, Middleburg virus; SHUV, Shuni virus; SINV, Sindbis virus; WNV, West Nile virus.
†Percentage of total number of specimens tested.
‡Percentage of total number of confirmed WNV-positive cases. Confirmed cases were those that tested positive by PCR plus those that tested positive by WNV IgM Capture ELISA Test (IDEXX Laboratories, Montpellier, France) followed by neutralization assay.

Source: Venter et al. (2017)

Human cases of mainly WNV fever, have been consistently diagnosed in South Africa, with the largest outbreak in the Karoo in 1974, with 50–80% seroconversion (McIntosh et al., 1976) and a subsequent outbreak in 1984 in Gauteng (Jupp, 2001). Approximately 5-15 cases are reported annually by the National Institute for Communicable Diseases (NICD), including some neurological cases and a fatality due to hepatitis syndrome (Burt et al., 2002). The diagnosed cases lead to a subsequent study identifying South African veterinarians as a group with likely similar exposure risk as horses to WNV. The study found that 7.9% of the samples from veterinarians tested positive for antibodies against WNV, their distribution being approximately similar to that of WNV-positive cases detected in animals (Van Eeden et al., 2014).

2.3. EPIDEMIOLOGY

2.3.1. Distribution of WNV

Presently, WNV has a wide geographical range including parts of Europe, Asia, Africa, Australia and the Americas. Even before its relatively recent entry into the Western Hemisphere, it had been one of the most widespread flaviviruses, extending throughout Africa, the Middle East and southern Eurasia (John et al., 2000). Substantial WNV epidemics have occurred in the Mediterranean and Eastern Europe, North Africa and Asia (Petersen and Roehrig, 2001). WNV was identified as a cause of death in horses, birds and humans in New York, USA, since August 1999.

Presumably it was spread by migratory birds, accidental importation of infectious mosquitoes by aeroplane, or the legal or illegal importation of infected wild bird/s as the lineage 1 strain sequence associated with the outbreak was shown to be almost identical to an Israeli WNV strain (Lanciotti et al., 1999; John et al., 2000; OIE, 2018). A serologic survey disproved the theory that the source of the outbreak had been infected, imported birds in the Bronx Zoo, New York (Ludwig et al., 2002), despite viral activity causing morbidity and mortality amongst the resident New World species of birds and the zoo being located close to the epicentre of the outbreak.

Subsequently the disease spread to large parts of North and South America, including Canada, Mexico, as well as to parts of Europe and the Middle East (OIE, 2018). According to the Centers for Disease Control and Prevention (CDC), annual cases in humans and horses occurred since 1999 in the USA with sporadic epidemics during 2002, 2003 and 2012. During 1999–2017, more than 48,000 human WNV infection cases were reported in North America, of which approximately half were neuroinvasive with a 9% case fatality rate (CDC, 2018a). The American Association for Equine Practitioners (AAEP) reports that more than 27,600 horses in the USA have been confirmed with WNV neuroinvasive disease with a 30–40% estimated case fatality rate during the same period (AAEP, 2019).

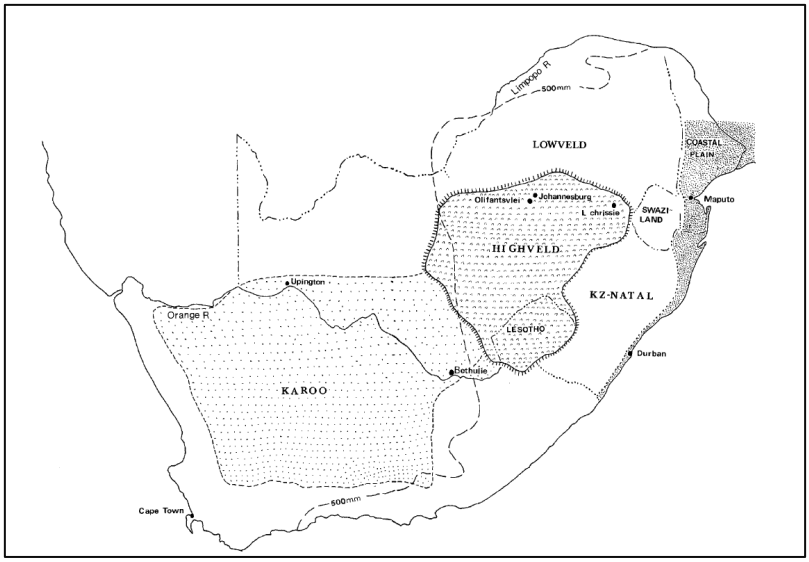

Figure 1: South African inland plateau formed by the Karoo and Highveld, as well as the KwaZulu-Natal coastal plain. The 500 mm isohyet bisects the country into Eastern moist and Western arid parts. Source: Jupp (2001).

Studies on WNV neutralizing antibodies in humans were undertaken on the inland plateau of South Africa (Figure 1), which is formed by the semi-arid Karoo (average annual rainfall <500 mm) and the cooler grassland Highveld (annual rainfall 500–700 mm), which is an area with a temperate climate and elevation of 400–2000 meters above sea level. The KwaZulu-Natal coastal plain which has a moist subtropical to tropical climate was also included in the study (Jupp, 2001). Neutralizing antibodies against WNV were detected in humans at 11 localities on the inland plateau (Karoo 17.1% and Highveld 8%) and 2 localities on the KwaZulu-Natal coast (2%) (Jupp, 2001). The former is consistent with the locations of the largest South African human WNV outbreaks in 1974 in the Karoo (McIntosh et al., 1976) and 1984 in the Highveld (Jupp et al., 1986). Both outbreaks occurred after periods of unusually high rainfall and flooding in those areas (Jupp, 2001).

The location of the human outbreaks was also attributed to the highly efficient and ornithophilic *Culex univittatus* as main WNV mosquito vector in South Africa *(*also to a lesser degree *Culex theileri, Culex pipiens* and *Aedes caballus*) populating the higher moisture Highveld areas in comparison to the less efficient, less ornithophilic KwaZulu-Natal coastal lowlands (only 2% seroprevalence was detected in two localities in KwaZulu-Natal coastland) mosquito *Culex neavei (*as well as *Aedes circumluteolus*) (Jupp, 2001, 2005). Given the right climatic conditions of heavy rains and higher than usual temperatures, *Cx. univittatus* has been responsible for significant WNV outbreaks in humans, despite having a low human feeding rate. The human outbreaks, therefore, are also closely associated with avian infection (Jupp, 2005). Their eggs being very sensitive to desiccation, *Culex* spp. mosquitoes preferably lay their eggs in standing water and survive dry winters by quiescent larvae and pupae or dormant adult females, preferring temporary to semi-permanent rain flooded grassland, swamps or other permanent water collections with emergent vegetation as breeding sites (Jupp, 2005).

During the outbreak in 1974 in the Karoo, which covered a 2500 km^2 area from the Orange river in the north (Upington area), Laingsburg to the south, Beaufort West to the east, and up to the West Coast, an average of 55% human sera tested WNV positive and 18,000 people were affected although neither human deaths nor equine disease were reported (McIntosh et al., 1976; Castillo-Olivares and Wood, 2004).

The 1983–1984 outbreak of WNV in Gauteng province of the Highveld area also occurred after unusually high rainfall and floods followed by high temperatures in late summer. In this case, however, SINV infections were more frequently diagnosed than WNV in humans, despite field mosquito infections indicating outbreaks of both viruses (Jupp et al., 1986; Jupp, 2001). The reason for the limited WNV transmission to humans was unclear. Up to 2001, only four diagnosed human WNV cases had presented more serious than the usual mild WNV fever (Jupp, 2001). Human WNV-positive diagnoses in RSA had remained constant at 5–15 cases per year since 1985, but only a proportion of cases were subjected to laboratory testing (Venter et al., 2005) and people with neurological signs were not routinely tested for WNV. However, during 2008-2009, WNV was detected in 3.5% of unsolved cases of human neurologic disease in Gauteng provincial hospitals, indicating that WNV is underdiagnosed in human neurological cases. This may be partly, at least, due to the lack of the medical practitioners' awareness of its pathologic potential (Botha et al., 2008; Zaayman and Venter, 2012). Internationally, human WNV encephalitis was rarely encountered prior to early 1990s (OIE, 2018) but since then human WNV disease outbreaks of increased severity, from new viral strains, likely of African origin, have occurred in part of Russia, southern and eastern Europe, Romania, Russia, Israel and Greece; subsequently also affecting the western hemisphere since 1999 causing substantial human disease incidence (Petersen et al., 2013). This recent higher proportion of neuroinvasive WNV in humans (as well as horses) may possibly be attributed to emergence of both WNV lineage 1 and 2 strains with increased virulence. Alternatively, previously existing neurovirulent strains may have been underestimated or become more prevalent particularly in highly susceptible, immunologically naive populations (Burt et al., 2002; Botha et al., 2008).

Research in 2000–2001 was performed in an attempt to estimate the rate of seroconversion to WNV in South African Thoroughbred horses. Paired serum samples were collected from a cohort of 488 yearlings and 243 dams, in which it was found that on serum neutralization tests, 11% of the Thoroughbred yearlings had already seroconverted relative to sera collected approximately 12 months prior. 75% of their dams had also seroconverted, and yet no neurological clinical signs had been reported in any of these horses (Guthrie et al., 2003). This is consistent with typical WNV occurrence world-wide, as most of the WNV infected horses do not display overt

clinical signs and viral encephalitis is seen in only a small percentage of infected horses (OIE, 2018). Thoroughbred stud farms which participated in that study were widely distributed geographically throughout South Africa and the latter serum samples were collected at the 2001 National Yearling Sales in Johannesburg, Gauteng (Guthrie et al., 2003). This certainly supports the general distribution of WNV in South Africa, as a large proportion of Thoroughbred stud farms are located not on the Highveld, but rather along the coastal regions away from the inland plateau, where previous WNV cases were seen (Figures 2&3).

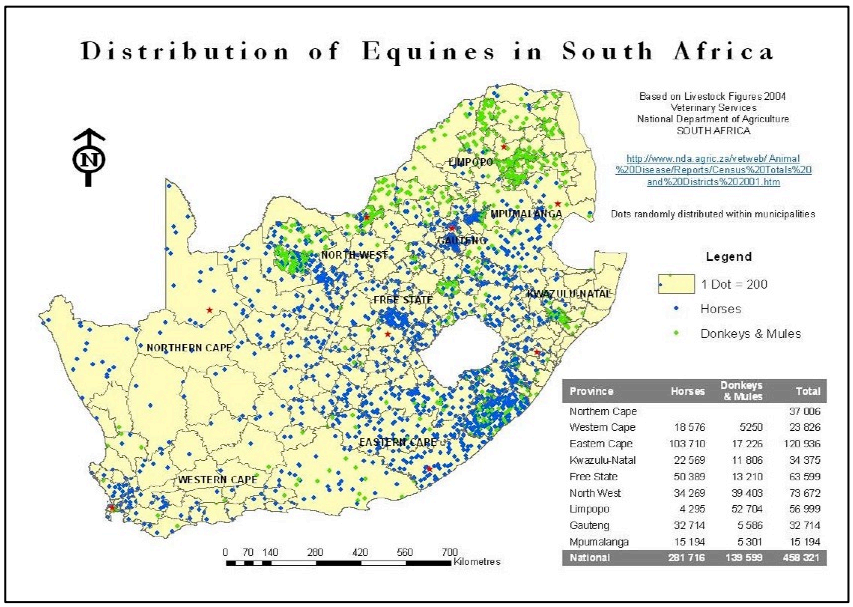

Figure 2: Distribution of general population of equines in South Africa as determined from National Veterinary Services Livestock Figures in 2004. Source: DAFF (2016).

More recent WNV detection in febrile and/or neurological horses, livestock and wildlife in South- Africa (Figure 3), also indicates a consistently high level of WNV-positive cases, particularly in Gauteng. Other Highveld and surrounding areas, central to southern KwaZulu-Natal, Eastern parts of the Karoo and Eastern Cape as well as Cape Town and surrounding areas were also involved (Venter et al., 2017). According to the 2004 National Veterinary Livestock figures from the DAFF African Horse Sickness Season Report for 2016, this correlates somewhat to the general distribution of equines in South Africa (Figure 2). However, there are large parts of South Africa where equines are located from which few or no WNV-positive cases have been

reported (DAFF, 2016). This may be due to climatic differences not favouring the breeding habits of the predominant *Culex* spp. mosquitoes which act as main vectors for WNV, as the largest proportion of cases was consistently found in the temperate to warm, predominantly grassland zones (Figures 3), or it may be due, partly at least, to a lack of equine veterinarians in the rural areas, who may be able to readily recognize typical WNV clinical signs.

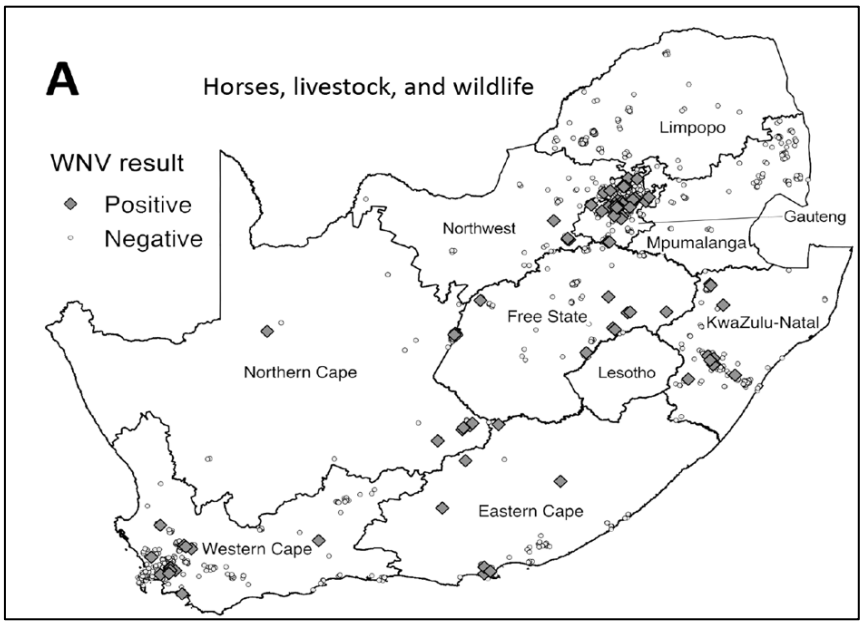

Figure 3: Distribution of WNV cases in South Africa as detected by ZARV among horses in 2008–2015 and livestock animals and wildlife species in 2010-2015. Source: Venter et al. (2017).

2.3.2. Transmission and host range of WNV

WNV is classified as an arbovirus because it is transmitted by blood-sucking mosquitoes, mostly but not exclusively *Culex* spp. (Castillo-Olivares and Wood, 2004; OIE, 2018). It is maintained endemically in nature by an arthropod–avian cycle, and most African bird species, as reservoir hosts, do not show distinct clinical signs of infection, most likely due to genetic resistance (Jupp, 2001; Burt et al., 2002). WNV viraemia was demonstrated in a number of wild bird species in RSA, both from natural and viral inoculation in laboratory, amongst others cattle egrets (*Bulbulcus ibis*), doves (*Streptopelia senegalensis* and *S. capicola*), masked weavers (*Ploceus velatus*), red bishops (*Euplectes orix*), sacred ibis (*Threskiornis aethiopicus*) and yellow-billed

ducks (*Anas undulata*). Both fowls and pigeons have also been successfully used as sentinels to monitor virus transmission in the field (Jupp, 2001). It has, however, been reported in the Northern Hemisphere, specifically in the USA and Europe as of 2004, as a cause of severe illness or death in birds (Castillo-Olivares and Wood, 2004; Williams et al., 2014). Certain avian species such as blue jays (*Cyanocitta cristata*), crows (*Corvus* spp.) and raptors, mostly red-tailed hawks (*Buteo jamaicensis*) and great horned owls (*Bubo virginianus*) in the USA (Saito et al., 2007; OIE, 2018) and goshawks (*Accipiter gentilis*) in central Europe (Erdélyi et al., 2007), display fatal infections.

In South Africa, about 30 avian species have been demonstrated to be involved without significant mortality, assumedly due to genetic resistance, displaying viraemia after both viral inoculation and natural infection with WNV (Jupp, 2001). The virus is maintained in an enzootic transmission cycle mostly between wild birds and the ornithophilic mosquito *Cx. univittatus* (Jupp, 2001, 2005). *Cx. univittatus* is usually not highly anthropophilic, but due to its high susceptibility to and efficient transmission of WNV, it may also transmit the virus to humans given sufficiently high population levels. The genetic similarity of the South African WNV lineage 2 strains suggests that migratory birds may not play a significant role in South African outbreaks, but that the virus might rather be maintained in specific areas in resident wild birds during the relatively mild winters on the inland plateau (Jupp, 2001). Migratory or imported birds may have, on occasion, been the reservoir host responsible for the less common lineage 1 WNV-infections detected by the CVZ in South Africa (Venter et al., 2011; Williams et al., 2014; Venter et al., 2017). In the Northern Hemisphere, migratory and especially passerine birds, particularly the house sparrow (*Passer domesticus*) have been identified as a likely source of trans-border infection. The house sparrow, a wild, ubiquitous passerine, which is distributed over large parts of the world, acts as an amplifying host for WNV because they are able to maintain a sufficiently high and long viraemia (5 to 6 days), during which mosquito infection may occur (Rappole and Hubalek, 2003; Castillo-Olivares and Wood, 2004).

For a mosquito to become infected with and transmit WNV, it has to first feed on a vertebrate host which is able to sustain a high enough viraemia to infect the vector.

Sufficient oral ingestion of the virus results in viral replication inside the mosquito's salivary glands. It may then take around 2 weeks before a susceptible host may be infected by the vector. Both the mosquitoes' development rates (the gonotrophic period) and the WNV replication within them (the extrinsic incubation period of the virus) are very temperature dependent and can also be influenced by other environmental factors such as precipitation, hydrology and humidity (Cornel et al., 1993; DeFelice et al., 2018). The complex interaction between environmental factors and mosquito interspecies ecology differences influence WNV transmission and dynamics, and consequently the risk of infection to humans and other species (DeFelice et al., 2018). There is also a highly seasonal variation in WNV transmission and disease outbreaks, which tend to occur in late summer or autumn in temperate regions, although they may occur throughout the year in the warmer tropical regions (Castillo-Olivares and Wood, 2004). It is, therefore, difficult to predict disease outbreaks, particularly in humans, which often occur in a complex, nonlinear way (DeFelice et al., 2018).

Whilst birds are the amplification hosts, humans and horses are the main mammalian species showing clinical disease, and acting as incidental or dead-end hosts because they cannot serve as a source of infection of additional mosquitoes (Jupp, 2001). Horses generally do not develop viraemia of sufficient level or duration to infect uninfected mosquitoes with WNV, and thus are unlikely to serve as natural amplifying hosts for the disease (Bunning et al., 2002; Venter, 2015).

Many other vertebrate species have also been diagnosed with WNV infection in recent years, including livestock and various species of wildlife (Venter et al., 2017; OIE, 2018). The study on phylogenetic relationships of Southern African WNV isolates (Burt et al., 2002) was done on samples originating from 1958 to 2001 taken from humans, a long-billed crombec, pigeon, ostrich, dog, horse, hamster and several species of mosquitoes (Burt et al., 2002). Serological surveys undertaken on the inland plateau have also shown neutralizing antibodies in cattle, sheep and horses (Dickinson et al., 1962). Experimental inoculations have shown that dogs are possibly able to maintain a viraemia sufficient to act as a minor reservoir host (Blackburn et al., 1989; Jupp, 2001). Experiments also demonstrated the potential intrauterine teratogenic effects of WNV giving rise to lesions such as hydranencephaly in the lambs of pregnant ewes

(Jupp, 2001). WNV may similarly have potential teratogenic effects in human pregnancies (Petersen et al., 2013).

Human infections usually occur through natural mosquito transmission, but there have been cases of WNV transmission by blood transfusion, organ transplantation, breast milk and laboratory infections (Petersen et al., 2013; Weese, 2017; OIE, 2018). In utero WNV infection, or infection at the time of parturition in humans, are potentially feasible although most infants from mothers infected during pregnancy do not show conclusive evidence of congenital infection nor malformations linked to WNV (Petersen et al., 2013). Although horses are “dead end” hosts, there is a risk of transmission to humans by direct contact when handling WNV infected tissues, especially brain, spinal cord and CSF (Venter et al., 2010). Thus, appropriate protection and precautions should be taken by practitioners, especially during post-mortem examinations (Weese, 2017).

2.4. PATHOGENESIS, CLINICAL SIGNS AND PATHOLOGY OF WNV

The incubation period for WNV infection in equine cases is estimated to be 3–15 days, after which a low-level titre may precede clinical onset, and a small percentage of equids develop viral-induced encephalitis or meningoencephalitis. The incubation period for experimental intrathecal induction of viraemia in horses has been determined to be 8–13 days post challenge with a mean duration of 3.9 days (Siger et al., 2006).

Fever, particularly as the main syndrome, is an inconsistent finding in WNV-affected horses, especially when compared to other South African arboviruses such as SINV and MIDV (Van Niekerk et al., 2015). It seems to be the only clinical sign in equine WNV infection that is not an exclusive reflection of the CNS pathology and may rather be attributed to the horse’s immune response to the viral infection. The virus’ capacity to cause disease depends on its ability to survive *in vivo*, infect vital cells and evade immune system recognition and/or capacity to antagonize the host immune response. WNV is cytolytic and induces apoptosis in a relatively diverse spectrum of cell types, including neurons (Samuel and Diamond, 2006). Approximately 90% of diagnosed WNV-positive cases develop neuroinvasive disease (Venter et al., 2017), resulting in

a high (30–40%) case fatality rate particularly in previously uninfected horses (Ward et al., 2006). It is, however, feasible that the most severely affected neurological cases would be most likely to be diagnosed. Horses may show typical encephalomyelitis signs which may range from mild incoordination to severe ataxia, recumbency and death. Neurological signs can generally be classified as related to damage to the three areas of the CNS, as follows (Cantile et al., 2000; Castillo-Olivares and Wood, 2004; Siger et al., 2006; OIE, 2018):

- **Spinal cord pathology**: weakness, ataxia, reluctance to move, paresis or paralysis affecting one or more limbs, skin- or muscle fasciculations, muscle tremors and muscle rigidity. Paralysis of hindlimbs is aptly described in layman's terms as "dog-sitting"; progressive paralysis of all four limbs usually ends in recumbency.
- **Brain pathology:** damage to medulla oblongata, pons, thalamus, reticular formation, cerebellum and brain cortex may manifest as ataxia, dysmetria, hyperaesthesia and abnormal mentation (ranging from somnolence and depression to agitation and hyperexcitability, even aggression).
- **Cranial nerve deficits:** facial nerve paralysis (including droopy lip or muzzle deviation), tongue weakness or paresis, head shaking, head tilt, lip twitching, fine tremors of the face and neck muscles and dysphagia.

Onset of neurological signs is usually sudden and the course progressive, with 30% of cases experiencing increased severity of clinical signs within 7–10 days after onset. Occasionally horses may show signs of colic (Taylor et al., 1956; Weese, 2017), and gross nonspecific visceral lesions may be seen (Williams et al., 2014) but currently the virus is not known to specifically affect the gastro-intestinal system.

Case mortality rates range from 33–40% of clinically affected, unvaccinated horses (Bunning et al., 2002; Ward et al., 2006; Venter et al., 2017; OIE, 2018; AAEP, 2019) and approximately 40% of the horses which initially survive acute WNV infection may display post recovery residual effects such as gait and behavioural abnormalities (AAEP, 2019).

Currently no specific treatment is available against WNV infection but AAEP guidelines recommend supportive treatment and nursing care aimed at reducing the CNS inflammation, preventing self-inflicted trauma and providing nutrition and oral and intravenous fluid therapy as deemed necessary. Therapy of recumbent horses should be more aggressive, including dexamethasone, mannitol, antibiotics (to prevent secondary bacterial infections from cellulitis, other wounds and pneumonia) and tranquilization as they are usually mentally alert and thrash causing not only injury to themselves but also potentially to personnel (Long et al., 2002; Castillo-Olivares and Wood, 2004).

Histologically, affected horses typically display a non-suppurative polioencephalomyelitis with characteristic T-lymphocyte and macrophage infiltration, plus involvement of the ventral horns of the thoracic and lumbar spinal cord, where focal gliosis and haemorrhage may also occasionally be apparent. Lesions occur mostly in the spinal cord, rhombencephalon and mesencephalon, the cerebral cortex being less likely to be involved (Cantile et al., 2000; Siger et al., 2006). In other studies, besides hematogenous and infected monocytic spread, the olfactory neurons, endothelial cells, choroid plexus and infected peripheral neurons have all been implicated in the ability of WNV to cross the blood-brain barrier and may explain some of the histopathology seen (Samuel and Diamond, 2006).

CNS pathology observed post-mortem in South African WNV-positive horses has resembled the non-suppurative polioencephalomyelitis seen in the Northern hemisphere lineage 1 cases. RSA lineage 2 cases have displayed a considerable amount of variation in CNS lesion severity, type and distribution and the RSA lineage 1 case examined histologically, resembled some of the milder lineage 2 cases pathologically. On occasion meningitis, leucomyelitis, asymmetrical ventral motor spinal neuritis and olfactory lobe or cortex involvement have been seen (Williams et al., 2014).

2.5. DIAGNOSIS OF WNV

Due to a fleeting viraemia in horses with WNV infection, that is estimated to be at the most 1–3 days long and experienced 3-5 days after experimental infection (Bunning

et al., 2002), attempts to detect viral RNA by reverse transcription polymerase chain reaction (RT-PCR) from the serum or EDTA blood from clinical cases is usually not successful except early on in disease. RT-PCR, however, may be used after necropsy with greatest success on CNS tissue samples in horses (Kleiboeker et al., 2004; Williams et al., 2014). Organ samples such as kidney, heart, liver, spleen and intestine may also be used (OIE, 2018). Horses with neurological WNV infection, however, was found to diagnose positive for WNV RNA on CNS samples, rather than other organ samples (Kleiboeker et al., 2004; Williams et al., 2014). During the 1999 WNV outbreak in USA, experimental challenge and natural infection indicated that IgM isotype anti-WNV antibodies become detectable 8–10 days post-infection and were likely to persist for less than 2 months (Ostlund et al., 2001), making the IgM antibody capture-ELISA (MAC-ELISA) the test of choice for detecting initial immune response to infection. In studies using mice, the level of WNV-specific IgM four days after infection was even used as a prognostic indicator for recovery (Samuel and Diamond, 2006).

The diagnosis can therefore be made with the following serological tests to detect antibodies against WNV (OIE, 2018):

- IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA) which indicates a current/recent infection,
- Haemagglutination inhibition (HI) and IgG ELISA for which a rise in antibodies over 2 time points is needed to indicate a current infection or cause of disease,
- Plaque reduction neutralization (PRNT) or virus neutralization (VNT) tests are done to confirm IgM or IgG assays as WNV, and to rule out cross-reactivity to other flaviviruses.
- According to the OIE, in endemic countries, IgM followed by PRNT or VNTs are the standard for confirming WNV as the likely cause of disease. In RSA, however, RT-PCR on blood or tissues and/or WNV specific IgM are considered the standard and diagnostic tests, both of which are routinely done at the CVZ. Virus isolation, although time-consuming, is also considered diagnostic.

Due to the fleeting viraemia, agent identification in live, clinically ill horses presents some difficulty. Brain and spinal cord samples are the preferred tissues for successful,

post-mortem agent identification in horses, generally using RT-PCR to detect viral nucleic acid, with the possibility of later or simultaneous cell cultures and viral isolation. Immuno-histochemistry is generally a poor test in horses for uncertain reasons, despite the presence of severe inflammation (Bunning et al., 2002; Castillo-Olivares and Wood, 2004; Kleiboeker et al., 2004; Williams et al., 2014). WNV is less readily isolated from horses and mosquitoes than from diseased birds, in which a variety of tissues may be used for successful agent identification (OIE, 2018).

It is advised that all horses suspected to have died or which have been euthanised due to WNV infection should be submitted for necropsy, to be performed using biosafety precautions. This should be done in order to confirm WNV infection and rule out other potentially zoonotic viral infections that might pose a risk to public or veterinary health in general such as rabies and other viral encephalitic diseases such as Eastern, Western and Venezuelan equine encephalitis in USA (Long et al., 2002). In RSA, emerging viruses such as SINV, MIDV, SHUV, WSLV may also be considered as potential risk (Venter et al., 2010; Van Eeden et al., 2014; Van Niekerk et al., 2015) along with equine encephalosis virus (EEV) and equine herpes virus (EHV), as possible differential diagnoses for equine encephalitis.

2.6. PROPHYLAXIS FOR WNV

Currently there is no specific, effective treatment protocol for WNV infection in horses. Treatment is mostly supportive and depends on the severity of the clinical signs. Oral or intravenous fluid therapy, steroidal or non-steroidal anti-inflammatories, sedatives, tranquilizers and antibiotics may be incorporated in addition to general nursing (Long et al., 2002). The control of the disease depends mainly on prophylactic vaccination to stimulate a protective immune response and mosquito management to avoid exposure to infected mosquitoes (Long et al., 2002; Castillo-Olivares and Wood, 2004; Siger et al., 2006).

Control efforts in the USA were stunted during initial outbreaks because of lack of knowledge about the biology of WNV in the resident ecosystems, delays in mosquito

control and public objection against using insecticides (Long et al., 2002). These are valid reasons and may very well be global issues, potentially pertaining to South Africa.

Vector management may include the following (Weese, 2017):

- Frequent use of insect repellents especially during periods of rain. The CDC and the United States Department of Agriculture (USDA) particularly recommends insect repellents containing diethyltoluamide (DEET) or Picaridin for human use. Other active ingredients for mosquito repellents may include Insect Repellent 3535 (IR3535), oil of lemon eucalyptus, para-menthane-diol or 2-undecanone (CDC, 2018b).
- Stabling of horses at night
- Minimization or complete elimination of standing water
- Populating tanks or ponds with mosquito feeding fish
- Eliminating all equipment and general yard paraphernalia where standing water can collect and mosquitoes might breed, such as brush piles, gutters, old tyres and litter

Chaskopoulou *et al*. reports current WNV vector control in Italy, France and Greece against mosquito larvae using *Bacillus thuringiensis israelensis* (*B.t.i.*) and diflubenzuron products. For adult mosquito control pyrethroid products such as deltamethrin, permethrin and d-phenothrin is used, but less regularly. Adulticides are used largely as an emergency response to human WNV infections (Chaskopoulou et al., 2016).

Table 2: Types of commercially available WNV vaccines currently registered for use in horses in the USA.

Type of vaccine	USDA Licensing
Inactivated whole virus vaccines	Aid in prevention of viraemia or viraemia and mortality, as well as an aid in reduction of severity of clinical disease
Recombinant canary pox vaccine	Aid in prevention of disease, viraemia and encephalitis
Inactivated flavivirus chimera vaccine	Aid in reduction of disease encephalitis and viraemia

Source: Adapted from AAEP (2019)

During the 1999 USA WNV outbreak, the introduction of an inactivated WNV vaccine had a significant effect on the reduction and control of WNV infections in horses (Roehrig, 2013). It was also shown that a recombinant DNA vaccine had a protective effect on vaccinated versus unvaccinated horses, in which only the unvaccinated horses developed detectable viraemia, fever or neurological signs (Davis et al., 2001). According to the AAEP, WNV vaccination is recommended in North America as a part of the core vaccines and essential standard of care. There are three equine WNV vaccines currently licenced by the USDA according to their protective ability (AAEP, 2019) (Table 2). Internationally, formalin-inactivated vaccines derived from tissue culture, a live canarypoxvirus-vectored vaccines, DNA and chimeric vaccines are available and licensed for use in horses (OIE, 2018).

In South Africa, an inactivated WNV vaccine is distributed by Zoetis (Duvaxyn), and a WNV recombinant canarypox virus vaccine by Merial / Boehringer Ingelheim (Proteq West Nile) and were licensed after epidemiological studies showed that WNV lineage 2 was associated with fatal neurological disease in horses (Venter et al., 2009) and a vaccine trial in mice that showed that a lineage 1 vaccine (Duvaxyn) cross protected against lineage 2 (Venter et al., 2013). Both WNV vaccines are available in 1 ml doses to be administered by intramuscular injection in the neck and are safe to use in foals over 5–6 months old as well as during pregnancy and lactation. The vaccination schedules are also similar and involve an initial vaccination, followed by a booster 3–6 weeks later and an annual booster to maintain immunity. Immunity with the inactivated vaccine is achieved after 3 weeks, and for the recombinant canarypox virus vaccine after 4 weeks. Vaccinations may cause transient local reactions in the form of swelling and pain at the injection site and occasionally mild depression, anorexia and fever for up to 2 days. According to the vaccine package inserts, both vaccines may interfere with sero-epidemiological surveys but they also state that a positive IgM ELISA test result should rather be considered as characteristic of a natural WNV infection, as the IgM antibody response as a consequence of vaccination rarely occurs (Long et al., 2002; Zoetis, 2016; Boehringer-Ingelheim, 2017).

Both South African registered vaccines are indicated to reduce the number of viraemic horses, thus reducing the duration and severity of clinical signs and likelihood of

mortality in infected vaccinated horses (Zoetis, 2016; Boehringer-Ingelheim, 2017). The inactivated vaccine was proven to provide complete protection against both South African lineage 1 and 2 WNV strains in mice. All the vaccinated mice stayed healthy, compared to all the unvaccinated mice which showed severe neurological signs, gross and microscopic lesions and a 75% fatality rate due to WNV. WNV was only detected in the brains of the unvaccinated mice following the virus challenge (Venter et al., 2013).

It was previously demonstrated that the canarypox vaccine provided protective immunity in horses during clinical trials. Infection was achieved by WNV-infected mosquitoes (Siger et al., 2004) as well as by intrathecal administration of a virulent WNV strain into the cisternal space of the atlanto-occipital joint (on day 49) of 10 recently vaccinated (on day 0 and 35) and 10 unvaccinated control horses (Siger et al., 2006). During the intrathecal challenge study 8/10 of the unvaccinated control horses developed encephalomyelitis and displayed typical WNV neurological signs and the other 2 control horses developed only fever (total of 9 controls had fever >38.8°C). None of the vaccinated horses showed post-intrathecal challenge viraemia and only one vaccinated horse developed a fever, and one displayed mild muscle fasciculations at a single observation (Siger et al., 2006). In an earlier study, 9 horses that received a single dose of recombinant canarypox WNV vaccine on day 0, and 10 unvaccinated control horses, were challenged with the bites of WNV-infected mosquitoes on day 26 (Siger et al., 2004). One out of 9 vaccinated horses and 8 out of 10 unvaccinated control horses developed post-challenge viraemia. All horses seroconverted, although anamnestic responses were detected earlier in the vaccinated horses than in the untreated control horses (Siger et al., 2004).

These studies also showed that there is a similar magnitude and duration of disease induced naturally by WNV-infected mosquito and experimentally by intrathecal administration of WNV strain. It took, however, at least 7 days for pathological changes to occur in the central nervous system before clinical signs were evident after intrathecal administration, despite the virus having been isolated from the control horses' blood as early as 24 hours post-intrathecal injection, indicating blood-brain-barrier disruption (Siger et al., 2006). None of the horses had detectable antibodies

against WNV or St. Louis encephalitis virus before the onset of the trial. After the intrathecal challenge, all control horses had seroconverted while the vaccinated horses developed an apparent anamnestic response with detectable antibodies as soon as 7 days after the first vaccination. The authors concluded that the canarypox vaccine showed significant protection from even a single dose of vaccine and that protective immunity lasted for a year after a course of two injections (Siger et al., 2006).

A comparative vaccine study found that vaccination, irrespective of which commercial vaccine was used, followed by intrathecal WNV challenge with a virus obtained from the brain of an infected crow, resulted in protection against the onset of WNV encephalitis and viraemia, with 100% survival at 21 days post challenge and only mild histological inflammatory lesions in a few of the vaccinated horses (Seino et al., 2007). Three commercial vaccines were used in 6 horses each: the chimera-vaccinated horses were challenged at 28 days post-vaccination, while those vaccinated with the commercial inactivated and recombinant canarypox vaccines, had received a primary vaccination at day 0 and a second booster vaccination at day 28, and were consequently challenged on day 56. All control horses were euthanised before the end of the study (the study ended 21 days after the intrathecal challenge) due to moderate to severe encephalitis, revealing moderate to severe histopathologic changes in the brain and spinal cord. Those vaccinated with the chimeric and canarypox vaccines showed significantly fewer and milder clinical signs, with only mild inflammatory changes post-mortem, than did the control horses. Four of the six horses vaccinated with the inactivated vaccine, showed mild to moderate neurological signs with mild inflammatory changes in the brain and spinal cord. Also, none of the vaccinated horses displayed any injection site reactions nor were post-vaccination systemic effects observed bar a few horses with mild increases in rectal temperature after the second inactivated and canarypox booster vaccinations (Seino et al., 2007).

According to the historical summaries of WNV infection in Kentucky, USA, a total of 744 horses were confirmed WNV cases from 2001–2017, of which 30% died. Of these 744 horses, 96% were not adequately vaccinated and 1% had unknown but dubious vaccination histories (KDA, 2019). Only 3% of WNV cases had been reported by the treating veterinarian as “current” on vaccination. The outcome of the vaccinated WNV

cases was also not specified. Available data did not provide sufficient opportunity to define the number of exposed, vaccinated horses in Kentucky that did not develop clinical signs of WNV. The recommendation by the Kentucky Department of Agriculture (KDA) is that these numbers provided evidence to support the logical conclusion that timely vaccination against WNV provides good defence against disease and mortality and is thus beneficial (KDA, 2019). Thus it is clear that vaccination against WNV, by whichever registered and correctly applied vaccine is used, provides protection against WNV viraemia and decreases the severity and/or incidence of clinical signs as well as the mortality rate seen in horses affected with WNV (Long et al., 2002; Siger et al., 2004; Siger et al., 2006; Seino et al., 2007; OIE, 2018; AAEP, 2019; KDA, 2019).

2.7. SOURCE OF DATA FOR THE PROJECT

The data for the current project was sourced from a passive surveillance programme for neurotropic arboviruses in humans, horses and other animals in South Africa which was set up and facilitated by the ZARV program at CVZ, University of Pretoria, in 2006 (Venter et al., 2017). The program recruited the participation of private veterinarians, state veterinarians and clinicians at veterinary training institutes, to submit, along with completed specific submission forms, diagnostic samples from animals under their care showing fever of unknown origin and/or suspected neurological viral disease and/or death. Neurological disease included signs such as ataxia, paresis and/or paralysis. Occasional samples from clinical cases with unknown diagnosis and with varying clinical signs were also have been submitted for testing. These were, however, excluded from the subpopulation of controls used in this MSc study as they did not fit the case description of typical WNV syndrome. The ZARV program was described in a previous publication (Venter et al., 2017).

Samples required were serum for IgM serology, EDTA blood for PCR (and plasma potentially used for IgM detection) and/or or post-mortem samples of brain, spinal cord / cerebrospinal fluid from horses with neurological signs for RT-PCR and possible viral culture. Spleen was later also requested when discovery of arboviruses other than WNV occurred in order to ascertain if a virus was present in both CNS and circulating

systemically. Samples of lung and other tissues were later accepted by ZARV when the search for respiratory viruses became pertinent in some species (these latter cases were not included in the current study). Samples were collected and sent to the ZARV on ice via medical couriers, together with the test requisition form, requesting information regarding the owner, attending veterinarian or pathologist, horse and clinical presentation, amongst some other pertinent information, which had to be completed by the veterinarian at the time of sample collection.

Testing was done at no expense to the owner or veterinarian, due to the funding secured through research grants by the ZARV program throughout the programme, which continues to date (2019). The test requisition form, which was regularly updated, always contained a disclaimer in which the veterinarian agreed that the information be used for research purposes; as well as an acknowledgement that the local state veterinarian would be informed. This was included after recommendation by DAFF although WNV and the other arboviruses discovered during the project are not currently notifiable in RSA.

Virological testing at the ZARV was performed on submitted samples, in the DAFF-compliant BSL3 laboratory using a genus-specific real-time RT-PCRs (rtRT-PCR) for alpha and flaviviruses followed by specific PCRs for WNV, SHUV, MIDV and SINV, WSLV and EEV as described in (Venter et al., 2017). WNV IgM ELISA (IDEXX WNV IgM Capture ELISA test, Idexx Laboratories, Montpellier, France) was performed on all serum and plasma from EDTA samples followed by neutralization assays. Virus isolation for WNV positive cases was attempted for further characterisation. Cases were regarded as positive if the sample tested positive for WNV either by the IgM ELISA or the rtRT-PCR test or both. The WNV rtRT-PCR also genotyped cases according to lineage using hydrolysis probes as previously described (Zaayman et al., 2009). All rtRT-PCR positive cases were confirmed by Sanger sequencing and phylogenetic analysis used for typing the genetic lineage of cases to lineage 1 or 2.

Table 3: CVZ equine sample submission data 2016–2017. Samples may refer to one or more biological specimens submitted to the CVZ from individual equine cases at different temporal intervals for follow-up tests. Duplicate sample submissions were excluded for the WNV-positive totals and therefore these numbers refer to actual individual equine cases.

2016–2017	Samples (n)	Samples %	WNV-positive (n)	WNV-positive % per province samples
Gauteng	264	45%	19	7%
Western Cape	109	19%	5	5%
KwaZulu-Natal	84	14%	14	17%
North West	27	5%	2	7%
Northern Cape	24	4%	11	46%
Free State	24	4%	3	13%
Mpumalanga	20	3%	0	0%
Eastern Cape	15	3%	0	0%
Limpopo	8	1%	0	0%
Not Provided	11	2%	0	0%
Total	586		54	

Table 3 shows the number of equine sample submissions to the CVZ according to province, during 2016–2017. A total of 586 samples were submitted, of which 54 tested positive for WNV. Most of the samples were submitted from Gauteng and Western Cape, in both of which less than 10% of the samples tested positive for WNV. KwaZulu-Natal had the third most sample submissions, but a higher proportion of samples (17%) tested positive for WNV in this province. In comparison, there were very few samples submitted from the Northern Cape, yet almost half of the sample submissions tested positive for WNV.

CHAPTER 3: OBJECTIVES AND HYPOTHESES

3.1. OBJECTIVES OF THE STUDY

1. Investigate and describe demographic, management and environmental factors as predictors of WNV infection in horses during 2016–2017. Data required:

    - Animal demographic data such as breed, age and sex.
    - Vaccination status for WNV, African horse sickness (AHS) and equine influenza virus (EIV).
    - Illness or stressful events within 4 weeks prior to sample submission, including long distance traveling or recent vaccination.
    - Environmental predictor variables:
        - Geographic location of cases
        - Monthly temperature ranges
        - Average monthly rainfall
        - Annual rainfall patterns
        - Altitude of location of subject

2. Investigate and describe the clinical presentation of cases arising from passive surveillance for West Nile disease in horses during 2016–2017 in South Africa. The association of clinical signs with WNV infection was also determined by comparing the cases from both years to WNV-negative controls originating from the same database during the same time period.

3.2. HYPOTHESES

1. The null hypothesis is that acute neurological clinical signs and death were not associated with WNV infection in horses in the WNV endemic country, South Africa, during 2016–2017.

The alternative hypothesis is that acute neurological clinical signs or death were associated with WNV infection in horses in South Africa, during 2016–2017.

2. The null hypothesis is that there is no association between predictor variables such as age, breed, sex, vaccination status and environmental factors, and the occurrence of fever, neurological disease, death, or WNV infection in horses sampled during passive surveillance for febrile and neurological disease.

   The alternative hypothesis is that there is indeed an association between one or more predictor variables such as age, breed, sex, vaccination status and environmental factors, and the occurrence of fever, neurological disease, death, or WNV infection in horses sampled during passive surveillance for febrile and neurological disease.

CHAPTER 4: METHODS AND MATERIALS

4.1. ETHICAL APPROVAL

The applicable research ethics approvals were obtained for this study. The protocol was submitted to and approved by the University of Pretoria (UP) Animal Ethics Committee and the Faculty of Veterinary Science Research Committee, reference number V080-18 [Appendix A].

The UP Animal Ethics Committee had also previously given permission for the testing of the samples during 2016–2017 for ZARV program at the CVZ, reference number H01216, and DAFF Section 20 approval had been obtained [Appendix B].

Veterinarians and owners involved were informed of the purpose of the questionnaire [Appendix D] and had assented either verbally or electronically (as well as by written permission on the test requisition form [Appendix C]) to the information being used for research purposes; they were also informed that no personal information would be made public. Interviews with owners also involved general awareness of WNV explaining common signs, potential pathological course, occurrence and distribution in South Africa as well as the availability and use of vaccines against it.

All information submitted regarding cases was treated as confidential, and no individual persons or animals, who contributed information to this study, were identified in the publication. No experimental animals were used.

4.2. CVZ ARCHIVES

The dataset, in the form of a Microsoft Access database, as well as the submission form and test results, were maintained at and retained in possession of ZARV program in the Centre for Viral Zoonoses, at the Prinshof Campus, University of Pretoria. All electronic information was kept confidential and password protected, and all original hard copy test requisition and result forms were archived and protected under lock

with restricted access. Information for each case submitted contained the following information fields in Microsoft Access:

- Unique identification of animal (usually name)
- ZARV case number. All biological specimens (e.g. EDTA, organ samples etc.) submitted for a particular equine case in one consignment were assigned the same ZRU case number, and thus regarded as a single submission. Follow-up samples submitted for the same equine case at a later stage, were noted as such on the database.
- Age, sex, species, type and breed of animal
- Date of onset of clinical signs
- Date animal died, if relevant
- Died or euthanised
- Main clinical signs (fever, neurological, respiratory)
- Other signs: Anorexia, anaemia, icterus, hepatitis, rectal prolapse, ataxia, paresis, hindleg or foreleg paralysis, recumbency, head tilt, nystagmus, tongue paralysis, paddling, seizures, blindness, congested or cyanotic mucous membranes, nasal discharge, respiratory rate, cough, dyspnoea, pulmonary oedema, pneumonia, abortion, foetal deformity, arthrogryposis, haemorrhagic manifestations
- Results of all diagnostic tests performed by the ZARV on the sample such as viral rtRT-PCR, IgM and/or VN and culture tests
- Other diagnostic tests that samples were submitted to at other labs such as African Horse Sickness or EEV at OVI, ERC
- Recent vaccination details of the animal
- Owner name and contact details
- Veterinarian (sender/sample collector) name and contact details
- Location and GPS coordinates of the animal
- Sample type received
- Date sampled
- Date received
- If sample was received on ice (cold chain maintained)
- Condition sample arrived in

- Storage details of sample at ZARV lab and studies used for
- Date sample was tested
- Person performing the test
- Person entering the data into database

4.3. EXPERIMENTAL DESIGN

This was a case-control study (Table 4), with the cases defined as the horses in the ZARV database which tested positive for WNV infection in 2016 and 2017. All available cases in horses were used. For each case, at least two control horses were randomly selected from the same population of ZARV sample submissions which complied to one or more of the following criteria:

- WNV-negative on rtRT-PCR and serology tests
- Pyrexia without neurological signs (>38.5°C)
- Acute neurological signs with or without pyrexia
- Death (including euthanasia)

Table 4: Sample size for the case-control study of WNV in horses during 2016–2017, South Africa. [Again – an explanation of sample referring to an equine case, and not actual numbers of submitted samples –since that was often more than one per case – would be clarifying]

	2016	2017	Total
Total horse sample submissions at ZARV 2016 -2017.	136 sample submissions	447 sample submissions	583 sample submissions
Cases: Horses that tested positive for WNV (rtRT-PCR or IgM ELISA) out of sample submissions for specific year	6 WNV-positive cases	48 WNV-positive cases	54 WNV-positive cases
Controls: Horses randomly selected from sample submissions to ZARV, which did not test positive for WNV	24 WNV-negative controls	96 WNV-negative controls	120 WNV-negative controls

Neurological disease was characterized as horses displaying one or more of the following clinical signs: ataxia, blindness, facial paralysis, hyperreactivity or hyperaesthesia, incoordination, nystagmus, paresis, partial or complete paralysis,

recumbency, seizures, tremors and muscle fasciculations, tongue paralysis and/or weakness, lip twitching, head tilting and/or dysphagia.

Any incomplete information (e.g. sex, age, breed and location) on the sample requisition forms submitted for the equine clinical cases which presented with fever, various neurological signs and/or death in 2016 and 2017, was obtained from the veterinarians who sent the samples for the cases or the owners of the horses, either by telephonic or electronic communication such as email. In addition, the following information was obtained:

- Whether the horse had recovered from the disease, with or without retained neurological signs; or had died or was euthanised.
- Whether the horse had been stabled at night prior to and at time of sample submission. Horses which had open sided enclosures in their camps or were free to move in and out of their stables during the night, were not considered stabled.
- Whether the horse had been vaccinated against AHS (OBP registered vaccine), EIV and WNV in the 12 months preceding the sample submission (2015–2017). Owners were not asked to specify which of the registered vaccines were used against EIV or WNV. The small number of horses that were vaccinated against AHS with only the unregistered Disease Control Africa vaccine were not considered as vaccinated against AHS, in this study.
- Whether the owner or other owners at the same yard/stud subsequently vaccinated their horses against WNV after sample submission (2017–2018).
- Whether the horse had been, in the owner's opinion, severely stressed (in general) in the 4 to 6 weeks prior to sample submission. Owners were asked, in particular, about long-distance transport of the horse to a different area or province, strenuous training or competition, change of ownership and management system (e.g. moved to different stable yard), recent disease or injury (e.g. colic episodes), weaning, or recent AHS vaccination.

4.4. DATA ANALYSIS

Comparisons between years and various categories were done using all available data from the 583 CVZ sample submissions of horses during 2016–2017. Analysis of

the case-control study was done using the 174 subjects on which complete data had been obtained (54 cases and 120 randomly selected controls which met the relevant inclusion criteria).

Variables for analysis were divided into 2 groups: the exposure or potential risk factors that may or may not have contributed to the likelihood of a horse developing WNV syndrome were used to develop a multiple logistic regression model; secondly, the possible consequences or outcomes (partial/full recovery, death/euthanasia) as a result of developing WNV syndrome, which mainly consisted of the recorded clinical signs, were correlated with the presence or absence of WNV infection by means of univariate analysis and descriptive statistics.

Univariate analyses of risk factors were performed using cross-tabulation and two-tailed chi-squared or Fisher's exact tests. For continuous variables the assumption of linearity was assessed by plotting the Pearson and Deviance residuals against the value of the predictor, in a simple logistic regression model, and evaluating the linearity of the resulting pattern. The predictors were also categorised into quartiles and the quartile midpoints plotted against their estimated log odds to furthermore evaluate linearity.

Some categorical variables were recoded to increase statistical power by combining categories with few observations. The following changes were made:

- "Month" was recoded into three bimonthly levels (January–February, March–April, May–June) plus a fourth level that included July-December, due to the seasonality of WNV infection which leads to very low number of WNV cases in the latter half of the year (Table 16,17).
- "Biome" was recoded into 3 levels by combining the Fynbos and Succulent Karoo categories, as well as the Grassland and Nama Karoo levels, as these were located close to each other, but some of which had very small numbers of cases (Figure 15,Table 16).
- "Altitude" was recoded into quartiles because it showed a non-linear relationship with the outcome. Altitude of subject locations was divided into equal sized quartiles according to number of total observations:

16–1056 m, 1057–1292 m, 1293–1466 m, 1467–1784 m (Table 16,17).

- “Age”, “Rainfall in mm”, “Maximum temperature” and “Minimum temperature” were used as continuous variables in the logistic regression model as they showed an approximately linear relationship with the outcome (Table16,17). Total rainfall and average minimum and maximum temperatures in the month prior to the sample submission to ZARV, from the respective weather stations closest to the subject location, were obtained from the South African Weather Service (SAWS).
- “Breed” was initially categorized into 6 levels according to the type of horse, but to increase statistical power they were recoded into only 3 levels according to perceived hybrid vigour: highly purebred breeds included purebred Arabians, Thoroughbreds and American Saddlers. Mixed or cross and local breeds included any mixed or cross bred horses as well as local horse breeds, such as Boerperd and Nooitgedachter. The intermediate category contained all other breeds of intermediate genetic variety, which may have a combination of Thoroughbred, imported or local bloodlines, such as SA Warmblood and Anglo-Arabian horses. The reasoning behind this was that horses with increased hybrid vigour may display increased resistance against developing WNV syndrome (Figure 7,Table 11,16,17).

“Province” was not included in the maximum model due to some provinces having very few or no WNV-positive cases; it was decided to use variable “Biome” instead of “Province” to indicate location of case. It was also considered that “Biome” might be more biologically meaningful (Table 16).

Multivariable analysis was done using multiple logistic regression. For the maximum logistic regression model, all univariate risk factor variables with the likelihood ratio test (LRT) $p<0.2$ were included and the least statistically significant variables were eliminated using a backward stepwise procedure. Finally, all independent variables were re-included one by one and retained if significant, or if inclusion resulted in substantial (>20%) changes in the coefficients for other variables in the model. Analysis was done using Stata 15 (StataCorp, College Station, TX) and NCSS

statistical software 2007 edition. Significance was set at $p<0.05$. Goodness of fit was evaluated using the Hosmer-Lemeshow goodness-of-fit statistic.

Main syndromes and clinical signs displayed were evaluated mainly by descriptive statistical measures by tabulating the counts and percentages of both WNV-positive cases and negative controls. The associations of clinical signs with the outcome of subjects were assessed using a two-tailed Fisher's exact, odds ratios and 95% confidence interval, and sorted according to prevalence in the WNV-positive cases.

GPS coordinates of subject location were determined using Google Earth (Google AfriGIS Ltd) and the area in which the subject was located at time of initial clinical signs (or the veterinary practice which diagnosed if the subject location area was not known) was used as location rather than the owner's actual address, to protect the owners' privacy. Geographical location of cases was mapped using ArcMap 10.6 (ESRI Corp.; Redlands, CA, USA) to determine spatio-temporal patterns and thus WNV hotspots in South Africa. The geographic coordinate system used was GCS_WGS_1984. World map, South African provincial and biome maps were obtained from ESRI Living Maps, ArcGIS online. Altitude point locations of subjects were obtained using GPS coordinates and ArcToolbox with South African elevation layer from Diva-GIS (Hijmans, 2019). Information for the geographic information system (GIS) layers regarding the environmental factors such as rainfall, temperature and altitude was obtained from the Department of Geography, Geoinformatics and Meteorology at the University of Pretoria and from SAWS. Weather data provided by SAWS were as follows: monthly rainfall in millimetres, and minimum and maximum monthly average temperature in degrees Celsius for specific weather stations located in RSA. Subjects were assigned to closest weather stations as provided by the SAWS; alternatively, if the weather data were not available for the particular month needed, another close weather station would be assigned. Weather data used for each subject was for the calendar month prior to sample submission, to allow for mosquito breeding cycle as well as incubation period of WNV. Historical rainfall maps for RSA were created by SAWS website (SAWS, 2019).

CHAPTER 5: RESULTS

5.1. CLINICAL SIGNS

A total of 54 cases and 120 controls were included in the analysis. All equine sample submissions to the CVZ were tested using genus-specific rtRT-PCRs for alpha and flaviviruses followed by specific PCRs for WNV, SHUV, MIDV and SINV, WSLV and EEV. WNV IgM ELISA (EIDEX) were performed on all serum and EDTA samples followed by neutralization assays. Virus isolation for WNV positive cases were used for further characterisation. A WNV-positive case was considered diagnosed positive if it was positive for WNV on either WNV real-time RT-PCR (rtRT-PCR) or IgM ELISA or both tests. Most of the WNV cases were diagnosed with IgM ELISA (47/54, 87%) of which 2 cases tested positive on both rtRT-PCR and IgM ELISA (2/47, 4%). In total only 17% (9/54) of the cases tested positive for WNV on rtRT-PCR.

Table 5: ZARV equine WNV-positive cases, summarized results of study, 2016–2017.

Year	Total WNV +	WNV rtRT-PCR +	WNV IgM ELISA +	Neuroinvasive	Deaths	Co-infections	Co-infecting viruses
2016	6	2	4	5	3	0	-
2017	48	7	43	43	18	8	4 MIDV 4 EEV
Total	54	9	47	48	21	8	
Percentage		17%	87%	89%	39%	15%	

In total 85% (46/54) of WNV-positive cases in 2016–2017 were infected with only WNV and 15% (8/54) had co-infections with another virus (4 cases were co-infected with MIDV and 4 cases with EEV) (Table 5). Of the WNV-negative controls which were used for the study, 76% (91/120) tested negative for all of the viruses on the ZARV program testing panel, 12% (14/120) tested positive for MIDV and 12% (15/120) tested positive for EEV. There were no SINV, SHUV, WSLV or AHS co-infections diagnosed with either WNV-positive cases or WNV-negative controls used in the study, in 2016–2017. Total co-infections for WNV-positive cases in 2016–2017 were 15% (8/54). An increased co-infection rate with both MIDV and EEV (4 cases

each) was seen during 2016–2017 when compared to previous years (Venter et al., 2017).

Neurological signs were significantly higher in the WNV cases than in the WNV-negative controls (Table 6). Most of the cases in 2016–2017 (48/54, 89%) displayed some neurological signs of which 54% (26/48) had only neurological signs without fever. Approximately half of the WNV cases (28/54, 52%) had a fever >38.5°C with or without neurological signs (35% in 2008–2015) (Table 7). Fever as clinical sign did not show a significant association with WNV cases when cases were compared to controls (Table 8).

Table 6: Summary of main syndromes and fatalities per year in WNV-infected horses during 2016–2017.

Year	Fever main syndrome	Fever and Neuro	Neurological only	Fatalities	Elective Euthanasia
2016	1	1	4	3	2
2017	5	21	22	18	14
Total	6	22	26	21	16
Proportion of Total WNV-positive Cases (n=54)	11%	41%	48%	39%	30%

Only 11% of WNV-positive cases (6/54) had fever as main syndrome and 41% (22/54) had fever as well as neurological signs, but the largest category were cases with only neurological signs. 39% (21/54) of WNV cases in 2016–2017 died, of which 76% of deaths were due to elective euthanasia (16/21). When comparing cases to controls, it is clear that there were distinctly fewer WNV-positive cases with only fever (6/54, 11%), compared to the controls (43/120, 36%). Approximately a third of the subjects in the control group were in each of the various main syndrome categories, of which the least control cases had displayed both fever and neurological signs (33/120, 28%).

Table 7: Disease outcome: deaths and recovery of WNV-positive cases and WNV-negative controls by main syndrome, displaying percentage of each outcome, 2016–2017.

	Disease outcome	*Fever main syndrome*	*Neuro main syndrome*	*Fever and neuro main syndrome*	*Total*
WNV positive cases	Deaths	1	14	6	21 (39%)
	Recovered	5	12	16	33 (61%)
	Total per syndrome	6 (11%)	26 (48%)	22 (41%)	54
WNV negative controls	Deaths	7	27	9	43 (36%)
	Recovered	36	17	24	77 (64%)
	Total per syndrome	43 (36%)	44 (37%)	33 (28%)	120

Total proportions of death and recovery (Table 7) were similar for both cases (died 39%, recovered 61%) and controls (died 36%, recovered 64%). In both groups, subjects with only neurological signs had the highest fatality (cases 14/26, 54% vs. controls 27/44, 61%), while as expected, those with only pyrexia had the fewest fatalities in both groups: cases (1/6, 17%) vs. controls (7/43, 16%). Only one equine patient of those that were only pyrexic, became progressively weak, recumbent and was subsequently euthanised. There was a marginally significant association (p=0.057) between WNV-positive cases with pyrexia (with or without neurological signs) and recovery. Statistical significance also differed for fatalities amongst the main syndromes for the cases (p=0.108) and controls (p<0.001) and were probably due, in part, to sample size variation.

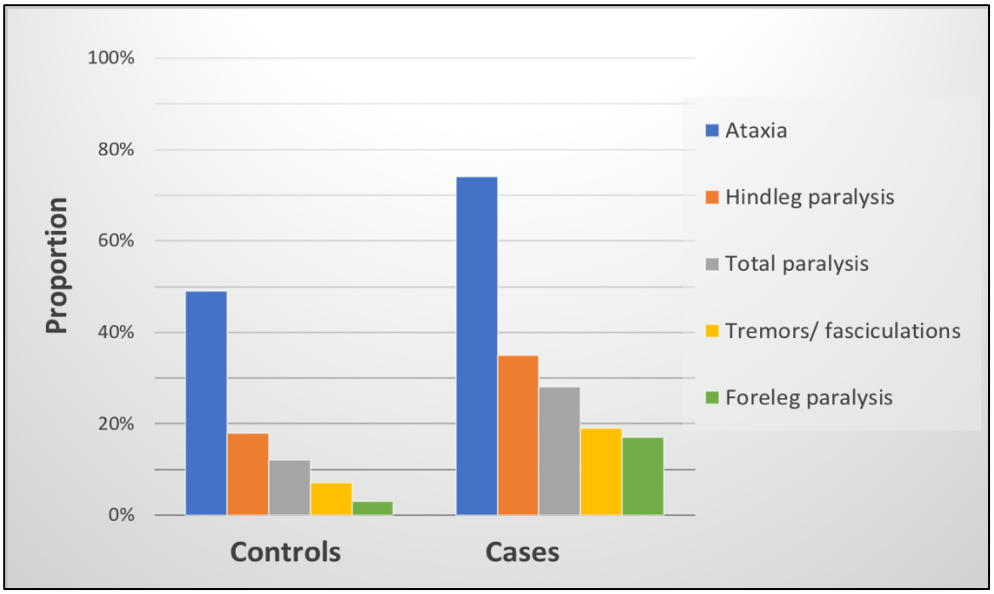

Figure 4: Proportions of horses showing neurological signs amongst WNV-positive cases and negative controls, 2016–2017.

In the clinical signs analysis (Table 8 & Figure 4) significant associations and larger proportions of neurological signs were seen WNV-positive cases when compared to negative controls, displaying the several clinical manifestations of the neurological damage caused by WNV. These were ataxia (40/54, 74%), hindleg paralysis (19/54, 35%), paresis (16/54, 30%), complete paralysis (15/54, 28%), tremors or muscle fasciculations (10/54, 19%) and foreleg paralysis (9/54, 17%).

Of the WNV cases which died, a larger proportion showed hindleg paralysis (9/21, 43%) and total paralysis (8/21, 38%) than foreleg paralysis (2/21, 10%) and tremors (2/21, 10%). Of the WNV-positive cases which recovered, approximately similar proportions were seen of these clinical signs: hindleg paralysis (10/33, 30%), tremors (8/33, 24%), total paralysis (7/33, 21%) and foreleg paralysis (7/33, 21%) . Similar proportions in both recovered (24/33, 73%) and dead (16/21, 76%) WNV-positive cases had ataxia (Figure 5).

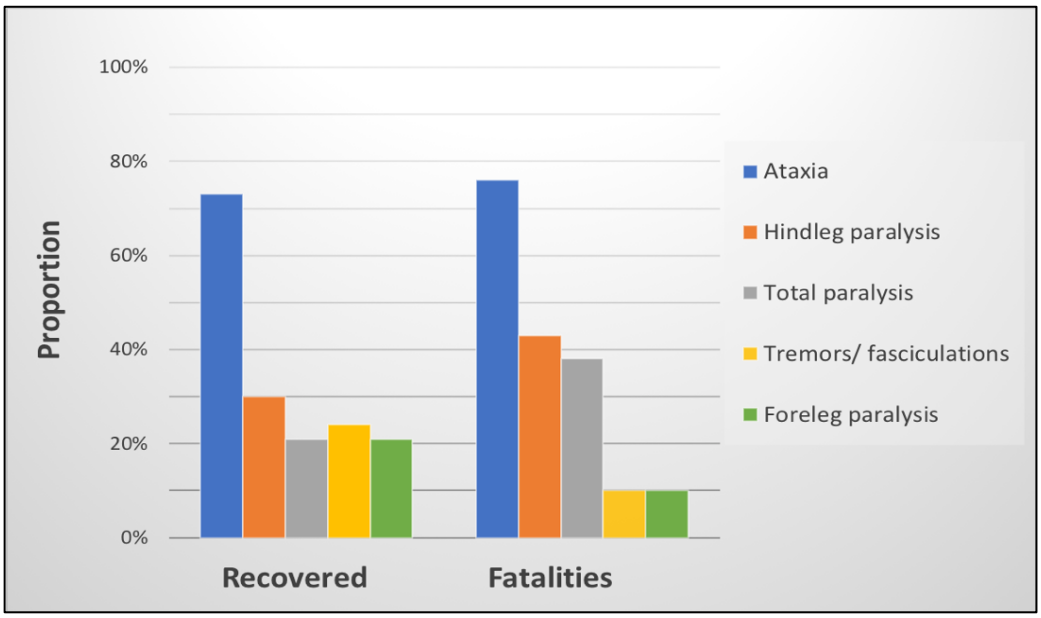

Figure 5: Proportion of horses showing neurological signs amongst WNV-positive cases which recovered vs. those which died, 2016–2017.

Table 8: Most important clinical signs in the 54 equine WNV-positive cases vs. the 120 WNV-negative controls, 2016–2017.

Clinical Signs	WNV-positive		WNV-negative		*Odds ratio*	*95% CI*	p-value*
	n	*%*	*n*	*%*			
Died	21	39%	43	36%	1.14	0.55, 2.32	0.824
Euthanized	16	30%	25	21%	1.61	0.71, 3.51	0.284
Retain signs post recovery	6	11%	3	3%	4.50	0.98, 31.02	0.053
Neuro signs	48	89%	77	64%	4.19	1.70, 13.72	0.001
Ataxia	40	74%	59	49%	2.89	1.39, 6.48	0.003
Fever	28	52%	76	63%	0.63	0.31, 1.26	0.208
Hindleg paralysis	19	35%	22	18%	2.40	1.09, 5.30	0.028
Recumbent	18	33%	24	20%	2	0.90, 4.35	0.091
Paresis	16	30%	18	15%	2.37	1.02, 5.51	0.045
Paralysis	15	28%	14	12%	2.88	1.18, 7.14	0.018
Icterus	11	20%	28	23%	0.86	0.34, 1.94	0.823
Tremors, fasciculations	10	19%	8	7%	3.12	1.05, 9.87	0.041
Foreleg paralysis	9	17%	4	3%	5.41	1.51, 26.78	0.008
Anorexia	8	15%	28	23%	0.59	0.21, 1.42	0.278
Laminitic stance/ footsore	5	9%	1	0.83%	8.85	1.29, -	0.023
Hyperreactive/ Hyperaesthetic	5	9%	3	3%	3.73	0.73, 26.38	0.124

* Two-tailed Fisher's exact test

Neurologic signs were significant and present in 89% of WNV-positive cases (OR 4.19, 95% CI 1.70, 13.72). Fever was only present in 52% of WNV-positive cases (OR 0.63, 95% CI 0.31, 1.26) but not significantly associated with WNV infection ($p < 0.05$) when

compared to the control subjects. All types of paralysis (hindleg, front leg, paresis and total paralysis) as well as ataxia (OR 2.89, 95% CI 1.39, 6.48) and tremors/fasciculations (OR 3.12, 95% CI 1.05, 9.87) were significantly associated with WNV. Recumbency (n=18, 33%), icterus (n=11, 20%), anorexia (n=8,15%) and hyperreactivity / hyperaesthesia (n=5, 9%), although present in several of the cases, were not associated with WNV infection when compared to the control group. Unlike the previous ZARV study findings (Table 1), only a small, non-significant number of the WNV-positive cases in 2016–2017 were reported to have had seizures (n=3, 6%) and none exhibited tongue paralysis. Various other clinical signs were also listed (Table 9) which were not significant but still worth mentioning as they might be directly or indirectly related to WNV infection. Laminitic stance / sensitivity in the feet was an interesting clinical sign noted in 9% (n=5) of the cases and only one of the controls (p=0.023).

Of the 54 WNV-positive cases, 16 horses were euthanized due to a poor prognosis, of which minimum survival time was 0 days (euthanised same day as clinical signs started) and median survival time was two days until elective euthanasia. Retained neurological signs were seen in a marginally significant number of the cases after recovery (n=6, OR 4.50, 95% CI 0.98, 31.02) (Table 8), mainly related to ataxia or neurological instability. Three of these horses, an American Saddler and two Thoroughbred horses, were euthanized more than a month after recovery (54-469 days survival time) ranging in ages: 4 months, 6 years and 18 years old. The 18 year-old Thoroughbred horse was also diagnosed at the time of euthanasia with a cardiac tumour. One of the three other horses, a Thoroughbred, was significantly affected by the retention of some degree of neurological signs to such an extent that it was retired and sold as a pleasure hack even before competing in racing. Two other Thoroughbred horses were retired after unsuccessfully attempting some racing, one of which was raced and soon after retired (after 8 months' recuperation) and the other which had a very unsuccessful racing career. These three Thoroughbred racehorses' ages ranged from 2.5–3 years old.

Table 9: Other, less important or less often seen, clinical signs in the 54 WNV-positive cases vs. the 120 WNV-negative controls, 2016–2017.

	WNV positive		*WNV negative*		Odds Ratio	95% CI	p-value*
Clinical Signs	*n*	*%*	*n*	*%*			
Anaemia	4	7%	11	9%	0.85	0.18, 2.85	0.953
Facial Oedema	4	7%	8	7%	1.18	0.24, 4.41	1.00
Weak	4	7%	4	3%	2.31	0.41, 12.91	0.417
Seizures	3	6%	11	9%	0.65	0.10, 2.34	0.630
Congested mucous membranes	3	6%	2	2%	3.22	0.38, 42.37	0.348
Depressed	3	6%	6	5%	1.20	0.17, 5.48	1.00
Facial nerve paralysis	3	6%	4	3%	1.76	0.24, 10.45	0.751
Lethargy/ listless	3	6%	8	7%	0.90	0.14, 3.62	1.00
Colic	2	4%	3	3%	1.60	0.12, 13.47	0.989
Lame (single limb)	2	4%	3	3%	1.60	0.12, 13.47	0.989
Blindness	1	2%	5	4%	0.59	- , 4.03	0.789
Circling/ Paddling	1	2%	5	4%	0.59	- , 4.03	0.789
Dyspnoea/ respiratory distress	1	2%	4	3%	0.73	- , 5.72	1.00
Stumbling	1	2%	2	2%	1.33	0.02, 21.81	1.00

* Two-tailed Fisher's exact test

The highest proportion of WNV-positive cases was seen in younger horses, especially those less than 5 years old (n=30, 55%) (Figure 6, Table 10). No associations were seen between the different WNV-positive cases' age groups and neuroinvasive disease, death and euthanasia. The 9–12 years old WNV-positive group seemed to have the highest proportions of neuroinvasive disease (9/9, 100%), death (5/9, 56%) and euthanasia (4/9, 44%), but these numbers are insignificant when evaluated in terms of the small numbers of cases (Table 10).

Table 10: WNV-positive cases relating to neurological disease and fatalities by age groups, ZARV equine cases, South Africa 2016 to 2017.

Age Group	WNV positive	WNV neuroinvasive		WNV Deaths		WNV Euthanised	
	Cases	n	% Neuro per age group	n	% Dead per age group	n	% Euthanised per age group
0 to 2 years	13	12	92%	4	31%	3	23%
2 to 5 years	17	15	88%	6	35%	5	29%
6 to 8 years	10	8	80%	4	40%	3	30%
9 to 12 years	9	9	100%	5	56%	4	44%
13 to 18 years	5	4	80%	2	40%	1	20%
Total 2016–2017	**54**	**48**		**21**		**16**	

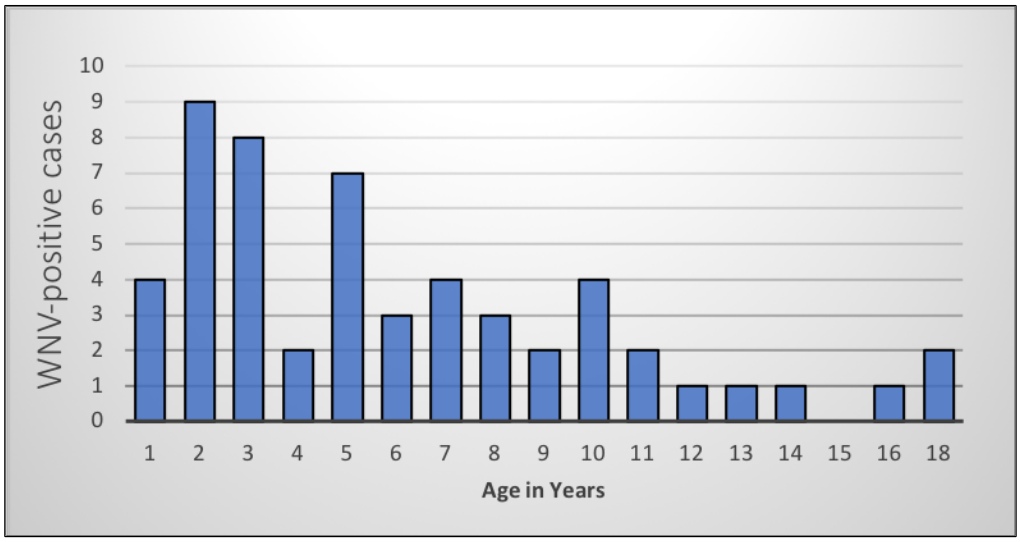

Figure 6: WNV-positive cases in horses by age in years, 2016–2017.

The minimum age of West Nile virus positive horses during 2016–2017, was 4 months old and the maximum 18 years old. The mean age was 6 years and the mode was 1.5 years old (Figure 6). Amongst the West Nile virus negative controls used for the study, the minimum age was 2 months old and the maximum age was 27 years old. The mean age was 8.6 years and the mode was 6 years old.

Table 11: Main syndromes (neuro- and non neuroinvasive syndromes and death) in WNV-positive horses, by breed, 2016–2017. Horse categories assigned to breeds were 1-Hot blooded horses, 2-Cold blooded horses, 3-Warmblood horses, 4-Light horses, 5-South African or indigenous horses and 6-Crossbreed horses of unknown pedigree.

Breed (category)	*WNV cases*		*Non-neuro*		*Neuroinvasive*		*Fatalities*	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Thoroughbred (1)	26	48%	2	8%	24	92%	12	46%
Warmblood (3)	9	17%	1	11%	8	89%	2	22%
Arab (1)	7	13%	0	0%	7	100%	4	57%
Mixed breed (6)	3	6%	0	0%	3	100%	0	0%
American Saddler (4)	2	4%	0	0%	2	100%	1	50%
Percheron (2)	2	4%	2	100%	0	0%	0	0%
Boerperd (5)	1	2%	0	0%	1	100%	1	100%
Clydesdale (2)	1	2%	0	0%	1	100%	1	100%
Friesian (2)	1	2%	0	0%	1	100%	0	0%
Gypsy Cob (4)	1	2%	1	100%	0	0%	0	0%
Nooitgedachter (5)	1	2%	0	0%	1	100%	0	0%
Total	**54**		**6**	**11%**	**48**	**89%**	**21**	**39%**

Individual breed results are listed in Table 11. The breeds mostly represented in WNV-positive cases for 2016 -2017 were the Thoroughbreds (n=26, 48%), Warmbloods (n=9, 17%) and Arabian horses (n=7, 13%). This contrasts starkly to the various cross or mixed breeds (n=3, total 6%) and the South African breeds Boerperd and Nooitgedachter which in combination only represented 4% of the cases (n=2). Between the most represented breeds, neuroinvasive proportions tended to be similar (90–100%) but fatalities seemed to be less in the Warmblood horses (22%). Due to the small numbers in individual breeds, statistical associations could not be made.

Breeds were divided into generally accepted horse categories in an attempt to increase statistical power (Figure 7,Table 11), but these did not show a significant association in the univariable analysis. Another classification of breeds according to perceived hybrid vigour was statistically significant, with the mixed, local and cross

breed category having significantly fewer cases than the highly purebred horses (p=0.009).

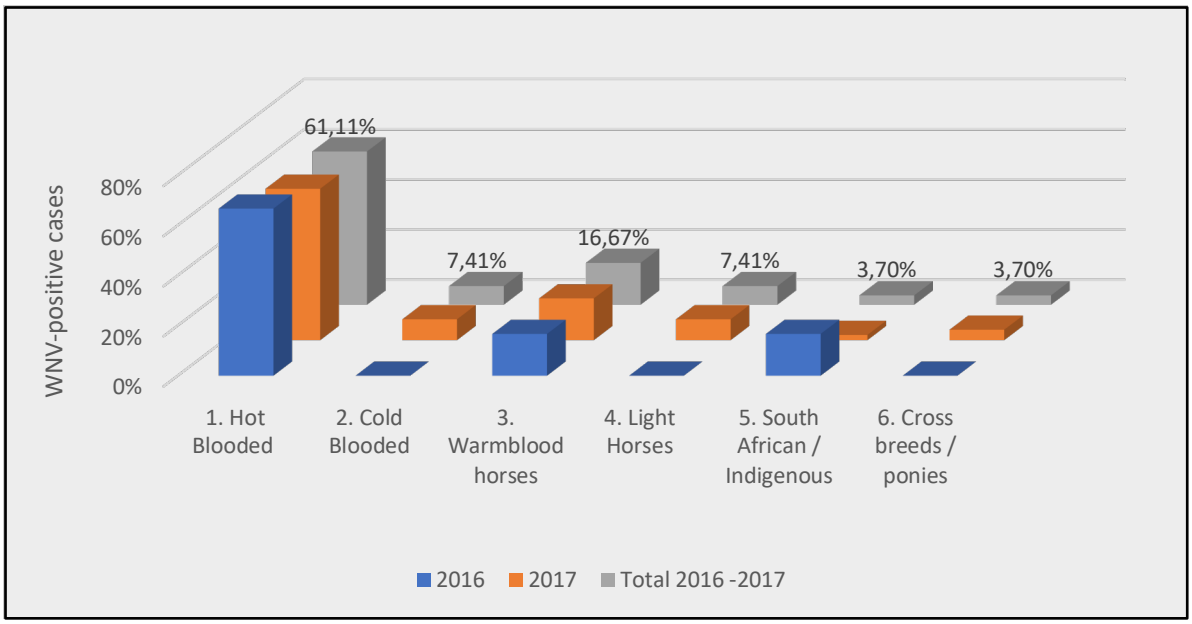

Figure 7: Summary of proportions of WNV-positive cases in South Africa by breed category and year, 2016, 2017 and total 2016–2017.

5.2. CASE DISTRIBUTION

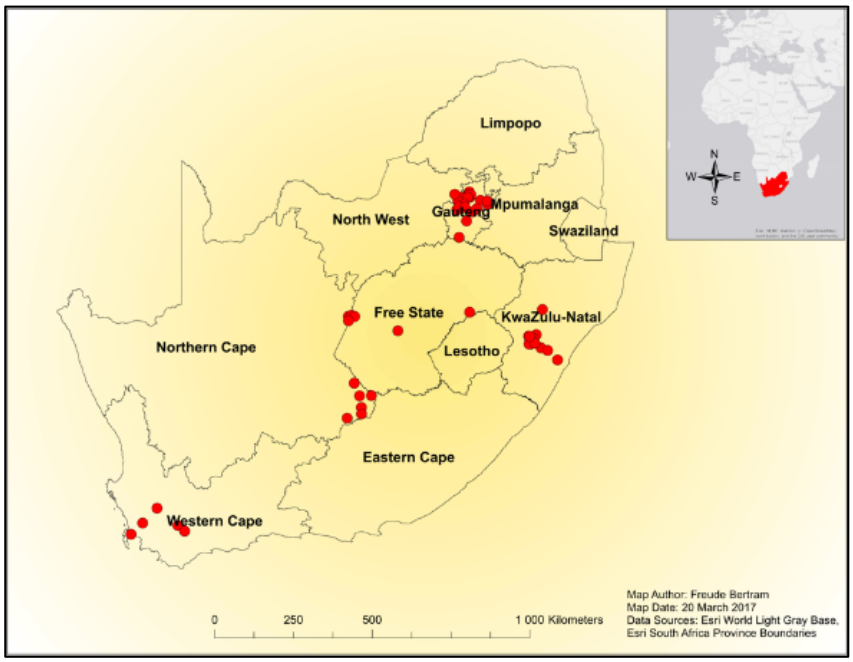

Figure 8: Distribution of total WNV-positive cases in horses in South Africa, 2016–2017. Each marker may represent one or more cases at the same location.

Spatial distribution of WNV cases (Figure 8) was similar to previous findings in 2008–2015 (Venter et al., 2017), in which most of the cases were located in Gauteng province (n=19, 35%), KwaZulu-Natal (n=14, 26%) and the Northern Cape (n=11, 20%). In the current study, fewest cases were seen in the Western Cape (n=5, 9%), Free State (n=3, 6%) and North West provinces (n=2, 4%), with no cases in Mpumalanga, Limpopo and the Eastern Cape (Tables 8,12).

Table 12: Summary of case distribution, deaths and neuroinvasive WNV-positive cases, by province in South Africa, 2016–2017.

	Cases	Neuroinvasive cases		Fatalities	
Province	*n*	*n*	*%*	*n*	*%*
Gauteng	19	14	74%	8	42%
KwaZulu-Natal	14	14	100%	6	43%
Northern Cape	11	11	100%	5	45%
Western Cape	5	5	100%	1	20%
Free State	3	2	67%	0	0%
North West	2	2	100%	1	50%
Total RSA	**54**	**48**	**89%**	**21**	**39%**

The differences in neuroinvasive WNV infection between the provinces were not statistically significant (p=0.077), being the lowest in the Free State (2/3, 67%) and Gauteng (14/19, 74%). The fewest fatalities were also seen in the Western Cape (1/5, 20%) and the Free State (0/3, 0%), but no association was found between fatality and provinces, due to small numbers resulting in lack of statistical power (Table 12).

Table 13: South African Thoroughbred stud farm survey 2017. Source: The Thoroughbred Breeders' Association of South Africa (Hartley, 2019).

	Province	*Western Cape*	*KwaZulu-Natal*	*Eastern Cape*	*Northern Cape*	*Gauteng*	*Total*
Studs	n	27	14	7	2	3	**53**
Horses	n	4692	1599	502	372	81	**7246**
Horses	**%**	65	22	7	5	1	**100**

According to a stud farm survey done by the Thoroughbred Breeders' Association early in 2017 (Hartley, 2019), 65% (4692/7246) of the Thoroughbred stud horses were located in the Western Cape province, 22% (1599/7246) in KwaZulu-Natal, and 1% (81/7246) in Gauteng (Table 13). Of those specified on participating stud farms, 52% (3300/6346) were classified as youngstock under 3 years old and 45% (2858/6346) as adult breeding stock. Only 4% (258/6346) were reported to be non-breeding adult Thoroughbred horses at stud with a negligible number of horses of other breeds (<1%). Of WNV-positive cases diagnosed in Thoroughbred horses during 2016–2017, 46% (12/26) were youngstock less than 3 years old and 15% (4/26) adult breeding stock at stud. 38% (10/26) were either racing, pleasure horses or retired but not resident on stud farms at the time.

Table 14: Distribution of WNV-positive cases in horses by month and province, 2016. Total annual cases (n=6) and fatalities (n=3) included.

2016

Province	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	Fatalities
Gauteng	0	1	1	0	0	0	0	0	0	0	0	0	2	1
Western Cape	0	0	1	1	0	0	0	0	0	0	0	0	2	0
KwaZulu-Natal	0	0	1	0	0	0	0	0	0	0	0	0	1	1
North West	0	0	1	0	0	0	0	0	0	0	0	0	1	1
Total cases per month	**0**	**1**	**4**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**6**	**3**

A total of six WNV-positive cases were identified in 2016, of which three were fatal. Cases were located in the main centres namely Gauteng (n=2), Western Cape (n=2) and KwaZulu-Natal (n=1) and occurred from February–April 2016 (Table14). As indicated by Figure 9, randomly selected controls were submitted from most of the provinces where WNV-positive cases were previously detected in 2008–2015.

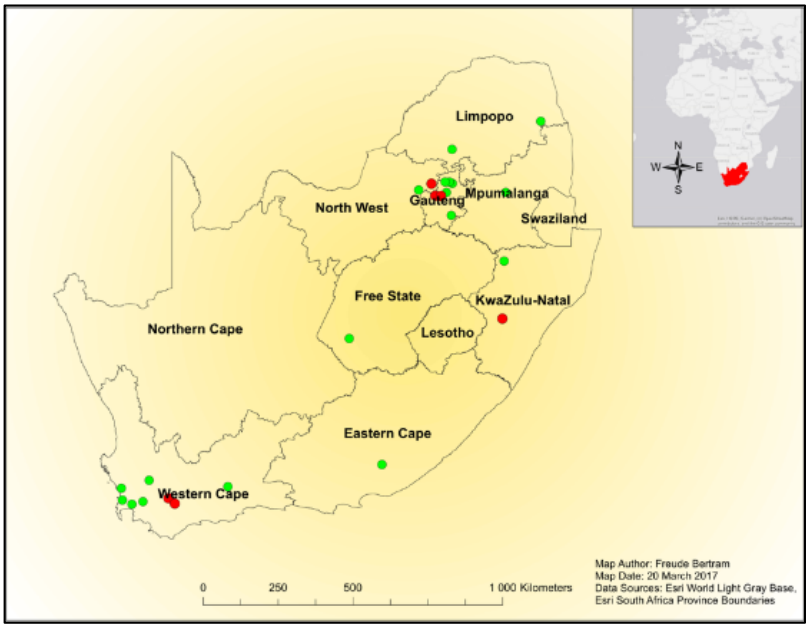

Figure 9: Distribution of ZARV WNV-positive cases (red) and negative controls (green) in horses by province, 2016. Each marker may represent one or more cases at the same location.

In 2017 equine WNV-positive cases occurred from January–June, with a single case in December. March was the month which had the highest number of cases in both years (n=28, 52%) and the most cases occurred from February–April (n=46, 85%). WNV-positive cases in 2017 were located mainly in Gauteng (n=17), KwaZulu-Natal (n=13) and the Northern Cape provinces (n=11) with hotspots of cases clustered mainly in and around Gauteng, the KwaZulu-Natal Midlands, around the borders of the Free State and with a few cases in Cape Town and surrounding areas (Figure 10).

Table 15: Distribution of WNV-positive cases in horses by month and province, 2017. Total annual cases (n=48) and fatalities (n=18) are included.

2017

Province	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	Fatalities
Gauteng	1	2	8	4	0	2	0	0	0	0	0	0	17	7
KwaZulu-Natal	0	1	4	5	2	1	0	0	0	0	0	0	13	5
Northern Cape	0	1	10	0	0	0	0	0	0	0	0	0	11	5
Free State	1	1	1	0	0	0	0	0	0	0	0	0	3	0
Western Cape	0	0	1	1	0	0	0	0	0	0	0	1	3	1
North West	0	0	0	1	0	0	0	0	0	0	0	0	1	0
Total cases per month	**2**	**5**	**24**	**11**	**2**	**3**	**0**	**0**	**0**	**0**	**0**	**1**	**48**	**18**

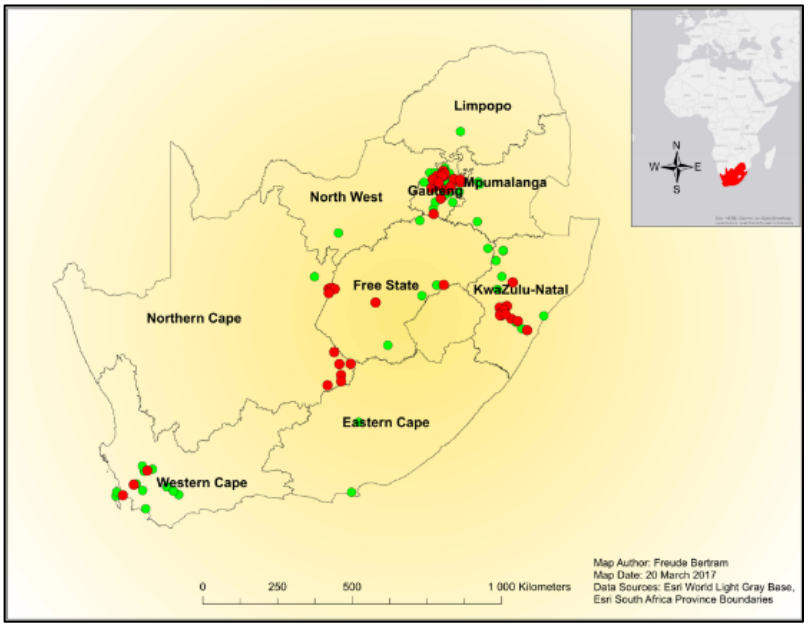

Figure 10: Distribution of WNV-positive cases (red) and WNV negative controls (green) in horses used in the study, by province, 2017. Each marker may represent one or more cases at the same location.

5.3. ENVIRONMENTAL DATA

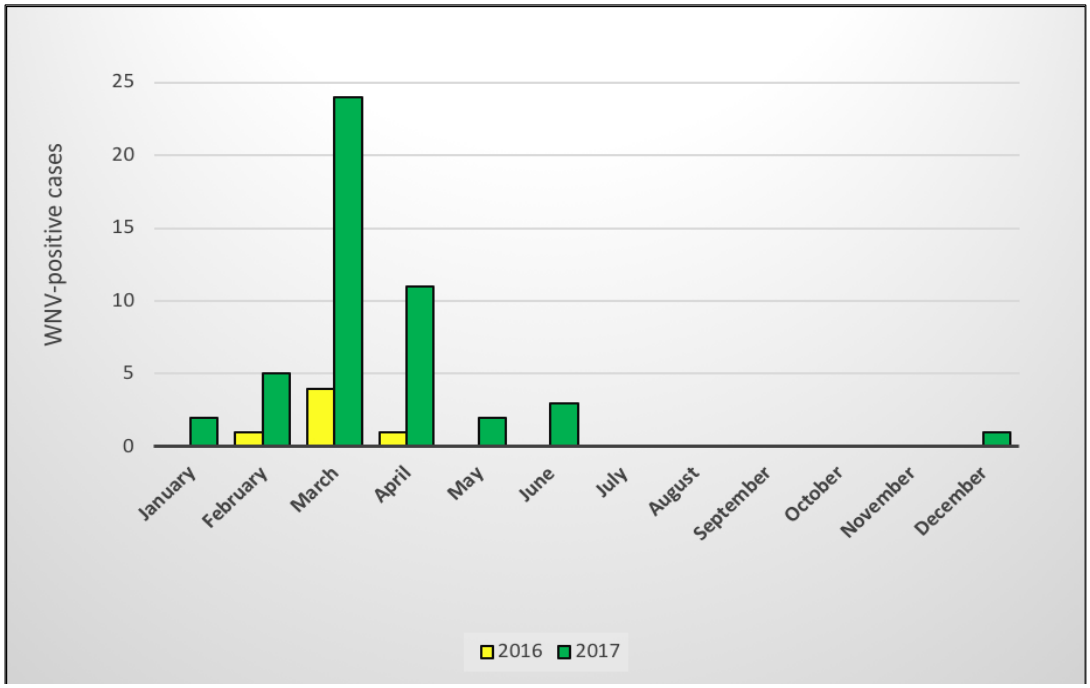

Figure 11: WNV-positive cases in South Africa by the month in which the initial signs were displayed, 2016–2017.

Temporal distribution of WNV-positive cases (Tables 14,15) was mainly in the late summer / early autumn months for both years, as already described. Only one case occurred in early December 2017 in the Western Cape province (Figure 11).

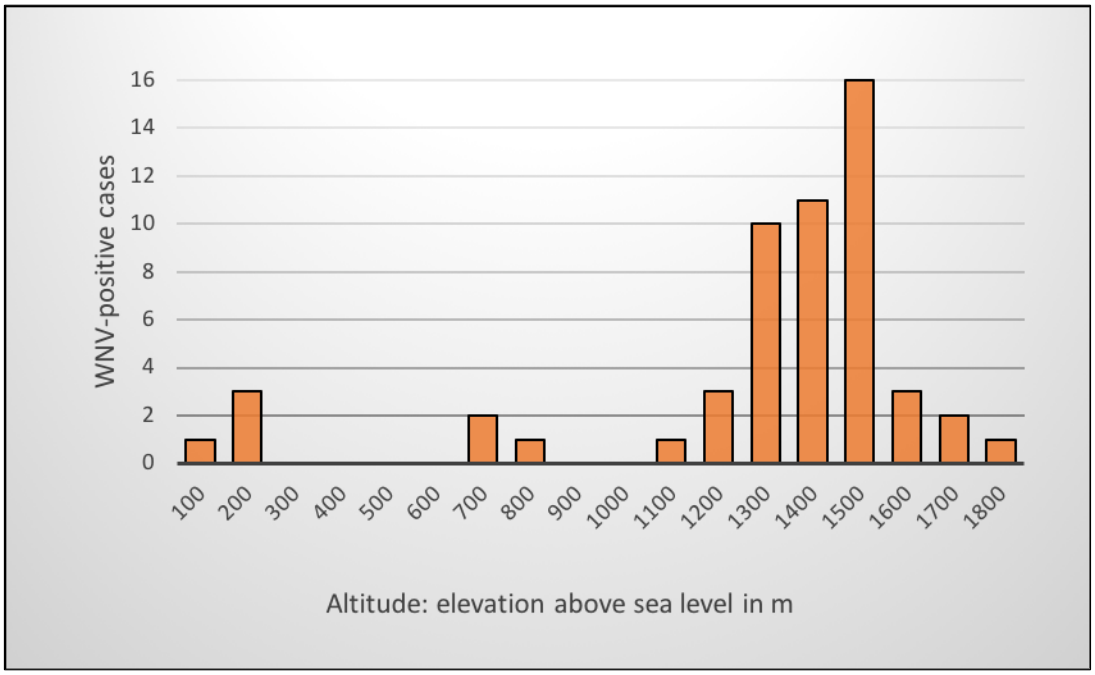

Figure 12: WNV-positive cases in horses by altitude of location, 2016–2017.

Elevation above sea-level (altitude) of the locations of WNV-positive cases (Figure 12) was categorized into quartiles due to being non-linear and was significant in both the univariable analysis and the final regression model. The largest proportion of cases occurred at altitudes 1060–1470m (n=37, 69%) with the single quartile with most cases being 1290–1470m (n=25, 46%) (Table 16,17).

Overall, RSA had been experiencing a severe drought since 2015 (SAWS, 2019). Especially during the early summer months of 2015/2016 there was very little rain and extremely high temperatures, followed by sudden high rainfall in the late summer months of 2016. The eastern parts of the country, in particular, had high rainfall in 2017, varying between 75% to 200% of the normal rainfall (Figures 13,14).

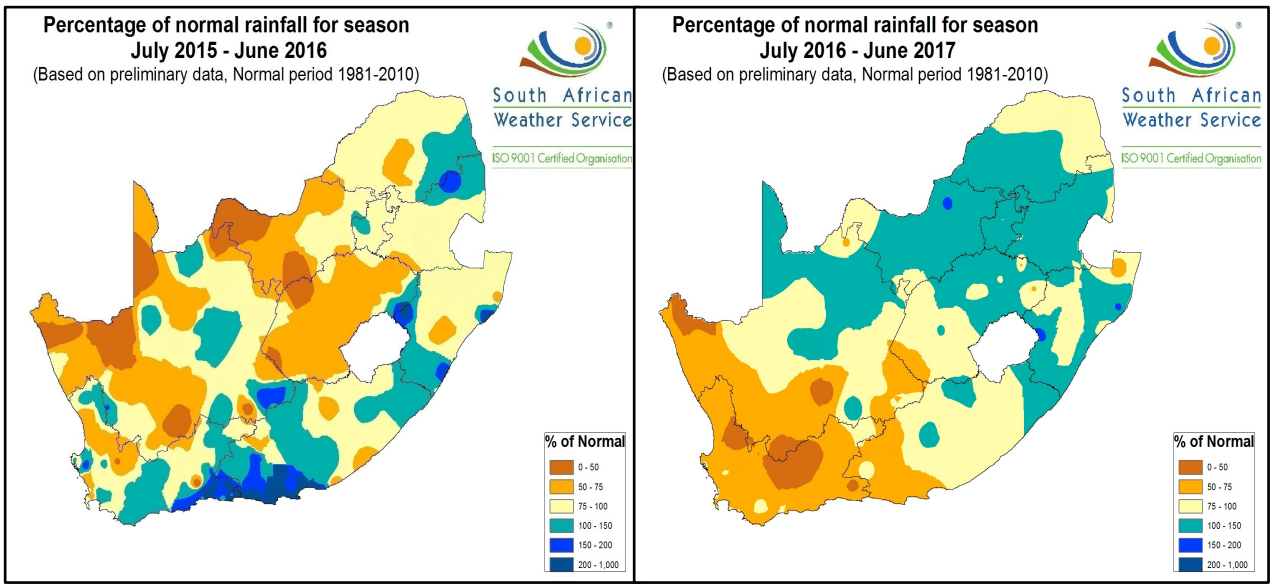

Figure 13: Percentage of normal rainfall for seasons July 2015–June 2016 and July 2016–June 2017 (SAWS, 2019).

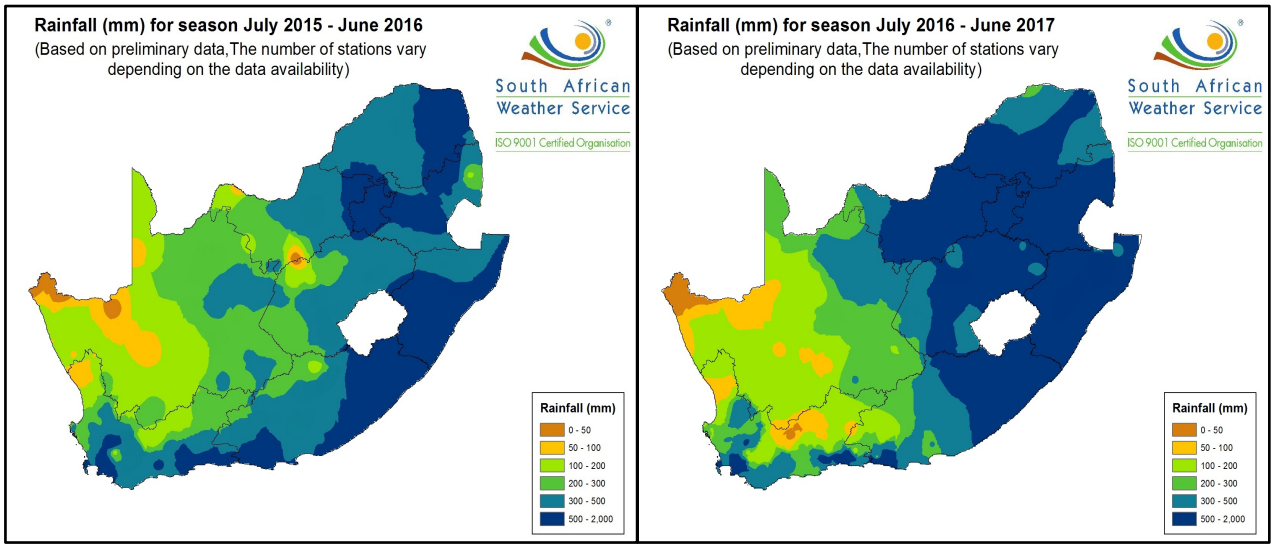

Figure 14: South African rainfall summaries (in mm) for seasons July 2015–June 2016, and July 2016–June 2017 (SAWS, 2019).

Table 16 shows that rainfall in the month prior to WNV case diagnosis was significant in the univariable analysis. The largest proportion of cases occurred in areas with previous month total rainfall of 11–291 mm (n=50, 93%) with the largest number of cases in a single quintile 134–291 mm rainfall, which also corresponded to the highest rainfall quintile (n=18, 33%). The largest proportion of cases occurred above previous month average maximum daily temperatures of 25°C (n=48, 89%) and the quintile with most cases was 28–30°C (n=13, 24%). The largest proportion of cases occurred at previous month average minimum daily temperatures above 12°C (n=49, 91%) and the quintile with most cases 17–21°C (n=16, 30%).

The biome with the largest proportion of WNV-positive cases (Figure 15) was the Grassland biome (n=33, 61%), with second largest number of cases identified in the Savanna biome (n=9, 17%) and third largest in the Nama-Karoo biome (n=7, 13%). Combined, these three biomes form the largest part of Eastern RSA and contained 49 of the cases (91%). For the univariate analysis most of the variables that related to climate or location proved to be significant (Table 16). WNV case location in biome was preferentially used in the final logistic regression, rather than provincial location, due to small numbers in certain provincial categories (Table 17).

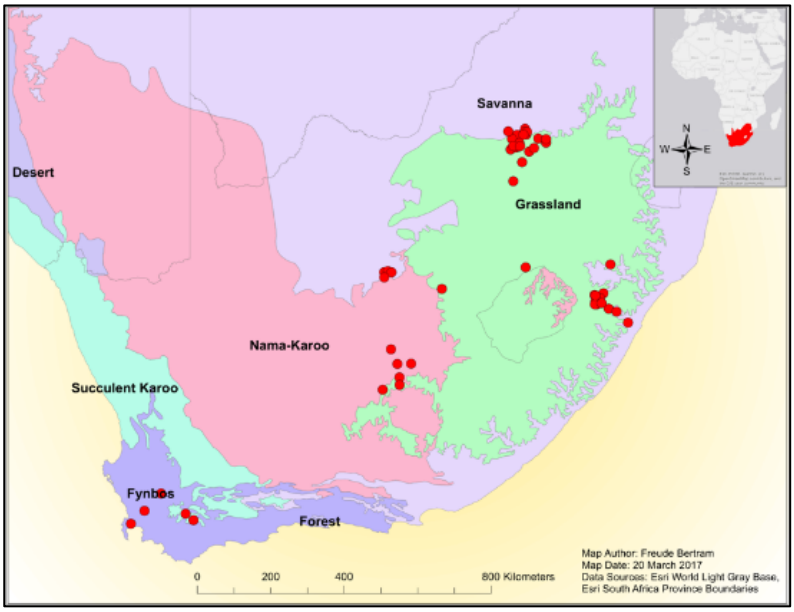

Figure 15: Total equine WNV-positive cases, by biome, during 2016–2017.

In summary, the climatic variables associated with the WNV-positive cases in horses during 2016–2017 were as follows: the largest proportion of cases in both years were diagnosed in early autumn, in the Grassland, Savanna and Eastern Nama-Karoo biomes, at altitudes of approximately 1000–1500 m above sea level; with rainfall above 134 mm, maximum daily temperatures above 25°C and minimum daily temperatures above 12°C, in the month before WNV case diagnosis. WNV cases were located in the more temperate, higher rainfall areas, especially those with summer rainfall in the Eastern parts of the country, and only a few in the Western Cape coastal areas.

5.4. RISK FACTOR ANALYSIS

Univariable associations of potential risk factors with WNV infection are shown in Table 16.

Table 16: Univariable risk factor analysis as measured against outcome of WNV-positive or negative in simple logistic regression model in NCSS. Some of the categories were subsequently reduced to fewer levels or completely dropped if not significant or if highly correlated to other more significant category, from final model.

Exposure Factor	Levels	Cases		Controls		Fisher's Exact P value (2 tailed)
		n	%	n	%	
Year: in which sample submitted for testing	2016	6	11%	24	20%	–
	2017	48	89%	96	80%	
Month: in which signs were initially displayed	January	2	4%	10	8%	0.019
	February	6	11%	16	13%	
	March	28	52%	30	25%	
	April	12	22%	19	16%	
	May	2	4%	13	11%	
	June	3	6%	4	3%	
	July	0	0%	4	3%	
	August	0	0%	4	3%	
	September	0	0%	4	3%	
	October	0	0%	5	4%	
	November	0	0%	6	5%	
	December	1	2%	5	4%	
Breed: classified according to hybrid vigour groups	Highly Purebred	35	65%	56	47%	0.009
	Intermediate	14	26%	29	24%	
	Mixed and Local	5	9%	35	29%	
Breed: classified according to groups as indicated	Hot blooded	33	61%	54	45%	0.244
	Cold Blooded	4	7%	9	8%	
	Warmblood	9	17%	19	16%	
	Light horses	4	7%	11	9%	
	South African/ Indigenous	2	4%	13	11%	
	Mixed breeds and ponies	2	4%	14	12%	
Sex	Male	23	43%	66	55%	0.143
	Female	31	57%	54	45%	
Age: in years, grouped according to quintiles	0.2 to 2	13	24%	23	19%	0.143
	2.5 to 5	17	31%	21	18%	

	6 to 8	10	19%	26	22%	
	9 to 12	9	17%	26	22%	
	13 to 27	5	9%	24	20%	
Province: in which the subject was located at time of sample submission	Gauteng	19	35%	54	45%	< 0.001
	KwaZulu-Natal	14	26%	18	15%	
	Northern Cape	11	20%	2	2%	
	Western Cape	5	9%	23	19%	
	Free State	3	6%	7	6%	
	North West	2	4%	5	4%	
	Eastern Cape	0	0%	3	3%	
	Limpopo	0	0%	3	3%	
	Mpumalanga	0	0%	5	4%	
Biome: in which subject was located at time of sample submission	Grassland	33	61%	63	53%	0.013
	Savanna	9	17%	33	28%	
	Nama Karoo	7	13%	2	2%	
	Fynbos	3	6%	13	11%	
	Succulent Karoo	2	4%	9	8%	
Altitude Quartiles: Elevation above sea level of subject location	16–1056 m	8	15%	36	30%	< 0.001
	1057–1292 m	12	22%	31	26%	
	1293–1466 m	25	46%	19	16%	
	1467–1784 m	9	17%	34	28%	
Average maximum temperature: during the month prior to sample submission at weather station closest to subject location.	17.4–24.4°C	6	11%	28	23%	0.386
	24.5–26.7°C	11	20%	24	20%	
	26.8–28.1°C	12	22%	25	21%	
	28.2–30.4°C	13	24%	21	18%	
	30.5–33.6°C	12	22%	22	18%	
Average minimum temperature: during the month prior to sample submission recorded at weather station closest to subject location	2.2–11.9°C	5	9%	28	23%	0.018
	12.0–14.7°C	9	17%	28	23%	
	14.8–15.6°C	15	28%	22	18%	
	15.7–17.0°C	9	17%	26	22%	
	17.1–21.4°C	16	30%	16	13%	
Total rainfall in mm: measured at weather station closest to subject location, during the month prior to sample submission	0–10.4 mm	4	7%	30	25%	0.008
	10.5–35.4 mm	10	19%	25	21%	
	35.5–74.0 mm	11	20%	25	21%	
	74.1–134.0 mm	11	20%	24	20%	
	134.1–290.8 mm	18	33%	16	13%	
Vaccinated against AHS: during 12 months prior to sample submission with registered OBP vaccine	Yes	48	89%	108	90%	0.794
	No	6	11%	12	10%	

Vaccinated against WNV: in 12 months prior to sample submission	Yes	1	2%	9	8%	0.176
	No	53	98%	111	93%	
Vaccinated against EIV: during the 12 months prior to sample submission	Yes	43	80%	88	73%	0.449
	No	11	20%	32	27%	
Stabled: The horse was stabled at night during the month prior to sample submission	Yes	29	54%	69	58%	0.741
	No	25	46%	51	43%	
Stressed: The horse was **generally, highly stressed** during 4 to 6 weeks before sample submission	Yes	18	33%	37	31%	0.860
	No	36	67%	83	69%	
Stress: The horse **travelled long distance** during 4 to 6 weeks prior to sample submission	Yes	11	20%	19	16%	0.517
	No	43	80%	101	84%	
Stress: The horse received **AHS-vaccination** during 4 to 6 weeks prior to sample submission	Yes	0	0%	7	6%	0.101
	No	54	100%	113	94%	

90% of the WNV cases for this study occurred in 2017 (Table 16). Both the univariable association of the individual months and the final model with grouped months were significant. Months were grouped for the multivariable analysis due to few or no cases in some of the months.

Breeds were divided into generally accepted categories (Figure 7,Table 11), but did not show a significant association with WNV infection in the univariable analysis (Table 16). By categorizing the breed variable into only 3 levels according to perceived hybrid vigour, statistical power was greatly increased, and was significant in both the univariable analysis and the final logistic regression model. Highly purebred breeds category included purebred Arabians, Thoroughbreds and American Saddlers, and this category contained the largest proportion of cases (n=35, 65%) compared to the mixed and local breeds category which included any mixed or cross bred horses as well as local breeds such as Boerperd and Nooitgedachter horses, which contained

the smallest proportion of cases (n=5, 9%). Accurate numbers are not presently available but it is estimated that a large proportion of the South African equine population are formed by local and mixed breed horses, likely up to 50%, particularly in the rural areas.

There was no significant association between WNV infection and sex despite 57% (n=31) of WNV cases being female (p=0.14), but odds of WNV infection significantly decreased with increasing age, with the largest proportion of cases diagnosed in horses less than 5 years old (30/54, 56%). Province, which was significant in the univariable analysis, was substituted for location in biome, which was significant in both the univariable and the logistic regression model, due to higher numbers in the different categories (Table 16,17).

Owners who participated in the study were also questioned on their horse's vaccination status. Only 1/54 cases (2%) were reported to have been vaccinated against WNV in the 12 months before sample submission in 2016–2017, in comparison to 9/120 controls (8%). Owners were also asked in the questionnaire whether they, or owners at their yard, had subsequently vaccinated their horses against WNV, in 2017–2018. Because some of the owners had multiple submissions to the dataset (such as some of the Thoroughbred studs), to account for possible clustering effect, their responses were grouped into herds based on location rather than individual horses. Of the 149 herds interviewed in total (cases and controls), only 9 herds had vaccinated their horses before sample submission in 2016–2017 (6%). Of the same 149 herds, 30 had subsequently vaccinated their horses in 2017–2018 (20%).

Stabling was not associated with WNV infection in the univariable analysis (Table 16). 54% of horses that were diagnosed with WNV infection were stabled at night compared to 58% of the controls, which is approximately equal. Being vaccinated against AHS in the previous 12 months was not associated either, as approximately 90% of both cases and controls were vaccinated; however, 6% of the control horses (n=7) were vaccinated against AHS in the preceding 4–6 weeks before sample submission and this was regarded as a possible stress factor. This variable was not

included in the logistic regression because there were no WNV-positive cases present which had been vaccinated just prior to diagnosis.

The other two stress-related risk factors (generally stressed and long-distance travelling) also did not prove to be associated with WNV when compared to the negative controls. It is still notable that a fairly large proportion of the total subjects had, according to the owners, indeed experienced high levels of stress (cases n=18, 33% vs. controls n=37, 31%), specifically long-distance travelling (cases n=11, 20% vs. controls n=19, 16%) in the 4 to 6 weeks before the sample submission.

Table 17: Final logistic regression model of factors associated with WNV-infection in South African horses detected by ZARV program at the CVZ, 2016–2017.

Variable	*Level*	*Odds Ratio*	*95% CI*	*P-value*
Month	January–February	5.44	0.59, 49.91	0.134
	March–April	17.99	2.17, 149.51	0.007
	May–June	4.15	0.39, 44.85	0.241
	July–December	1*	–	–
Altitude Quartiles	16–1056m	1*	–	–
	1057–1292 m	1.16	0.35, 3.88	0.807
	1293–1466 m	5.97	1.87, 19.05	0.003
	1467–1784 m	1.22	0.34, 4.31	0.764
WNV vaccinated	Yes vs. no	0.10	0.01, 0.97	0.047
Age in years	Continuous	0.92	0.85, 1.00	0.041
Breed hybrid vigour	Highly Purebred	2.99	0.92, 9.69	0.068
	Intermediate	4.86	1.30, 18.22	0.019
	Mixed and Local	1*	–	–
Equine Influenza virus vaccinated	Yes vs. no	2.07	0.76, 5.64	0.153

* Two-t* Reference level

The final multiple logistic regression model (Table 17) included the following variables (significant at $p<0.05$) as significantly associated with WNV infection:

- Month of case diagnosis, specifically March–April vs. July–December (OR 17.99, 95% CI 2.16,149.51)
- Altitude of location quartiles, specifically 1293–1466 m vs. 16–1056 m (OR 5.97, 95% CI 1.87, 19.05)

- Breed according to hybrid vigour, particularly the intermediate breed category vs. mixed and local breeds (OR 4.86, 95% CI 1.30, 18.22)

WNV vaccination (OR 0.10, 95% CI 0.01, 0.97) and age in years (OR 0.92, 95% CI 0.85, 1) variables were shown to be protective against risk of WNV infection.

EIV-vaccination, although not significant (p=0.145), was retained in the final logistic regression model as a confounder, as its removal resulted in substantial changes to the coefficients of the WNV-vaccination and age variables. The Hosmer-Lemeshow test indicated adequate fit of the final model (p=0.094). This indicates that there is insufficient evidence to conclude that the number of cases predicted by the model in each decile of risk differs from the observed number.

CHAPTER 6: DISCUSSION

6.1. DISCUSSION

The previous ZARV study for WNV in horses in South Africa 2008–2015 (Venter et al., 2017) described a total of 79 cases in horses over a period of 8 years, thus an average of 10 diagnosed WNV cases per year (minimum of 3 in 2012 and maximum of 18 cases in 2010). The 6 WNV cases in 2016 in the current study is on par with that trend; however, the 48 cases diagnosed in 2017 indicates a marked increase in case numbers. This could possibly be due, in part, to increased awareness of WNV in RSA and sample submissions from suspected cases by owners and vets. Increased awareness was created over the past few years by pharmaceutical companies' product advertising, information disseminated via social media, continuing professional development (CPD) events such as veterinary congresses, scientific publications on the research results, and owner targeted talks. Some of these events were facilitated by Prof. Marietjie Venter and the ZARV program, who gave feedback on WNV-positive cases. The interviews conducted with owners, managers and veterinarians, by the author during the course of this study, also contributed to raising awareness. Very likely though, the sudden increase in WNV cases in RSA in 2017 was mostly due to the environmental factors at the time, as will be discussed.

Consistent with international findings, due to the fleeting viraemia (OIE, 2018); most of the WNV cases were diagnosed by IgM ELISA. In only a small proportion of cases was agent identification successfully performed with rtRT-PCR, with even fewer cases diagnosed with both rtRT-PCR and IgM ELISA. This latter finding was fairly similar to 2008–2015, in which two thirds of cases were diagnosed with IgM ELISA and only 5% of cases with both tests. A much higher proportion of cases was, however, diagnosed by means of rtRT-PCR in 2008–2015 than in 2016–2017. Possible explanations could be that fewer post-mortem samples were submitted in the latter period, or EDTA samples were taken later in the course of the disease, missing the viraemic period, or that samples were suboptimally stored or handled during shipment. It is unlikely that there might have been some change in the test itself, as the same tests had been

used since 2008 and all tests are always run with control positive samples. Genetic variation in current circulating strains reducing the sensitivity of the tests was also unlikely as the test was designed to detect all flaviviruses and variation in the probe region for WNV would therefore be detected during gel electrophoresis. Recent increased exposure rates resulting in higher persistent IgM levels in convalescent cases, which may then be incorrectly diagnosed as acute cases, were also unlikely, as the WNV-positive cases which were used in this study fit the typical clinical description of acute WNV-positive cases.

Despite SINV and WNV having similar ecology in South Africa (Jupp, 2005) and SINV being responsible for 20% of co-infections in WNV positive horses in 2008–2015, there were no equine SINV co-infections detected in 2016–2017. Total co-infection rate for 2016–2017 approached the 18% level previously reported (Venter et al., 2017). An increased co-infection rate with both MIDV and EEV was observed during 2016–2017 when compared to previous years. Amongst the randomly selected WNV negative controls most tested negative for all the virus PCRs, but a small proportion were MIDV and EEV-positive. These findings may be related to environmental factors favouring MIDV and EEV circulation. SINV also has a short viraemia and the CVZ testing panel does not currently include an IgM test for SINV, so SINV infections may be underdiagnosed. Overall, reasons for these specific co-infections are not clear but it indicates the need for further research concerning the epidemiology of WNV and co-infecting viruses, especially regarding the ecology of the vectors in RSA.

Total death and recovery proportions were not significantly different for both cases and controls. Two thirds of the WNV-positive case deaths were due to elective euthanasia, presumably due to either a grave prognosis or economic restraints affecting treatment, or a lack of diagnosis to indicate the possibility of recovery. The extremely short median survival time until elective euthanasia is likely attributable to the rapidity of onset of severe clinical signs. Differences in the statistical significance for fatalities amongst the main syndromes in the cases and controls, were probably due to sample size variation. Case fatality proportion of 39% in WNV-positive cases in 2016–2017 was fairly similar to the 34% reported in South Africa in 2008–2015 (Venter et al., 2017) both of which are also consistent with the international case fatality proportions of 33–40% (Ward et al., 2006; WHO, 2018; AAEP, 2019).

The most important clinical signs displayed during 2016–2017 were very similar to those described for equine WNV-positive patients in general, which consisted mainly of various neurological signs with or without fever (Castillo-Olivares and Wood, 2004; Weese, 2017; OIE, 2018; WHO, 2018) and, as with the previous ZARV study (Venter et al., 2017), neurologic signs were present in most WNV-positive cases and significantly associated with WNV infection. The main syndromes of the previous ZARV study in horses agreed fairly well with the findings of this study. Almost all of the WNV cases in 2016–2017 displayed some form of neurological signs, of which less than half had neurological signs accompanied by fever. Fever, when present, was presumably due to a general inflammatory reaction to the WNV (Samuel and Diamond, 2006) and did not show a significant association with WNV when compared to the controls. Increased proportions of WNV cases with fever compared with cases during 2008–2015 (Venter et al., 2017) may be due to the co-infecting viruses or simply increased awareness resulting in greater or earlier sample submissions, especially of less severe clinical cases. Viruses such as MIDV, SINV and EEV may be more often associated with pyrexia than WNV; SINV and EEV also seem to be less often associated with neurological disease than WNV (Van Niekerk et al., 2015; Tirosh-Levy Sharon et al., 2017; Venter et al., 2017). Thus, it may at least partly explain why a greater proportion of controls than WNV cases had only fever as a main syndrome than in 2008–2015.

In both WNV cases and controls, subjects with neurological signs alone had the highest odds of fatality, which is likely due to severity of pathology in the CNS, resulting in a grave prognosis and death. In both groups, subjects with only fever had the fewest fatalities. As in human patients (Petersen et al., 2013) uncomplicated equine WNV cases usually recovered fully. Surprisingly, three quarters of WNV cases which had fever either with or without neurological signs, recovered. The association between WNV cases with fever and recovery was marginally significant, which may be due to small numbers. In general, due to the transient viraemia of WNV infection (Bunning et al., 2002), horses displaying only mild fever without neurological symptoms, may have been missed by owners who do not regularly monitor their horses' temperature as part of their daily routine, resulting in under-recognised cases. Thus, the number of WNV-positive cases without neurological signs, may in reality be underdiagnosed.

Studies have shown that numerous attributes of the innate and adaptive immunity are required to successfully counteract the viraemia and mitigate pathogenesis in the CNS. Particularly T-cell mediated immunity is crucial in regulating neuroinvasive WNV, as well as factors such as elevated viraemia and deficiencies in the complement system, interferon alpha/beta and gamma, WNV-specific IgM and IgG producing B-cells, haematologic malignancies and impaired T-cell function may contribute to earlier neuroinvasion and fatality (Samuel and Diamond, 2006). Thus, the presence of pyrexia may be an indication of the horse's ability to mount an effective general immune response to the initial viraemia, which may be crucial and indicative of the ability of the immune system to both prevent and control neuroinvasion of WNV. Therefore, presence of pyrexia in WNV cases could potentially serve as a prognostic indicator for recovery.

All neurological clinical signs such as paresis, paralysis, ataxia, tremors or muscle fasciculations were significantly associated with WNV infection when compared to the negative controls. This is consistent with both local and international findings which correlate the neurological signs in WNV to the pathology in the brain and spinal cord (Castillo-Olivares and Wood, 2004; Williams et al., 2014). It was interesting that the fatal WNV cases had higher proportions of hindleg and total paralysis than foreleg paralysis and tremors or fasciculations, relating to the degree and location of spinal cord pathology. In one of the vaccine studies, only one vaccinated horse developed a fever, and one displayed mild muscle fasciculations at a single observation but no fatalities were seen (Siger et al., 2006). Thus, an effective initial humoral immune response by the horse clearly limits the potential degree of CNS pathology and the presence of certain clinical signs may serve as an indicator of severity or presence of pathology and potentially assist as prognostic indicators.

Laminitic stance / sensitivity in feet was an interesting clinical sign noted in a small but statistically significant number of the cases and only one of the controls. It is not generally described in literature as a sign of equine WNV infection even though one study mentioned 'reluctance to move' as possible clinical sign (Siger et al., 2006). Some human WNV patients may experience severe pain in their limbs just before or during the onset of weakness, which may be causally associated with demyelinating

neuropathies, motor axonopathy, axonal polyneuropathy, involvement of ventral spinal roots, myasthenia gravis and brachial plexopathies (Petersen et al., 2013), suggesting a possible neuropathic cause for the perceived pedal sensitivity in some of the equine patients. All five of these horses also displayed neurological signs such as ataxia, of which one was reported to display only weakness and two became paralysed. Both of the latter were euthanised, one shortly after onset of signs due to poor prognosis (recumbency and seizures) and the other a year after recovery due to retention of neurological signs, indicating residual damage to the CNS as a result of the WNV.

Retained clinical signs were seen in a small, marginally significant number of cases. In the USA it is estimated that up to 40% of recovered WNV horses may show some form of persistent neurological deficit, either gait or behavioural abnormality, post recovery (Ward et al., 2006; AAEP, 2019). It is possible that there would have been a higher proportion of retained clinical signs in South African horses if fewer economic constraints and more awareness of the disease would have allowed a longer treatment period for WNV horses due to fewer horses being euthanised. Owners who participated in the study often expressed their regret at WNV only being diagnosed post-mortem and would have preferred knowing the diagnosis before opting for elective euthanasia.

In human WNV infection it is described that clinical signs are expected to be more apparent and severe in very young and very old patients, with neurological signs especially in the old (Taylor et al., 1956; Samuel and Diamond, 2006; Petersen et al., 2013), and the AAEP expects a similar trend in horses (Weese, 2017). Other literature, however, reports that, in contrast to human disease, older horses do not seem to be preferentially affected with neurological and more severe disease (Castillo-Olivares and Wood, 2004; Venter et al., 2017), similar to what was found in the results of this study. About two thirds of South African WNV cases in 2016–2017 occurred in horses less than five years old while very few cases were seen in horses older than 13 years. Compared to the control subjects, the WNV cases generally occurred in much younger horses, similar to the findings in the previous ZARV study in 2005–2018. In fact, a significant, consistent decrease was seen in absolute numbers of WNV cases with increasing age, possibly due to an increased immunological resistance from repeated long term, low grade exposure to WNV. This theory is also supported by the

serological survey that was done in RSA which reported 11% WNV seroconversion in Thoroughbred yearlings and 75% in dams, of those tested (Guthrie et al., 2003). Despite this a consistently high percentage of neuroinvasion was seen in all age groups, which may be explained by the different mechanisms involved in viral dissemination and pathogenesis. While the innate and adaptive immune systems, mainly T-cells, B-cells, interferon and complement, are involved in controlling WNV infection, mechanisms by which the virus crossed the blood-brain barrier are largely unknown, but tumour necrosis factor alpha is implicated (Samuel and Diamond, 2006).

The main breeds represented with WNV infection were Thoroughbred horses and Warmblood horses, followed by Arabian horses. This contrasted starkly to the various cross or mixed breeds and the South African breeds Boerperd and Nooitgedachter which, in combination here were associated with a significantly lower odds of WNV infection in the multivariable model when compared to purebred and intermediate hybrid vigour groups. Accurate updated data is not available but an arguably large proportion of the general South African equine population, possibly around 50%, especially in the rural areas, should conceivably be comprised of non-purebred indigenous/local breeds or mixed breed horses. It stands to reason that these horses may show greater immunological resistance to endemic diseases, especially due to hybrid vigour, or some form of genetic adaptation such as seen in humans with certain gene mutations (Petersen and Marfin, 2002) or asymptomatic WNV infection in birds from endemic countries such as RSA (Jupp, 2001). The fact that a third of the randomly selected control cases fell in this high hybrid vigour category, contests the idea that the cross or mixed breed horses are underrepresented as WNV cases due to being of less economic value, and that owners would therefore be less likely to obtain a formal diagnosis from a veterinarian.

Regarding breed distribution, it was interesting to note that by far, Thoroughbred horses were most affected, comprising half of the clinical WNV cases in 2016–2017. This breed was previously postulated to not display overt clinical signs of WNV (Guthrie et al., 2003). It may have been possible that WNV infected cases were underreported in RSA in previous years (prior to 2007), due to lack of awareness of its pathological potential. The recent increase in WNV-positive cases in Thoroughbred horses may be attributed to the horses' high economic value causing increased

likelihood of reporting disease. Two thirds of the WNV cases in Thoroughbred horses from 2016–2017 were diagnosed in horses that were either at stud (most of which were young stock) or that were actively racing at the time and were likely of high economic value. Personal communication with some of the Thoroughbred stud managers in the Western Cape confirmed that there were, in previous years, several undiagnosed but distinctly neurological disease cases seen, particularly in the yearlings at these studs, specifically those located close to large bodies of water. These could potentially have been due to WNV infection. After initiating vaccination regimes, especially in their young stock, these studs had seen a decline in these unexplained cases.

Temporal variations of WNV cases were similar to those described in previous studies; they were seen mainly in late summer and early autumn (Jupp et al., 1986; Hayes et al., 2005; Venter et al., 2017) with cases occurring as early as December and continuing up to June. The slightly extended period of case distribution may have been attributed to the heavy rains and warmer temperatures of 2017, and due to the ecology of the vector which requires warm temperatures and moisture to breed.

Spatial distribution of the 54 WNV-positive cases in horses as detected by the ZARV program, CVZ, in 2016–2017, followed a similar pattern to those cases previously detected in earlier years by the CVZ in veterinarians as well as horses, livestock and wildlife species (Venter et al., 2017). As discussed in the introduction, the largest proportion of cases was detected on the Highveld, mainly in the warm to temperate zones in the Eastern parts of RSA. It is notable that in 2016–2017 there were no WNV positive equine cases diagnosed from the Eastern Cape. This may have been due to substantial parts of the Eastern Cape receiving normal or below normal rainfall during June 2016 to July 2017. As with the previous study, no cases were seen in Limpopo and Mpumalanga. This may be due to less favourable conditions for vector breeding, greater densities of donkeys than horses in the north-eastern part of the country and possibly less valuable horses as well as fewer equine veterinarians located in those provinces.

Due to the presence of genetically varying strains of WNV in South Africa (Jupp, 2001) differences in WNV case numbers may perhaps be attributed to the presence of less

neurovirulent WNV strains in certain areas (Beasley et al., 2001; Samuel and Diamond, 2006). Climatic circumstances may also not have been favourable for the development of large populations of the *Culex* spp. mosquitoes, increasing the likelihood of disease in vulnerable/immunocompromised individuals. In rodents, increased viraemia correlated with earlier neuroinvasion and increased WNV burdens in the CNS (Samuel and Diamond, 2006). Cape Town and surrounding areas, for instance, fall within the Mediterranean zone. With predominantly winter rainfall and warm, dry summers in the western parts of the country, overwhelming immunological challenge would not be as prevalent as would be expected in the more humid and hotter, higher summer rainfall areas such as Gauteng and KwaZulu-Natal. This is supported by the data from the 2017 Thoroughbred stud farm survey, which indicates that most of the Thoroughbred stud farms are located in the Western Cape. However, most of the WNV cases that were diagnosed in Thoroughbreds from stud farms during 2016–2017, were in KwaZulu-Natal and the Northern Cape, in which a much smaller number of the Thoroughbred stud horses resides. If one would reason that WNV would most often be diagnosed where the highest density of valuable horses is, without taking climatic factors into account, one would expect that most of the Thoroughbred WNV cases would be located in the Western Cape. This was not the case despite the Western Cape veterinarians submitting the second highest number of samples to the CVZ during 2016–2017. Only a very small proportion of the samples tested positive for WNV. Thus, it appears that both the climatic factors and vector ecology play a large role, and that the small number of WNV infections in the Western Cape should not be attributed to veterinarians, owners or studs under-recognising the disease or under-submitting samples.

Investigation regarding the South African environmental conditions during 2016–2017 was aimed at describing the basic climatic circumstances favourable for development of sufficiently large populations of *Culex* spp. as the main mosquito vectors involved in the spread of WNV. Increased environmental temperatures would also favour replication of the virus in the poikilothermic mosquito vectors, as well as decrease the subsequent length of the extrinsic incubation period and increase the efficiency of transmission of virus to susceptible hosts (DeFelice et al., 2018). Some mosquito species may readily breed in any standing water and *Cx. univittatus* and *Cx. neavei* prefer to deposit their eggs in open water and preferentially in temporary to semi-

permanent rain-flooded grasslands as well as the margins of either temporary or permanent marshlands, especially in the moister parts of the Highveld (Jupp, 2005). This area is also consistent with the location of the largest number of WNV-positive cases in 2016–2017. The increased WNV case numbers in 2017 were probably due mostly to climatic factors such as increased rainfall, promoting extensive breeding of the WNV vectors, with viral replication in the vectors. This included the drought and unusually high temperatures since 2015, which were followed by high rainfall and periodic flooding in late 2016 and early 2017 in large parts of RSA. The largest historic WNV epidemic in humans in 1973–1974 in the Karoo, and the more localized epizootic in 1983–1984 in the Gauteng area, were both thought to have been as a result of the abnormally high summer rainfall, flooding and elevated temperatures (Jupp, 2001).

In summary, the climatic data showed a significantly higher odds of WNV infection in horses in 2016–2017 in the final regression model during March to April and at altitudes of 1300–1500 m above sea level. In the univariable analysis significant variables were provincial and biome location with most cases in the Gauteng, KwaZulu-Natal and Northern Cape and in the Grassland biome, rainfall above 130 mm and average minimum daily temperatures above 15°C in the month before WNV case diagnosis. This is consistent with the ideal breeding habits for the vectors (Jupp, 2001, 2005).

Specific risk factors in terms of individual or management factors that pose a risk for exposure to WNV have not yet been identified and there seems to be a complex interaction of variables which eventually result in disease. An apparent protective factor was when horses were housed indoors at night (Long et al., 2002). However, in the results of the current study, stabling did not seem to be a significant risk factor for WNV as almost equal proportions of horses which were diagnosed with WNV were stabled at night when compared to the controls.

Despite being generally stressed and long-distance travelling not being significantly associated with WNV when compared to the controls, it is still important to note that a large proportion of the subjects (both cases and controls) had, according to the owners, experienced high levels of stress, specifically with long-distance travelling in

the 4–6 weeks before the sample submission. Research in rodents showed that increased stress levels promoted both immunosuppression, increased WNV replication *in vivo*, and increased neuroinvasion causing encephalitis and death (John et al., 2000; Ben-Nathan, 2013). Bearing in mind that all horses in this study, including controls, were diseased, this suggests that certain stressors may play a role in equine disease development in general. However, asymptomatic control subjects, in comparison to WNV-positive cases, would need to be used to further explore this assumption.

It is suspected that being vaccinated against EIV may have been acting as a proxy for other variables, possibly related to travelling, which was not covered in the initial questionnaire and thus the variable was retained in the final model as a confounder. Typically, competitive horses must be vaccinated against EIV, as societies such as the Federation Equestre Internationale (FEI) and the National Horse Racing Authority (NHRA) list it as a prerequisite for participation in events to prevent the spread of disease. Such horses would typically be more likely to travel than horses which are not vaccinated against EIV. Thus, it is possible that either the stress of competing, travelling, or exposure of an immunologically naïve horse to WNV or to different regional strains of WNV while in a different location, or some other unknown factor, or a combination of these factors, may predispose a horse to contracting WNV infection.

Only one of the 54 WNV positive cases was reported to have been vaccinated against WNV during the preceding year. This was a 3-year-old Warmblood that displayed neurological signs and was subsequently electively euthanised. Details on which vaccine was used and how long before the sample submission were, however, not ascertained at the time. It is advised that horses have a WNV booster 3 to 4 weeks after the initial vaccination and thereafter annually, to achieve best protection and be considered adequately vaccinated, and that a single vaccination will not provide sufficient protective antibodies (Siger et al., 2004; Siger et al., 2006; Seino et al., 2007; AAEP, 2019). This was also seen during the 2001 outbreaks in USA during which more than 100 of the over 700 encephalitis cases seen in horses, were reported to have received at least one vaccination but only three horses had completed a vaccination series in a time frame that could be expected to result in sufficient

immunity. The outcomes in these three vaccinated WNV cases were not specified (Long et al., 2002).

Of those interviewed by the author, there was a much higher proportion of WNV vaccinated herds after sample submission in 2017–2018 than before 2016–2017. It has to be noted though, that this number was only a reflection of the WNV cases and randomly selected WNV negative controls from the ZARV database, the selection of which was based on case descriptions specific for WNV. The owner would also have been informed at the time of diagnosis, by the attending veterinarian, of the various neurological viruses that would be tested for at the CVZ (of which only WNV registered vaccines are currently available) and that this subset was not necessarily an accurate reflection of the increased vaccination proportion countrywide. Personal communication with one of the pharmaceutical companies selling a WNV vaccine in RSA, has confirmed that there have indeed lately been increased sales of WNV vaccine, especially in 2018–2019. Unfortunately, privacy protocols precluded any numbers regarding product sales and names being released publicly.

Awareness campaigns in recent years, as already described, had created public recognition not only of the potential of WNV for severe neurological disease and death in horses and humans in RSA, but also greater insight regarding the prophylactic control measure of vaccination. It is strongly suspected that the increase in WNV vaccination had an effect on the sudden decrease in WNV-positive cases diagnosed at ZARV in 2018, during which only two WNV cases in horses were confirmed, while other neurological virus case numbers did not similarly decrease.

6.2. FURTHER ISSUES / QUESTIONS RAISED

Continued research efforts by the CVZ aims to describe the incidence of clinical disease caused by WNV, its epidemiology and its differentiation from other causes of febrile and neurological disease, to determine its importance in South Africa regarding international trade organizations and equine exportation to WNV non-endemic countries.

Future endeavours anticipated from the results of this study may include the implementation of an active surveillance programme aimed specifically at determining countrywide prevalence and incidence of WNV infection, continuing to create increased awareness of WNV amongst government officials, private veterinarians and veterinary training institutes in order to improve counts of accurate diagnosis countrywide; and increasing awareness in the general horse industry as to the true extent, dynamics and potential implications of WNV in South Africa. Further ongoing research is also needed in the epidemiology of WNV and co-infecting viruses, particularly the ecology of the vectors in RSA, to better explore these patterns.

Further research may be directed to investigating the relationship between immunity in horses and the risk of developing neuroinvasive WNV infection and mortality, as well as any association of EIV vaccination with increased risk of WNV due to some unknown factor associated with horses which are travelling and competing. Studies have shown that passive transfer of IgG protects against flavivirus infection in mice (Samuel and Diamond, 2006), and thus the use of hyperimmune plasma in treatment of horses with WNV is worth investigation. One study claimed a tendency in favour of WNV exposure occurring more in pleasure horses than in racing or breeding horses (Long et al., 2002) and thus both the discipline in which WNV cases participate, and economic value, may be considered in the likelihood of disease occurrence vs. prevalence for future studies.

A question that remains, due to limited information being available on long term immunity, is the duration of protective immunity against WNV after natural infection (Weese, 2017). Owners would often like to know whether it is necessary to vaccinate a horse which has recovered from WNV. Thus, further studies may be aimed at clinical trials that assess pre-infection and long term post-infection immunoglobulin levels, to determine the extent of immunity against WNV, taking into account the influence of possible repeated field exposure.

Since up to 40% of horses in North America retain some form of residual effect, such as gait and behavioural abnormalities, more than 6 months after recovery (AAEP, 2019), the possibility of “chronic” WNV infection in horses and viral persistence in equine CNS post recovery, should be considered. This could pose a risk of zoonotic

infection by, for instance, unprotected post-mortem technique, at a later stage. Viral persistence was noted in the brains and kidneys of WNV infected hamsters, as well as in the brains of immunologically deficient mice; however, one immunocompromised human patient remained viraemic for more than 60 days. WNV was cleared after two weeks from all tissues in wild-type mice which survived (Samuel and Diamond, 2006). One clinical WNV-vaccine trial found that WNV could neither be isolated from the brainstem nor from CSF samples of both control and WNV-vaccinated horses at the end of the study (21 days after the intrathecal WNV challenge) (Siger et al., 2006). Human patients may be infected with WNV by organ transplant from a previously WNV infected donor, indicating viral persistence or sequestration in selected organs post recovery (Petersen et al., 2013; OIE, 2018; WHO, 2018).

6.3. BENEFITS ARISING FROM THE PROJECT

Benefits which might arise from this project:

- Humans and horses are both dead-end hosts to WNV, and thus horses may act as sentinels to alert health authorities to WNV as a differential diagnosis during the presence of unexplained or undiagnosed human cases of typical viral encephalitis and myelitis, in the same regions as the equine cases.
- Increased awareness of the epidemiology and potential risk factors of WNV in South Africa not only furthers scientific knowledge but also presents an opportunity to potentially minimize further equine losses due to WNV by increased public awareness of WNV leading to increased vaccination, especially in high risk areas.
- International stakeholders will be informed of ongoing research and attempts to ameliorate the annual case load, and possibly alleviate current concerns that the OIE and international trade partners might have regarding the effect of WNV on the exportation of South African equines. WNV is an OIE notifiable disease, although DAFF does not recognise it as such in RSA.
- Continued surveillance and research activities will increase the level of confidence that the international community places in the South African

Veterinary services and will necessarily add to the improvement of South Africa's international sanitary, biosafety and trade status.

6.4. LIMITATIONS OF THE STUDY

Due to the nature of the study population, which was sourced retrospectively from a passive surveillance programme, inferences made from this study are not necessarily applicable to the general equine population in South Africa. The significance of the statistical tests strongly relates to the subpopulation that was used for the control cases, which were specifically chosen from the ZARV database as horses from 2016 and 2017 which had either fever, fever and neurological signs and/or death; the horses had to test negative for WNV but may have tested positive for other viruses. Thus the controls were not selected from a healthy subpopulation of horses, which should be kept in mind when interpreting the results.

There are several other reasons why the number of WNV cases in this dataset may have been an under-representation of the true number of WNV infections in horses in South Africa during 2016–2017:

- As passive surveillance, the ZARV program is dependent on private, academic and/or government veterinarians to submit samples from suspect cases, and thus relies heavily on practitioners' ability to clinically diagnose potential viral neurological cases, their knowledge of the available diagnostic services offered, and interest in reaching a specific diagnosis.
- Due to a previous, widely held assumption that WNV did not play a significant role in causing disease in animals, especially in horses, in South Africa, practitioners may be either ignorant of the recent history of WNV disease incidence or of the ability of the virus to cause significant clinical signs and death, hence resulting in a lower sample submission rate. This may be especially true of foreign practitioners who are not familiar with the tropical diseases found in South Africa.

- Economic constraints and the perceived value of a horse may play a role in the likelihood that an owner would consult a veterinarian. Therefore it is likely that more valuable horses may inadvertently have been overrepresented in the dataset. However, because the CVZ testing is done at no cost to the owner/veterinarian, this should not have influenced the veterinarian's decision to have the sample tested.
- A sample may have tested negative when in fact the horse had WNV, because there is a short period post-viraemia during which a serum sample may test negative for WNV by RT-PCR, but not yet positive on IgM ELISA.
- Sample condition is not always ideal on arrival at the laboratory, and haemolysed or necrotic samples in which the cold chain had not been maintained, may result in false negative diagnoses. WNV is a fragile virus and samples need to be transported rapidly on ice to the CVZ where they are stored at -80°C.
- Due to the wide range and level of severity of signs caused, WNV cases may be underdiagnosed in general, as it may be unrecognised and attributed to a transient illness of other or unknown cause.

CHAPTER 7: CONCLUSION

WNV positively diagnosed cases in horses in RSA in 2017 showed a remarkable increase from the average case numbers per year diagnosed in passive surveillance programs by the CVZ in 2008–2016. Increased co-infection rates were also seen with MIDV and EEV. Increased WNV case numbers were largely attributed to environmental factors but also partly to increased awareness of WNV. The largest proportion of WNV cases in equines in RSA during 2016–2017, was significantly associated with the temperate to warm, eastern inland plateau, at intermediate elevation above sea level, during March–April. A period of drought was followed by increased summer rainfall, flooding and high temperatures in this period, creating ideal breeding habits for the mosquito vectors, viral replication in vectors and thus heightened viral load inoculated into the hosts creating potentially overwhelming immunological challenge. Fewer equine WNV cases were seen in Western Cape and surrounding areas, most likely due to the Mediterranean climate and winter rainfall patterns being less favourable to vector development and not due to a lack of valuable horses or to underreporting of cases. No cases were reported in Eastern Cape, which experienced normal to below normal rainfall. Further research is needed in the epidemiology of WNV and co-infecting viruses, particularly the ecology of the vectors in RSA, to better explore these patterns.

WNV-associated case fatality rate of 39% and neuroinvasive disease proportions from 2016–2017 were consistent with those described in horses for 2008–2015 CVZ study as well as with international estimates. Approximately 90% of the equine WNV cases displayed neurological signs which were significantly associated with WNV. This commonly manifested as ataxia and partial or complete paralysis, mostly originating in the hindlimbs. Tremors or muscle fasciculations and laminitic stance (apparent sensitivity in feet) were also significantly associated with WNV compared to the controls. Approximately half of the WNV cases were pyrexic. Pyrexia in the cases was marginally significantly associated with recovery, whether the horse had neurological signs or not, while cases that exhibited only neurological signs without fever were more

likely to die. Thus, certain clinical signs such as fever may potentially be used as prognostic indicators.

A complex interaction of immune-related variables contributed to disease development: vaccination against WNV was significantly protective, and the risk of developing clinical WNV significantly decreased with increasing age, likely due to increased immunity from repeated long term, low grade field exposure. Half of the horses diagnosed with WNV in 2016–2017 were Thoroughbred horses; mixed, cross and local breeds were the least likely to develop WNV, presumably due to hybrid vigour. EIV-vaccination acted as a confounder, and proxy for some other, unmeasured risk factor in the multiple logistic regression model, and suggested that horses which travel long distances and/or compete may be at greater risk of WNV.

It is, therefore, advisable that owners with competitive horses or those younger than two to five years old, especially the highly purebred breeds (such as Thoroughbreds, Warmbloods and Arabians) residing in the Eastern temperate to warm parts of RSA with high summer rainfall, or travelling between provinces, should practice routine, complete vaccination against WNV. These vaccines should be given annually during spring, in order to prevent disease and death by timeously increasing immunological resistance against WNV.

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APPENDIX A: ANIMAL ETHICS COMMITTEE APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V080-18

9 October 2018

Prof. PN Thompson
Department of Production Animals
Faculty of Veterinary Science
(peter.thompson@up.ac.za)

Dear Prof. Thompson

Project V080-18
Retrospective analysis of the epidemiology and clinical representation of West Nile virus infection in horses in South Africa, 2016-2017 (F-M Bertram)

The application was discussed and approved by the Animal Ethics Committee of the University of Pretoria at the October 2018 meeting.

If you have any question, please feel free to contact the committee.

Yours sincerely

pp. Nuşai Bennett

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee
Copy F-M Bertram (Researcher)

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

APPENDIX B: ANIMAL ETHICS COMMITTEE APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Surveillance for zoonotic arboviruses and their epidemiology in South Africa**
PROJECT NUMBER	**H012-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. M Pretorius**

STUDENT NUMBER (where applicable)	----------
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

ANIMAL SPECIES	**Horses**	**LIVESTOCK** **Alpacas, Avian, Bovine, Canine, Caprine, Equine, Ovine, Porcine**	**WILDLIFE** **Antelope, Avian, Bat, Bovine, Carnivore, Crocodile, Elephants, Zebras, Giraffe, Warthogs, Primates, Rhinoceros, Snake**
NUMBER OF SAMPLES	**1266**	**145**	**288**
Approval period to use animals for research/testing purposes			**July 2016 – July 2017**
SUPERVISOR	**Prof. W Markotter**		

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - pleas
submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experimen

APPROVED	Date 25 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

APPENDIX C: ZARV SAMPLE SUBMISSION FORM

Centre for Viral Zoonoses (CVZ); Department Medical Virology
Faculty of Health Sciences

UNIVERSITEIT VAN PRETORIA
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INVESTIGATION OF ARBOVIRAL NEUROLOGIC & HAEMORRHAGIC DISEASE, ABORTIONS & CONGENITAL DEFORMITIES, AND VIRAL RESPIRATORY DISEASE IN FARM AND WILD ANIMALS

ZRU REFERENCE NUMBER					**DATE**		
Name of owner of animal					Cell nr		
Owner email address					Tel nr		
Location: disease occurred					GPS		
Name of referring veterinarian					Cell nr		
Practice name and address					Tel nr		
Vet email address					Fax nr		
Name/ID of animal					Sender Ref		
Species		Breed		Sex		Age	
Cloven hoofed animals*	Yes/no	*Must be submitted through Transboundary animal diseases (TAD)/OVR under red cross permit to ZARV					
Requested test:							
DATE SPECIMENS WERE TAKEN:			**FIRST SUBMISSION** ☐		**OR FOLLOW UP** ☐		
Specimen type	Blood samples:	☐ EDTA ☐ Clotted blood	☐ Animal Alive		☐ Animal Dead		
Postmortem Samples on ice	☐ Neurological signs:	☐ Brain	☐ Spinal chord		☐ CSF		
	☐ Respiratory signs:	☐ Lung	☐ Liver		☐ Spleen		
Date of death		☐ Died	☐ Euthanized		☐ Abortion/Fetus/Stillborn		
Other Details:							

CLINICAL SIGNS	**DATE ONSET OF CLINICAL SIGNS:**				
☐ Fever _____°C	☐ Anorexia	☐ Anaemia	☐ Icterus	☐ Hepatitis	☐ Rectal Prolapse
Neurological Signs	☐ Ataxia	☐ Paresis	☐ Hindleg Paralysis	☐ Foreleg Paralysis	☐ Recumbent
☐ Head Tilt	☐ Nystagmus	☐ Tongue Paralysis	☐ Paddling	☐ Seizures	☐ Blindness
Respiratory Signs	☐ Congested mucous membranes		☐ Cyanotic mucous membranes		☐ Nasal discharge
Respiratory Rate		☐ Cough	☐ Dyspnoea	☐ Pulmonary Edema	☐ Pneumonia
Other Clinical Signs	☐ Abortion	☐ Foetal Deformity	☐ Arthrogryposis	☐ Haemorrhagic manifestations	

(continue on back of form)

Treatment for Current Disease					
Were specimens submitted to another laboratory for diagnosis?			☐ OVI ☐ ERC ☐ Other(specify):		
☐ African Horse Sickness	☐ Equine encephalosis		☐ Equine Herpes 1 and 4	☐ Rabies	
☐ Brucella	☐ Bacterial culture		☐ Other (specify):		
Recent vaccinations	☐ AHS1 ☐ AHS2	☐ Equine Flu	☐ Tetanus	☐ Rabies	☐ West Nile Virus
Date last vaccination					
Other (specify):					

These investigations are performed as part of a surveillance and research programme and offered free of charge for collaborators. Specimens will be screened for relevance and quality. Only specimens accompanied by a fully completed ZARV submission form, that reach us **on ice within 3 days of collection or frozen** will be tested by PCR. Specimens must be taken within 10 days of onset of illness for PCR and virus isolation; Specimens taken > 10 days will be used for serology. Virus isolation cannot be performed if frozen at -20 °C. Serology and virus isolation results will be reported retrospectively if applicable or available only.

Submit specimens to: **Prof Marietjie Venter, Centre for Viral Zoonoses, Room 2-72, Pathology Building, 5 Bophelo Rd, Cnr Steve Biko and Dr. Savage, University of Pretoria Prinshof Campus, Pretoria 0001**. Contact: Prof Marietjie Venter +27(0)123192638; +27(0)832930884, **marietjie.venter@up.ac.za; zru@up.ac.za** OR Olivia lentsoane (lentsoane.mo@up.ac.za): +27 (0)12 319 2329 OR Megan Riddin +27(0)123192282; **megan.riddin@up.ac.za** (see separate information on taking of specimens and packaging).

NB: Please send specimens PACKAGED ON ICE by courier or via other laboratory services, not by post. Please use appropriate containers –no syringes or needles (see information sheet). **All cases submitted to this program need to be notified to the statevet before it will be tested.** Specimens from Cloven hoofed animals need to be submitted to TAD/OVR for RNA extraction under redcross permit and nucleicacids send to ZARV for testing (Attention Dr Liveo Heath for Prof M. Venter) and the statevet notified as per signature below.

We will share the results with the state vet if a controlled or notifiable animal diseases in terms of the Animal disease act, 1984 (Act No 35 of 1984) is detected as well as any other positive results." The test results remain the intellectual property of the CVZ and cannot be used for scientific publications without our consent. The identity of the animal and owner will not be revealed in scientific publications. The CVZ reserves the right to NOT test samples that arrive in poor condition and not sampled within viraemic phase. The CVZ requests also that it may follow-up telephonically or via email on cases where the CVZ viral PCRs proved negative in order to record if another diagnosis was made and may request a follow up specimen for research purposes.

I _____(submitter) confirm that I have personally notified the state veterinarian, Dr _____ email_____/cellphone_____ district_____ of this sample submission as the symptoms may be indicative of a suspected incidence of a controlled or notifiable animal disease in terms of the Animal disease act, 1984 (Act No 35 of 1984)".

Signature of acknowledgement of submitter: _____ Date: _____

**This is the most current updated submission form used since 2019, which is a composite of all the previous versions since 2008, compiled by the author at Prof Marietjie Venter's request in 2018 and used as guideline for the telephonic case follow-up.*

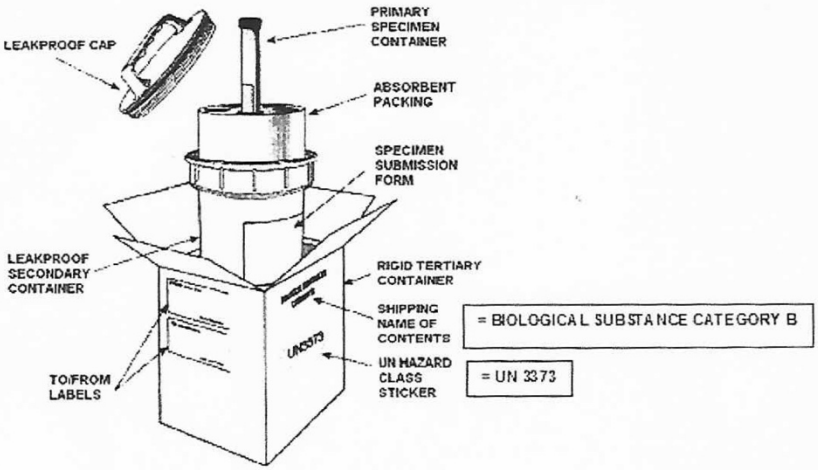
Zoonotic Arbo and Respiratory virus Program
Centre for Viral Zoonoses (CZV)
Department Medical Virology
Faculty of Health Sciences

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

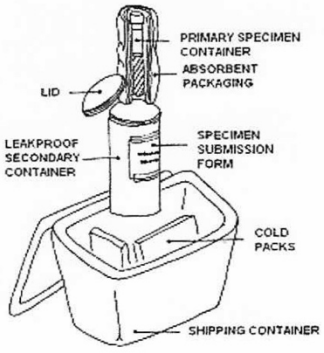
PACKAGING FOR TRANSPORT OF BIOLOGICAL SUBSTANCES,

NB: SEND SPECIMENS BY COURIER OR VIA OTHER LABORATORY SERVICES, NOT BY POST, WHICH IS ILLEGAL AND RESULTS IN DELAYS THAT RENDER SPECIMENS UNSUITABLE FOR TESTING.

Commercially available biosafety packaging e.g. Aulax (RSA), Saf T Pak (Canada) that conforms with IATA Regulations



Example of Improvised Biosafety packaging



Zoonotic Arbo and Respiratory virus Program
Centre for Viral Zoonoses (CZV)
Department Medical Virology

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

INFORMATION SHEET: INVESTIGATION OF NEUROLOGICAL DISEASE IN FARM AND WILD ANIMALS

Background. The Zoonotic arbo and respiratory virus program (ZARV) in the Centre for Viral Zoonoses (CZV) investigate whether West Nile and other **ar**thropod-**bo**rne **viruses** (**arboviruses**: viruses transmitted by blood-sucking arthropods such as mosquitoes, midges, sandflies and ticks), including Sindbis, Middelburg, Wesselsbron and Shuni viruses, account for cases of undiagnosed fatal and/or neurological disease in farm animals such as horses and cattle, as well as in wild animals such as rhinoceroses, buffaloes, warthogs, giraffes and crocodiles. Monitoring neurological disease, unexplained fatalities as well as abortion or fever outbreaks act as an early warning system of annual outbreaks or emerging and re-emergence arboviruses or new zoonotic viruses as part of a One Health approach. By submitting specimens for investigation you become a collaborator of the program and agree that we can use this in our research into zoonotic arboviruses. The ZARV may also investigate vectors or human cases around detected animal cases.

Research tests consist principally of molecular procedures (RT-PCR) to detect viral genetic material (nucleic acid) in blood, brain, or spinal cord samples. A DAFF compliant biosafety level 3 (BSL3) laboratory in the CVZ makes it possible to grow live virus from specimens in safety and identify and characterise emerging viruses. During acute illness the presence of virus is detected by RT-PCR, culture and virus discovery methods and later in the disease the diagnosis is established by demonstrating an IgM immune response.

The ZARV program specifically investigates zoonoses (diseases that humans can acquire from animals) and does not provide a general diagnostic service for veterinary pathogens, particularly controlled or notifiable diseases in terms of the Animal disease act, 1984 (Act No 35 of 1984). These diseases should be excluded by submitting specimens to the appropriate DAFF approved and SANAS accredited veterinary laboratories, with whom we collaborate.

All cases submitted to ZARV need to be accompanied by the DAFF approved submission form signed by the submitting veterinarian that the statevet had been notified that a specimen was submitted to ZARV with **symptoms that may be indicative of a suspected incidence of a controlled or notifiable animal disease in terms of the Animal disease act, 1984 (Act No 35 of 1984)". Please confirm this was done by signing** the submission form prior to submitting the sample. **All specimens of cloven hoofed animals need to be submitted directly to the Transboundary Animal disease program (TADP) at OVR under a red-cross permit for inactivation or RNA extraction with the completed ZARV submission form before nucleic acids will be sent to the ZARV. Send to TAD for attention Dr Liveo Heath for Prof Venter.** A veterinary import permit have to be obtained from DAFF for specimens submitted from countries outside of South Africa on a cases to case basis and the samples imported in compliance with the conditions of the import permit.

NB Include case details as requested on the accompanying submission form: species, age, sex, dates of onset, sampling and death, clinical signs, geographic location, plus contact details of persons from whom further information can be obtained, and to whom results can be reported. Indicate whether duplicate specimens have been submitted to a veterinary laboratory for the diagnosis of controlled and notifiable animal diseases **diseases in terms of the Animal disease act, 1984 (Act No 35 of 1984)** such as African horsesickness and rabies.

Additional specimens: Since neurologic disease may only be the extreme manifestation of infection, arrangements can be made to test blood samples from additional animals with milder signs on affected properties as part of the survey but the primary indication for testing is as stated above (neurological, fatalities, abortion not just single mild fever cases). The viruses are endemic, and detection of an IgG antibody titre alone is indicative of past infection, but is not evidence of current or recent infection. This necessitates a follow up specimen for IgM testing for specimens outside of the viremic period where virus RNA can be detected or to investigate a rise in antibody levels. For cases that test positive or negative by PCR we may also request a follow up blood for WNV IgM confirmation.

Costs. Investigations are performed free of charge and funded by research grants, which have been sourced by the principle investigator. The investigations may however be costly and for this reason, only appropriate specimens that fit the case definition and that were taken during the correct phase of disease will be accepted. Insurance cases or healthy animals due for export should be discussed with the head of the program, Prof Venter and the appropriate forms used.

Specimens. Wear appropriate protective clothing (gloves, gown/apron, mask, goggles) when performing autopsies.

Live animals with neurologic disease: EDTA plus clotted blood can be taken and sent on ice packs. If possible spin down the clotted tubes by centrifugation before shipping.

Fatal cases: Submit 2cm^3 blocks of relevant brain and cord tissue with viral transport medium in separate labelled containers with ice (see attached diagrams). Alternatively, submit caudal quadrant (includes cerebrum, midbrain, cerebellum and brainstem) plus spinal cord (especially lumbar) and blood (eg cardiac puncture), sent with ice packs. Visceral organs may also be submitted for further investigations.

Unidentified virus isolates or specimens from veterinary pathology laboratories can also be submitted if accompanied by our submission form. **Safety and quality control regulations require rejection of specimens that are degenerated/decomposed, or arrive in broken, leaking or otherwise unsuitable primary containers (syringes, plastic bags).**

Send specimens to: Prof Marietjie Venter , Centre for Viral Zoonoses, Room 2-72, Pathology Building, 5 Bophelo Rd, Cnr Steve Biko and Dr Savage, University of Pretoria Prinshof Campus, Pretoria 0001

Contact: Prof Venter: +27(0)123192282; marietjie.venter@up.ac.za; Olivia Lentsoane: **+27 (0)12 319 2329;** lentsoane.mo@up.ac.za;OR Megan Riddin: **+27(0)123192282;** megan.riddin@up.ac.za **to arrange for specimens to be received (see attached information on packaging). NB: send specimens by courier or via other laboratory services appropriate for specimen handling on ice and correctly packaged within 48 hours not by mail as legally required for infectious specimens. Avoid unnecessary delays that reduce specimen quality and practices that have a biosafety risk. Specimens to be sent in clearly marked tubes, indicating material type and preservative - no needles or syringes allowed.**

Questions can be directed to Prof Marietjie Venter at +27 (0)832930884, marietjie.venter@up.ac.za. For autopsies or histopathology on farm animals contact Dr June Williams, Section Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, +27 (0)832348886, June.Williams@up.ac.za, and for wild animals Dr Johan Steyl at the same address, +27 (0)823984823, Johan.Steyl@up.ac.za.

APPENDIX D: EXAMPLE OF QUESTIONNAIRE

Dear Dr _____

The Zoonotic Arbo- and Respiratory Virus (ZARV) at the Centre for Viral Zoonoses (CVZ), University of Pretoria, would please like to do case follow up on samples submitted to the CVZ for the horse _____ owned by/stabled at _____. Blood/postmortem samples from _____ was tested for neurological viruses on ____/____/_____. Test results were negative/positive for _____ virus/es. Kindly would you mind providing the following information which will only be used for statistical / research purposes. No personal information will be made public.

1. Age/year of birth of the horse.
2. Did the horse recover / die / was the horse euthanased? Please provide the date of death if deceased.
3. Breed of horse.
4. Date of onset of initial symptoms.
5. Location / address of horse at time of ilness.
6. Was horse vaccinated against AHS, Flu and West Nile Virus in 12 months prior to sample submission?
7. Is horse vaccinated against West Nile now or does yard/stud/owner vaccinate against WNV now?
8. Was the horse stabled at night in the month prior to diagnosis? If stabled, was the horse free to move in/out, please describe the type of construction if not regular stable e.g. shade cloth covered enclosure.
9. Was horse ill or severely stressed e.g. long distance transport or relocation or recent AHS vaccination in 4 - 6 weeks before sample submission?

Thank you for your kind assistance and please feel free to contact me for any inquiries.

Kind regards

Dr Freude Bertram