Seroprevalence and associated risk factors of West Nile virus in selected equine populations in South Africa

A dissertation submitted to the Faculty of Veterinary Science of the University of Pretoria in partial fulfillment of the requirements for the degree

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Declaration

I, Rebecca Jeal, hereby declare that this dissertation, to be submitted to the University of Pretoria for the degree MSc (Veterinary Science), has not been previously submitted by myself or other persons for the above-mentioned degree at the University of Pretoria or other such Universities. Contributions by others has been adequately recognised in the acknowledgements section or elsewhere stated, along with advice provided from my supervisors and other professionals in the Department of Veterinary Tropical Diseases. The work presented in this dissertation is that of my own and contributing materials have been sufficiently referenced.

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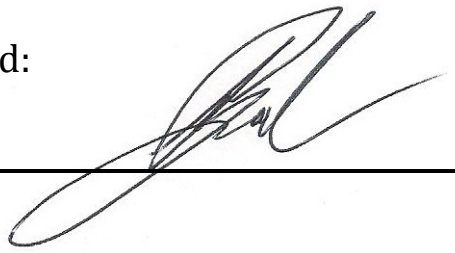

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Abbreviations

WNV	West Nile virus
SA	South Africa
RNA	Ribonucleic acid
CNS	Central nervous system
ELISA	Enzyme-linked immunosorbent assay
RT-PCR	Reverse transcriptase polymerase chain reaction
PCR	Polymerase chain reaction
SNT	Serum neutralizationtest
bp	Base pairs
DVTD	Department of Veterinary Tropical Diseases
$TCID_{50}$	Tissue culture effective dose 50
PBS +	Phosphate buffered saline containing calcium and magnesium
PBS	Phosphate buffered saline
SOP	Standard operating procedure
rpm	Revolutions per minute
OD	Optical densities
nm	Nanometres
NS	Non-structural
Vero	African green monkey kidney cells
HI	Haemagglutination-inhibition

Summary

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The family *Flaviviridae* consists of 94 species, which are distributed worldwide. Viruses in this family share aetiological properties such as membrane envelope proteins, resulting in diagnostic cross-reactivity. Flaviviruses are divided into two clades; arthropod-borne and non-arthropod borne viruses. The most predominant flavivirus seen in horses is West Nile virus (WNV), belonging to the arthropod-borne clade. West Nile virus has been divided into two lineages, of which, the second lineage is primarily found in South Africa.

West Nile virus is transmitted through vector species, of which mosquito species are most predominant. Two genera of mosquitoes in particular, *Aedes* and *Culex* are widely distributed and are fundamental in the transmission of the virus to both reservoir hosts and incidental hosts. Other vectors included in the transmission

cycle are *Culicoides* midges and ticks. Birds are the cornerstone hosts in the distribution and transmission of the virus globally due to migratory habits. Other reservoir hosts include rats and domesticated species such as livestock. Humans and horses are the incidental hosts in the transmission cycle and are symptomatically affected by the virus. As a zoonotic disease, WNV can be transmitted from horses to humans and propose a danger to personnel working with infected horses.

The virus is known to cause encephalomyelitis in infected horses. Documented symptoms include; ataxia, paralysis of the hind legs or complete body paralysis, grinding of teeth, fever, miosis of the pupils, hepatitis and jaundice. A few cases result in mortality, however, not all cases show clinical signs. Infected horses are treated symptomatically and preventative measures are undertaken with annual vaccination against the disease. There have not been a high number of mortalities documented in South Africa due to possible lack of knowledge of the disease and probable misdiagnosis with other notifiable diseases, such as African horse sickness.

The aim of the study was to determine the seroprevalence of WNV in a population of horses from different provinces of South Africa. Blood samples were collected from 112 properties in the nine different provinces of South Africa, amounting to a total of 1198 horses. A questionnaire was used to investigate the risk factors associated with prevalence in the country. The questionnaire survey was also used to determine the current knowledge of WNV amongst horse owners.

Blood samples were initially tested using the serum neutralization test (SNT). Documented positive and negative samples were then subjected to a capture IgG sandwich enzyme-linked immunosorbant assay (ELISA). The results of the two assays were compared with one another, proving to correlate efficiently, giving the resultant seroprevalence percentages for each province, and then were used in comparison with the outcome of the questionnaires to determine significant associated risk factors. Results were analyzed with both univariable and multivariable analyses, taking clustering into consideration, to determine both apparent and prevalence estimate for each province and significance of seropositivity with associated risk factors.

A small population of mosquitoes was collected in both Gauteng and Mpumalanga Provinces and were identified and separated into species. A nested SYBR green real-time PCR assay was conducted on the pooled species of mosquitoes. All species presented negative for the presence of WNV, which could be a result of a low number of mosquitoes or a low prevalence of WNV in each species. The species identified included: *Culex* spp., *Aedes* spp. and *Anopheles* spp.

The SNT were used to determine the apparent seroprevalence of WNV in the collected serum samples, thereafter, the prevalence estimate was calculated with a 95% confidence interval, taking clustering into consideration, for each province. The Free State Province had a high seroprevalence of 73% (95% CI 64-81%), the Western Cape Province had a seroprevalence of 65% (95% CI 51-79%) and Gauteng Province had a seroprevalence of 61% (95% CI 61-62%). Limpopo Province had a seroprevalence of 60% (95% CI 45-74%), followed by Northern Cape Province with 57% (95% CI 48-66%), KwaZulu-Natal Province with 54% (95% CI 43-65%) and Mpumalanga Province with 56% (95% CI 40-73%). The North West and Eastern Cape Provinces had lower seroprevalences of 43% (95% CI 34-52%) and 48% (95% CI 43-54%) respectively. Overall, the apparent seroprevalence for South Africa was 59% (95% CI 54-64) using the SNT.

The ELISA assays showed similar results to the SNT, with a 61% (95% CI 44-79%) seroprevalence of WNV for South Africa. Gauteng Province had a seroprevalence of 47% (95% CI 44-79%), KwaZulu-Natal Province had a seroprevalence of 24% (95% CI 44-79%), Northern Cape Province had 78% (95% CI 44-79%) seroposivitiy, Eastern Cape Province had a seroprevalence of 68% (95% CI 44-79%), North West Province with 59% (95% CI 44-79%) and Mpumalanga Province had a seropositivity of 79% (95% CI 44-79%). The results obtained using the ELISA had a moderate agreement with the SNT results (Kappa = 0.5).

The univariable analysis showed association of WNV seropositive horses with; various agricultural activities, contact with different animal species, presence of annual frost, assorted water sources, occurrence of standing water pools and presence of *Culicoides* midges. These variables were subjected to multivariable

analysis. The variables that indicated a p-value of less than 0.05 were considered significant. Among these values were agricultural activities, such as livestock in the Free State Province, forestry in Mpumalanga Province and vineyards in the Western Cape Province. Contact with small ruminants and other species were the only significant species associated with WNV seropositive horses. Both standing pools of water and river sources were associated with seroprevalence in different provinces. Lastly, annual frost was only associated with seroprevalence in the Limpopo Province. Of the medical history and symptoms, fever was the singular variable associated with seropositivity.

It is evident that many positive cases of infected horses are either not being reported or are not presenting with substantial clinical signs. The horses included in this study were from various age groups, different sexes and breeds and participated in various disciplines. Racehorses were excluded from the study due to their movement throughout the country making them bad sentinels for the study. The high seroprevalence of WNV in horse populations, determined in this study, indicates a subsequent high exposure rate throughout South Africa, varying amongst provinces. The risk factors associated with seroprevalence were all area specific, indicating the importance of habitats and the role it plays in transmission due to the presence of potential vectors. This study also noted a lack of knowledge about the transmission and prevalence of WNV amongst horse owners in South Africa

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Flaviviruses belong to the *Flaviviridae* family and consist of at least 94 various viruses able to infect a large number of hosts (Schoub & Venter 2009; Yeng *et al*. 2013). These viruses are enveloped; positive-sense single-stranded RNA viruses and all members share similar morphological components (Gillespie *et al*. 2010). Some flaviviruses are enzootic, causing disease in both humans and horses, making them dependent on an animal species to maintain their natural cycle (Cantile *et al*. 2000; Mackenzie *et al*. 2004). They can be transferred from animals to humans through blood contact, especially during necropsies, putting certain professionals at risk (Cantile *et al.* 2000; Mackenzie *et al.* 2004).

Members of the *Flaviviridae* family can be separated into vector-borne viruses or non-vector-borne viruses (Yeng *et al*. 2013). Vector-borne or arthropod-borne viruses rely on an arthropod species for transmission between animals and incidental hosts. This occurs through the arthropods sanguinivorous feeding habits, thus ensuring the completion of the viral life cycle (Mackenzie *et al*. 2004). These viruses replicate precipitously within both vector species and mammalian host species (Yeng *et al*. 2013). The principal vectors responsible for the transmission of flaviviruses (i.e. West Nile virus (WNV)) are mosquitoes; however other vectors include *Culicoides* midges and ticks (Mackenzie *et al.* 2004; Yeng *et al*. 2013). Aside from mosquito vectors, some avian species are also prominent in the intercontinental spread and transmission of flaviviruses (Parreira *et al*. 2012). Insect-related flaviviruses have previously been isolated from mosquitoes and genetically characterized; among these was WNV (Haddow *et al.* 2013). West Nile virus is the most prominent in horse populations across South Africa, causing encephalomyelitis in individuals (Romi *et al*. 2004).

The *Flavivirus* genus originated in Africa and emerged from an ancestral virus approximately 10 000 years ago (Mackenzie *et al.* 2004). The prefix 'Flav' originated from the term 'Flavis', meaning 'yellow', derived from the prototype yellow fever virus (Westaway *et al.* 1985; Monarth *et al*. 1990; Bredenbeek *et al*. 2003). Yellow fever virus originated from central Africa, where it remains endemic and has since moved into South America (Bredenbeek *et al.* 2003). The arthropod-borne flaviviruses diverged into different members of the *Flaviviridae* family approximately 3000 years ago (Mackenzie *et al*. 2004). The emergence of these viruses into various geographic regions has resulted from the increase in the human population and thus the growth in urbanisation, land use, agriculture, transportation and deforestation (Gould *et al*. 2003). Global warming and the vast changes in climate and the adaptability of competent insect vectors have resulted in a natural increase in the global distribution of flaviviruses (Maeda & Maeda 2013; Daep *et al.* 2014). The infection of millions of humans and animals has had an effect on the spread to previously non-endemic areas via the efficient colonisation of mosquito vector populations (Daep *et al.* 2014).

West Nile virus was originally isolated from a febrile human located in the West Nile region of Uganda in 1937 (Lanciotti *et al*. 2002) and forms part of the serocomplex, which includes; Japanese encephalitis virus, Murray Valley encephalitis, Kunjin virus and St Louis encephalitis virus (Lanciotti *et al*. 1999; Sejvar & Marfin 2006). Outbreaks and the successful spread of WNV in equines has since been documented in various countries, including South America, the United States of America, Asia, the Middle East, Europe and North and West Africa (Romi *et al*. 2004; Murgue *et al.* 2001; Ostlund *et al*. 2001; Blitvich *et al*. 2003). Maintenance of this virus is through the enzootic cycle between reservoir bird species and mosquitoes. Migratory and domestic birds facilitate transmission; however, global movement of horses has also contributed to the increased spread of the virus worldwide (Samuel & Diamond 2006). This poses an inevitable risk and challenge to the growth of the international equine industry as importation and exportation of horses for breeding and competition purposes continuously increases. Due to the adaptability of WNV, rapid transmission is possible as a result of the dynamics present between the host, vector, pathogen and other contributing environmental factors (such as; annual precipitation and seasonal temperatures) (Matters 2005).

West Nile virus is a neuropathologic virus, which causes encephalomyelitis, varying in severity from mild cases to potentially fatal cases in equine individuals (Romi *et al*. 2004; Venter *et al.* 2009a). This virus is the singular most predominant occurring flavivirus established in South Africa that affects horses (Weissenböck *et al*. 2002; Mathengtheng & Burt 2014). The equine industry in South Africa has recently experienced a significant increase both in sport activities, breed development and economically. Due to the exponential growth in exportation of equine athletes, viral diseases are of the utmost importance. This includes diseases such as African horse sickness, equine encephalitis (EEV) and WNV**.** The latter of which is in many cases most likely to be misdiagnosed as other potentially fatal and notifiable diseases, such as African horse sickness (Venter *et al*. 2010a). This also results in a greater exposure risk to veterinarians and horse owners working with infected cases.

This study intends to determine the seroprevalence of flaviviruses, specifically WNV, in horses in the different provinces of South Africa and the geographical distribution thereof. Benefits of this research will potentially lead to awareness of the disease in terms of regional prevalence and will emphasize some risk factors involved in the presence and transmission of the virus.

1.2 Aetiological agent

The *Flaviviridae* family is divided into three genera; *Flavivirus*, *Pestivirus* and *Hepacivirus* (Brinton 1986; Westaway *et al*. 1985). Flaviviruses all share common features, including size, morphology, nucleic acid content and structural appearance (Shi 2012). West Nile virus consists of a monopartite, positive-sense, single-stranded linear RNA genome that has 10-11 kilobases (Lanciotti *et al*. 1999; Bredenbeek *et al*. 2003; Munoz-Jordán *et al*. 2005; Shi 2012). The RNA molecule contains a 5’ capped end, however lacks a poly(A) sequence at the 3’ end (Westaway *et al.* 1985). The genomic coding for the structural proteins is located at the 5’ end of the RNA molecule (Westaway *et al*. 1985). The virions are pleomorphic and spherical, with a size of 40-65 nm in diameter and has a lipid envelope (E-dimer) containing structural proteins, which assist in infection and efficient replication once in the host (Figure 1.1) (Westaway *et al*. 1985; Mackenzie *et al*. 2004; Sejvar & Marfin 2006). The nucleocapsid

contains the viral genome and plays a crucial role in viral replication (Figure 1.1) (Samuel & Diamond 2006).

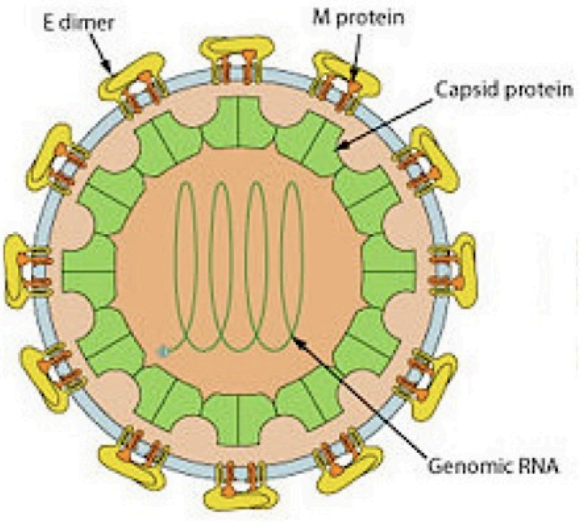

Figure 1.1. Animated structure of West Nile virus including structural annotations (Giri 2016)

1.2.1 Viral replication

Viral RNA replication occurs in the cytoplasma and is in close association with cellular membranes (Bredenbeek *et al*. 2003; Gillespie *et al*. 2010). RNA translation is modulated through the hairpin structure of the flavivirus genome (Bredenbeek *et al*. 2003). During viral RNA replication, the nucleocapsid is released into the cytoplasm through receptor-mediated endocytosis, where ribosomes interact with the nucleocapsid, transporting the RNA to the rough endoplasmic reticulum (Gillespie *et al*. 2010; Munoz-Jordán *et al.* 2005). The viral RNA is translated within the rough endoplasmic reticulum into particular protein precursors, which are further processed by proteases to produce both structural and non-structural proteins (Munoz-Jordán *et al*. 2005; Samuel & Diamond 2006; Avirutnan *et al.* 2011). Structural and nonstructural proteins allow for adequate translation of positive-sense RNA molecules, which are then enveloped into progeny virions and are transported through the trans-Golgi apparatus before being released through exocytosis (Samuel & Diamond 2006). There are three structural proteins; capsid (C), pre-membrane (prM) and envelope (E), and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lanciotti *et al.* 1999; Bredenbeek *et al*. 2003; Munoz-Jordán *et al*. 2005). The structural proteins

are responsible for viral genome translation (Westaway *et al*. 1985). Structural glycoprotein E is located in the peplomers of the envelope and is concomitant with the prM structural protein, which surrounds the C protein that encloses the RNA (Westaway *et al.* 1985; Sejvar & Marfin 2006). The E protein is based on the exterior of the envelope and facilitates connection to the cellular receptors located on the host cells, as well as membrane merging, neutralization, thus making it an important immunological protein (Samuel & Diamond 2006; Schlesinger 2006; Sejvar & Marfin 2006). Monoclonal antibodies formed by epitopes associated with the E protein allows for adequate testing through neutralization tests (Westaway *et al*. 1985). Genomic sequencing has previously been conducted using both the E protein and NS3 and NS5 nonstructural proteins, permitting for differentiation between various lineages, especially in the case of WNV (Sejvar & Marfin 2006; Mackenzie *et al*. 2004). The nonstructural proteins are imperative for RNA replication functionality, along with the coordination of morphogenesis and the mitigation of interferon and antiviral reactions within the host (Murray *et al*. 2008). The NS1 protein functions as a cofactor for the replicase enzyme and regulates complementary actions associated with organelle membranes (Young *et al.* 2000; Schlesinger 2006). The NS2A protein prevents host interferon responses, allowing for viral assemblage to occur, whilst NS3 permits for proteolytic activities to occur with the aid of NS2B as a cofactor (Samuel & Diamond 2006). Both NS4A and NS4B proteins function in the inhibition and modulation of interferon signaling, and the NS5 protein is responsible for the regulation of RNA polymerase and methyl-transferase, both of which are essential for the completion of viral RNA (Munoz-Jordán *et al.* 2005; Samuel & Diamond 2006).

The NS1 protein is the most important nonstructural protein when determining the concentration of the virus when testing for severity of infection. Schlesinger (2006) determined that there was a correlation between the abundance of NS1 protein and the severity of the disease (WNV). The ability of flaviviruses to inflict disease and survive in a host species is dependent on the viral ability to penetrate target cells whilst avoiding the immunological recognition system of the host (Samuel & Diamond 2006).

1.3 Epidemiology

1.3.1 Hosts

All flaviviruses are produced and spread through a cycle incorporating different vertebrate host species, with the exclusion of Dengue virus (Mackenzie *et al.* 2004). Reservoir or amplifying host species are crucial for the maintenance of flaviviruses within the transmission cycle between the host species and vector species and are responsible for the spacial distribution of flaviviruses globally (Mackenzie *et al*. 2004). Members of the *Flaviviridae* family, such as WNV, circulate through nature via wild animals and spillover into domestic species of animals, including rabbits, rats and pigs (Figure 1.2) (Romi *et al*. 2004; Weiver & Reisen 2010). The most common rat species that act as amplifying hosts include, *Rattus rattus, Rattus bowersi* and *Rattus sabanus* (Dobler 2010). Wild birds are important reservoir hosts that are typically asymptomatic, however, several cases have shown symptoms and mortality, thus allowing for efficient RNA replication and transmission worldwide through migratory activities (Romi *et al*. 2004; Weiver & Reisen 2010). Humans and domestic horses (*Equus caballus)* are the dead-end hosts or the incidental hosts (Figure 1.2) that are inflicted by the neurological diseases caused by flaviviruses, and thus do not play a role in the natural cycle of flaviviruses (Mackenzie *et al*. 2004). There are a few flaviviruses that commonly affect horses, of these include Murray Valley encephalitis, WNV and Kunjin virus (Lanciotti *et al.* 1999; Sejvar & Marfin 2006).

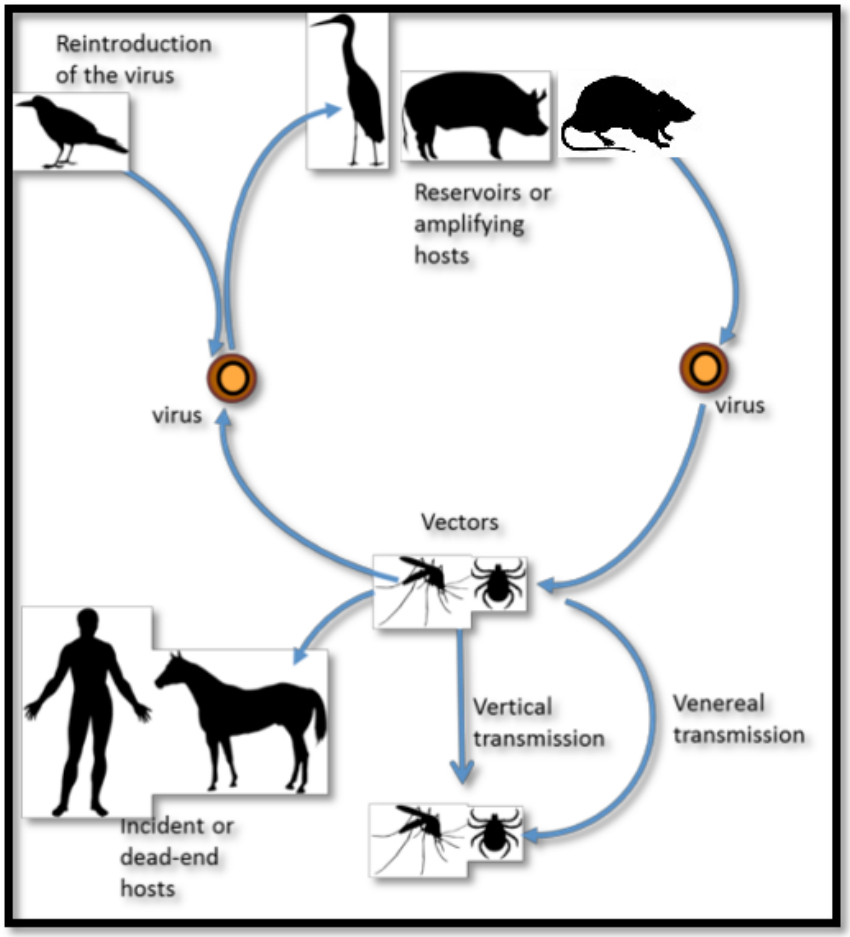

Figure 1.2. Natural transmission cycle of WNV indicating various host species (Guey-Chuen & Wei-June Chen 2013)

West Nile virus has previously been isolated from avian species, such as American crows (*Corvus brachyrhynchos*), and has also been identified in birds of prey (Anderson *et al*. 1999). Most predominant amplifying hosts of WNV are wild birds but also include porcines and cattle, and moderate viraemia has been seen amongst lemur species (Hubálek & Halouzka 1999). Frogs, in particular, *Rana ridibunda*, is another host for WNV in wetlands and other water-based niches. Certain reptiles have also been documented as amplifying hosts, such as; American Alligators (*Alligator mississippiensis*) in America and crocodiles (*Crocodylus niloticus*) in Isreal (Steinman *et al.* 2003; Klenk *et al.* 2004). The seroprevalence discovered in both amphibians and reptile species indicates their importance in the WNV lifecycle (Steinman *et al*. 2003). Transmission is facilitated through the feeding habits of mosquitoes when feeding on affected host species. Environmental conditions have a major influence on the rapid transmission between different species as it impacts the distribution and behavioural patterns of individuals within a species (host species), an example of this would be

locations of water sources and forage (Hubálek & Halouzka 1999). Thus there is a rapid transmission rate among horses, resulting in many documented cases in different countries, for example; North America, Europe, Asia and Africa (Romi *et al.* 2004; Weaver & Reisen 2010).

1.3.2 Transmission

Flaviviruses fit into two different clades, vector-borne and non-vector borne viruses (Gaunt *et al*. 2001; Cook & Holmes 2006). Research has predominantly been focused on the vector-borne clade due to the rate of transmission and spread between different regions and increased infliction of diseases in humans (Cook & Holmes 2006). Flaviviruses utilise various amplifying mechanisms in natural transmission cycles, ranging from virion replication within the gut and salivary glands of the vector species, to spillovers into domesticated animal species (Weaver 2005; Weaver & Reisen 2010). The most common form of transmission is through the sanguinivorous feeding habits of mosquitoes and the vertical transmission between generations through mosquito reproduction, which also serves as an overwintering mechanism (Figure 1.3) (Cook & Holmes 2006).

Mosquitoes are highly adaptable and are able to travel great distances and live in varying environmental conditions, allowing for the persistence of flaviviruses in many different regions (Cook & Holmes 2006). Transmission and spread of flaviviruses through mosquitoes has been commonly detected in two species of mosquitoes, the *Culex* species and the *Aedes* species (Gaunt *et al.* 2001; Cook & Holmes 2006). *Culex* mosquito species are generally associated with neurotropic viruses, which result in encephalitic diseases in livestock, and normally include an avian reservoir (Gaunt *et al*. 2001). The species *Culex pipiens* has a wider range of host species on which it feeds, thus is the most predominant species in the transmission of flaviviruses from birds to horses (Romi *et al*. 2004). *Aedes* mosquito species have multiple host species and are associated with non-neurotropic viruses (haemorrhagic diseases) and are the predominant species causing infection in humans (Gaunt et a*l*. 2001; Romi *et al.* 2004; Cook & Holmes 2006). *Culicoides* midges and ticks are other known vectors for flaviviruses; however, they do not have as great influence on transmission of

flaviviruses as compared to that of ornithophilic mosquitoes and their role in the transmission cycle is unknown (Romi *et al.* 2004).

West Nile virus in particular can be transmitted from a mare to foal through transplacental transmission, through organ transplants, blood donation and breast milk in humans (Westaway *et al*. 1985; Mackenzie *et al*. 2004).

Wild avian species are reservoir hosts pertinent to WNV and hence play a vital role in the transmission of the virus globally (Ayers *et al.* 1994). Avian species living in different ecoregions, such as wetlands and terrestrial environments, have a large influence on the presence and adaptability of WNV. West Nile virus is commonly distributed over greater geographical ranges by means of migratory aves (reservoir hosts). These birds show no clinical signs and a large persistent state of viraemia (McLean *et al*. 2001). Transmission between birds is indicated at its highest in cohabitating circumstances, where transmission normally occurs through the faecal-oral route (Figure 1.3) (McLean *et al*. 2001; Chancey *et al*. 2015).

Rats are also a known amplifying host for WNV, and occur globally due to their adaptability in different ecosystems (Dobler 2010). Rats are frequent pest species in equine areas due to the abundance in food resources (concentrate horse feed) and refugia (stables) and they often chew on horses' chestnuts and hooves at night, resulting in a greater oral exposure risk of infection (Tadich *et al*. 2016).

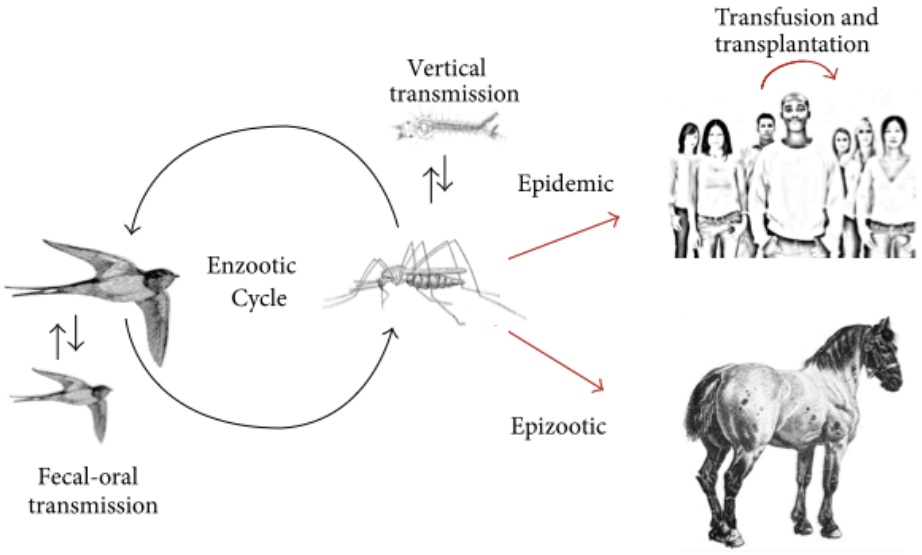

Figure 1.3. Transmission cycle of WNV between aves, mosquitoes and dead-end hosts, horses and humans. Illustrating transmission between avian individuals and mosquito vectors, and vertical transmission through production of mosquito progeny. Final stage of the cycle includes the epizootic transmission to the incidental hosts, humans and horses (Chancey et al. 2015)

1.3.2.1 Risk factors associated with transmission

Complex interactions between intrinsic (genetic composition of both vector and virus and competency of vector) and extrinsic (temperature, rainfall, biome, vegetation and land use) factors influence the transmission of vector-associated viruses (Ciota & Kramer 2013). Transmission of viruses through mosquito vectors occurs on a seasonal basis and is highest during the summer season due to the increase in rainfall, temperature and humidity (Markoff 1995). The change in climatic conditions plays a major role in the replication cycle of vectors, resulting in an increase in vector populations (Markoff 1995). Living conditions, such as concrete floors and presence of still standing water sources are considered as high priority when assessing the rate of transmission between individuals. Various avian species are responsible for the rapid spread of the disease through flight movements between regions according to seasonal changes and thus have an impact on the rate of spread globally. Along with the presence of birds, other species both wild and domesticated can act as carriers and as such are vital in understanding the epidemiology and various risk factors contributing to the infection rate within individuals of the dead-end host species. The susceptibility of vertebrate hosts, population dynamics, such as cohabitation and social behaviour,

between individuals of a host species and the immune status of hosts are cornerstones in the complex interrelationships that coordinate to ensure successful transmission of these viruses. A change in any of these factors could alter the entire transmission cycle (Ciota & Kramer 2013).

1.3.3 El Niño

Expansive distribution of WNV is largely facilitated by the change in global climate over recent years. An increase in transmission of viruses is seen in flooding phenomena that follow periods of drought, and visa versa (Weaver & Reisen 2010). El Niño-Southern Oscillation occurs due to the heating of the Indian Ocean, resulting in a lengthy drought period, followed by heavy rainfall (Weaver & Reisen 2010). This results in concentrations of vector populations around small water bodies during drought and an explosion of vector progeny due to rapid reproduction with the introduction of heavy rainfall, increasing the risk of infection to both humans and horses in the case of WNV (Weaver & Reisen 2010, Roche *et al*. 2013). Extreme changes in climate allow for easy distribution of vectors from northern regions to southern regions (Weaver & Reisen 2010). South Africa recently experienced an El Niño during 2015 and 2016, resulting in a period of severe drought and followed by heavy rains and flooding towards the end of 2016. This has a large effect on the location of horse populations as horses have had to move to different provinces in order to get relief from the drought, thus directly affecting the distribution and transmission of flaviviruses among horses in South Africa (eNCA 2015).

1.3.4 Occurrence

Japanese encephalitis virus, Kunjin virus, Murray Valley encephalitis virus, Louping ill virus and WNV are five flaviviruses that are pathogenic to horses (Cantile *et al*. 2001). These viruses are spread throughout different areas of the world. Japanese encephalitis virus has previously been isolated in Australia and Asia, Kunjin and Murray Valley viruses have been localised to Australia and Papa New Guinea (Cantile *et al*. 2001). West Nile virus has a far broader global distribution than the other four equine

affiliated flaviviruses and is found in Africa, Europe, Asia, India, Russia, South America and North America (Cantile *et al*. 2001; Weaver & Reisen 2010).

There are 10 known mosquito-borne flaviviruses that occur in South Africa, namely; Turkey meningo encephalitis, Bagaza virus, Wesselsbron virus, Banzi virus, Ntaya, WNV, Usutu virus, Uganda-S virus, AR 5189 (unidentified) and Spondweni virus (Barnard & voges 1986a). These viruses affect different animal species, e.g. Wesselsbron virus affects predominantly sheep, whereas WNV affects both horses and humans (Barnard & Voges 1986b).

There are 2 lineages of WNV, lineage 1 primarily experienced in the Northern hemisphere, as well as Australia, whilst lineage 2 is endemic to Africa, including Madagascar and South Africa (Venter *et al.* 2017). Further phylogenetic analysis has shown that lineage 1 can be subdivided into 3 clades, namely; clade 1a, including strains located in Europe, America, Isreal and Africa; clade 1b, which is predominantly experienced in India, from which clade 1c originated (Bondre *et al.* 2007). Neurological disease has occurred in horses in South Africa as a result of lineage 2 (Venter *et al.* 2017).

In 2007 in South Africa, WNV was discovered in three of the nine provinces; Gauteng, Northern Cape and the North-West Province (Venter *et al*. 2009a). It is expected that there will be a higher occurrence in areas of South Africa with an increase in climatic changes, such as temperature increases and the occurrence of droughts and subsequent flooding, land use and other human activities (Mackenzie & Williams 2009). For these reasons it can be expected that Gauteng, KwaZulu-Natal and the Western Cape provinces will have a higher occurrence of WNV due to the immense equestrian activity in these provinces.

1.3.5 Pathogenesis and clinical signs

The infection and pathogenesis of WNV is compiled of complex interactions and various host factors including age, sex, genetic susceptibility, pre-existing infection and immunity to heterologous agents (Monath 1990; Chambers & Diamond 2003). The

virus is neuropathogenic causing neurological disease in individuals, where it manifests and affects the central nervous system (CNS) (Chambers & Diamond 2003; Samuel & Diamond 2006). It is known that the younger the horse, the more susceptible it is to contracting WNV. Neonates are especially susceptible and often die from infection (Monath 1990). There are four known mechanisms used by WNV and other flaviviruses to move across the blood-brain barrier. One of the mechanisms entails passive transport of virions through the epithelial cells in the host; the second is infection via the olfactory neurons in the nasal passages (Samual & Diamond 2006). Infection via the nasal cavities is an alternative route of transmission, and is important due to the high exposure to aerosols in animals, especially when kept in herds and close proximity to one another (Monath 1990). Another mechanism consists of transportation to the CNS through infected peripheral neurons (Samuel & Diamond 2006). Replication also takes place in the mammary glands of pregnant mares, leading to the infection of neonates through the production of milk and succession of suckling (Monath 1990). Horses often develop interstitial myocarditis when infected with WNV (Monath 1990).

The virus enters the host through the bite of mosquito vectors. Virus in viral-infected saliva will subsequently infect vascular endothelial cells, fibroblasts or the reticuloendothial cells, and will then be transported to the CNS (Mackenzie *et al*. 2004). The inoculation of the virus into the skin (dendritic cells) via the bite of the vector, allows for replication in the region of local tissue and proximate lymph nodes, before entering the lymphatic system that carries the viral molecules towards the thoracic duct, and from there the virus is able to enter the bloodstream, where further infection of peripheral tissues occurs (Monath 1990; Samuel & Diamond 2006; Yeng *et al*. 2013). This form of pathogenesis is characteristic of extraneural spread, initiating infection from tissue sources other than neural tissues (Monath 1990; Chambers & Diamond 2003). West Nile virus exits secretory granules via exocytosis after the completion of RNA replication (Monath 1990). With an extraneural source of infection, viraemia levels are higher and thus have a faster infection rate throughout the rest of the body (Monath 1990).

Documented symptoms of WNV infections include; ataxia, weakness and in some cases paralysis of the hind legs and the front legs, complete body paralysis, chewing and grinding of teeth, seizures, fever, abortion in pregnant mares, partial blindness, miosis of pupils in one or both eyes, coma, hepatitis and jaundice (Benenson 1995; Venter *et al.* 2010b). Not all symptoms are present in every case. Most cases tend to result in mortality or humane euthanasia; if however, the horse survives the clinical symptoms, it needs to be rested for a long period of time before normal activities can be resumed (Venter *et al.* 2009b).

1.4 Diagnosis

Clinical symptoms of WNV are not pathognomic making diagnosis from clinical signs unreliable (Venter *et al*. 2009a; Williams *et al*. 2014). It is of great importance that diagnoses are made using conventional viral, serological and molecular-based tests. Flaviviruses can be detected post infection using plasma, serum and infected tissue (spleen or kidney). Virus isolation is a common test used to detect the presence of flaviviruses in the acute stages post infection using cell cultures. IgM and IgG enzyme-linked immunosorbant assays (ELISA) are used in the later stages of infection to detect antigens in serum samples (Kumarasamy *et al*. 2007). ELISA's are commonly used in diagnostics of flaviviruses, in particular the IgG and IgM ELISA (Bundo & Irigashi 1985; Venter *et al*. 2017). The ELISA inhibition method has previously been used in combination with the IgG ELISA to detect IgG antibodies in infected individuals. The results from different ELISAs are compared and correlated with results obtained by haemagglutination-inhibition tests to get accurate overall results (Maeda & Maeda 2013).

Molecular tests, which include reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR, provide sensitivity in terms of infection and are used to distinguish between primary and secondary flavivirus infections (Kumarasamy *et al*. 2007).

Plaque reduction neutralizing test has become the most recently favoured diagnostic test in the detection of different flaviviruses, due to the specificity using neutralising

antibodies. A titration is obtained which quantifies the reaction of antibodies to the virus within individuals (Maeda & Maeda 2013).

Flaviviruses are also identified through indirect immunofluorescence antibody tests, which utilise monoclonal antibodies. This test is utilised to distinguish between different flavivirus isolates from animal species, including amplifying hosts and vector species (Lanciotti *et al*. 1999).

1.4.1 Serological tests

Serological tests are commonly used in diagnosis of WNV and other arboviruses, particularly the ELISA and SNT (Paweska *et al*. 2003). The IgM antibody-capture ELISA measures the total quantity of IgM in the serum using specific antibodies pertaining to the particular host. Cross-reactivity has not been visualised with this particular assay; however, this has been observed with other testing methods including an indirect IgG ELISA (Kuno *et al.* 1985; Mathengtheng & Burt 2014). The IgG ELISA is commonly used in the detection of flaviviruses post infection using the E/M antigen-coating indirect IgG ELISA. An IgM/IgG ratio is used in laboratories in the diagnosis of primary and secondary WNV infection (Venter *et al*. 2017). The NS1 is used in a highly sensitive antigen capture ELISA to determine the presence of specific flaviviruses (Young *et al*. 2000). Most documented testing procedures use an IgM-capture ELISA, which identifies recent encounters with WNV but has not been used to differentiate between subtypes of flaviviruses.

For type-specific flavivirus testing, a SNT is used (Lanciotti *et al*. 2000). Due to the sensitivity of SNT's, they are used to distinguish between definite positives and negatives and the exposure to WNV among individuals (Lanciotti *et al*. 2000). The natural antibody response is utilized in these tests to deduce the presence of individuals' antibodies to viraemia, in so doing; titres for the neutralizing antibodies are determined using live virus antigens and cell cultures. Resultant titrations quantify infection in individual samples (Lanciotti *et al*. 2000).

1.4.2 Molecular tests

The PCR is a very sensitive test used to test for specific flaviviruses and has commonly been used to distinguish WNV in animal and human hosts (Harris *et al*. 1998; Lanciotti *et al.* 2000). Specific WNV primers used in PCR assays were originally derived from the Uganda originated WNV strain from 1939 (Lanciotti *et al.* 2000, Venter *et al.* 2011, Williams *et al*. 2014). The use of probes in both TaqMan and SYBR-green assays has a major diagnostic advantage in that different flaviviruses can be distinguished in a singular run assay (Lanciotti *et al*. 2000; Domingo *et al*. 2011). These assays have been tested on a wide variety of samples including that of mosquito pools, proving beneficial in testing for presence of viruses in vector species (Lanciotti *et al*. 2000; Domingo *et al*. 2011). Chao *et al*. (2007) successfully used a real-time RT-PCR to test for eight flaviviruses in spiked pools of laboratory-harvested mosquitoes, which involved Taqman fluorogenic probes. The use of this assay is beneficial for the detection of multiple viruses within mosquito populations in various regions for both medical and epidemiological purposes (Chao *et al*. 2007).

Evolutionary and phylogenetic studies have previously made use of the RT-PCR methods in determining the various lineages and genetic sequences (Domingo *et al*. 2011). Previously, nested RT-PCR's have been conducted specific for the NS5 protein using WNV-specific fluorescence resonance energy transfer probes and melt curve analyses, which allows for the efficacious deduction between lineage one and lineage two of WNV (Venter *et al.* 2009a; Zaayman *et al*. 2009). The outbreak of WNV in New York in 1999 was confirmed using a semi-nested RT-PCR, which made use of the NS5 protein region within the virus and a dendogram to illustrate predictability for the virus (Scaramozzino *et al.* 2001). West Nile virus in particular has shown to contain between 200 bp and 300 bp when further analyzed using agarose gel electrophoresis (Scaramizzo *et al*. 2001; Venter *et al*. 2009a).

1.5 Treatment and control

Vaccines against WNV are globally available for horses (Hathaway *et al*. 2003; Samuel & Diamond 2006). An inactive WNV vaccine for equines was developed by Hathaway *et al.* (2003), through the Fort Dodge Animal Health Institute. Vaccinations are an imperative prevention method in horses, where unvaccinated horses are highly susceptible to WNV in largely endemic areas, whilst vaccinated horses have efficient antibody responses and CD4+ responses when infected with the virus (Davis *et al*. 2008). The available vaccines in South Africa are Duvaxyn (Registration number: G4071) from Zoetis (Iyer & Kousoulas 2013) and Proteq West Nile from multiple distributors but mainly Lakato in South Africa, which consists of inactive WNV antigen for lineage 2 (Venter *et al*. 2013). To date this vaccine has proved successful in protection against the infection of lineage 2 in horses (Venter *et al*. 2013), however it is only recommended and not yet compulsory to vaccinate horses for WNV in South Africa.

Other control methods include the immediate environment, such as preventing the occurrence of standing or still water pools in which mosquitoes reproduce. The control of mosquito populations is of great importance in preventing the spread of WNV, as well as preventing individual exposure (Mackenzie *et al*. 2004). Insecticides are used in attempts to control the mosquito population and prevention of bites from mosquitoes; however, newer pesticide products are in need to avoid chemical tolerance in mosquitoes (Benenson 1995; Mackenzie *et al*. 2004). Other common measures include the use of flysheets and masks during the day to prevent biting insects from accessing individuals, shade netting over stables to prevent mosquitoes from entering the stables and the activity of stabling horses at night. .

Antiviral therapies are not present for infected horses, instead symptoms are commonly treated with anti-inflammatory and analgesic drugs and support is provided to prevent associated injuries caused during seizures, ataxia or the action of recumbency (thrashing, muscle twitches) (Monarth 1990, Venter *et al*. 2009b). In severe cases, tranquilizers and anti-seizure drugs are administered, along with subcutaneous fluids and nutritional supplements (Venter *et al*. 2009b).

1.6 Problem statement

The prevalence of WNV in different geographic regions in South Africa along with associated risk factors is unknown and with the growth in the equine industry, poses a necessary research topic. The prevalence of WNV in vectors is also unknown and the frequency of occurrence of mosquitoes is important in the investigation of the epidemiology of WNV in South Africa.

1.7 Hypotheses

Further knowledge of the number of seropositive WNV cases, will allow for a greater understanding of the epidemiology of the virus and the risk factors that contribute to the transmission and spread of WNV throughout South Africa.

- The seroprevalence of WNV is greater than 20% in the equine population of South Africa.
- There is a difference in seroprevelance between separate ecological regions and hence different provinces and ecosystems.
- West Nile virus will be detected in mosquito pools from two different provinces.

1.8 Research aims

Primary objectives for this study were:

- Determine the seroprevalence of WNV in a survey population of equines in different provinces and ecological regions of South Africa.
- Establish the presence or absence of WNV in mosquito species as the main vector.
- Perform a questionnaire survey to investigate the risk factors associated with the prevalence of WNV in South Africa.
- Determine the present knowledge and perceptions of WNV amongst equine owners in South Africa with the use of a questionnaire.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Study Design

A total of 661 blood samples were collected in 2013 from three provinces; Western Cape, Gauteng and KwaZulu-Natal. These were collected through a previous study on leptospirosis (Simbizi *et al*. 2016). The samples from this previous study were obtained from three equine hospitals in Summerveld, KwaZulu-Natal Province, University of Pretoria, Gauteng Province and in Milnerton, Western Cape Province. A further 566 blood samples were collected in 2015 and 2016 from individual yards and private holdings in the remaining six provinces; Mpumalanga, Limpopo, Free State, North West, Northern Cape and Eastern Cape. The sampling structure used by Simbizi *et al*. (2016) was used in this study during the collection of samples in 2015 and 2016, however the difference in years was not taken into consideration within the statistical model. The serum samples were tested for seropositivity using the SNT and capture ELISA. Risk factor questionnaires were completed during both studies by equine owners, which provided adequate demographic data. Seropositive and seronegative cases were correlated with the risk factor questionnaires. Of the 566 samples collected, a total of 5 horses were previously vaccinated for WNV, which was taken into consideration when analyzing the results. A limited number of vectors in 2 provinces were also collected in parallel to the serological study. Mosquitoes caught in Gauteng and Mpumalanga Provinces were tested using a real-time RT-PCR assay for the detection of WNV in pooled species.

2.2 Study Animals

Horses of varying ages and breeds, where a juvenile was considered as two years or younger, were included in the study. Included horses participated in different disciplines or were retired/not in work; however, racehorses were excluded all together from the study. This was due to movement between racing venues, thus

preventing the animals from being located at a single destination for an extended period of time. Therefore, racehorses would have made for poor sentinels of disease for a particular region or province. Horses showing clinical symptoms and illness were excluded from the study population in order to eliminate any potential bias.

Horses were stratified into groups based on their main disciplines or functional activity:

- Retired/not in work: both older horses that are no longer under saddle as well as foals and/or weanlings that are still growing, not yet in work.
- General pleasure: Horses used for hacks and general riding purposes.
- Performance horses: Horses that are used for the intention of competition be it in dressage, show jumping, eventing, western riding or polo. These horses commonly travel to different venues within their province and between provinces.
- Working horses: Horses mainly used in riding schools.

2.3 Sample Size

The sample size of equines for each province was determined using the following equation, adapted from Thrusfield (2005):

$$n = \left[Z^2{}_{\propto} P_{exp}(1 - P_{exp})\right]/d^2$$

Where:

n = Sample size

P_{exp} = Expected prevalence

d = Desired absolute precision

$Z^2{}_{\alpha} = 1.96^2$

Sample size (n) being the determinant, the expected prevalence was a value of 20%, while the absolute precision was 5% within a confidence interval of 95%, therefore, the sample size was calculated as

$n = [1.96^2.0.2(1 - 0.2)]/0.05^2$

Thus, sample size was calculated as 245 horses per province. The above values for expected prevalence, absolute precision and confidence interval were used by Simbizi *et al*. (2016) when calculating sample size.

2.4 Study Area

Sample sizes were determined for each of the nine provinces, thus separating ecosystems, which in turn could have an immediate effect on the maintenance of WNV and the prevalence of the virus in horses.

The Western Cape Province is well known for its Mediterranean type climate, providing a suitable environment for the famous fynbos vegetation, making it the most biodiversity-rich province in South Africa (Midgley *et al*. 2005). Microclimates and macroclimates are persistent throughout the shrubland and provide suitable conditions for insects, arthropods and small fauna to flourish and reproduce efficiently (Midgley *et al*. 2005). The Western Cape Province commonly experiences the majority of rainfall in the winter season and little to no rainfall in summer. Summer temperatures reach approximately 28°C and a lower value of 18°C in winter. Horse populations and properties are wide spread throughout the Western Cape, ranging from seas front properties to deep inland properties.

The KwaZulu-Natal Province is home to four endemic vegetation types, consisting of the Maputaland coastal belt, the Tembe sandy bushveld, the Maputaland wooded grassland and the northern coastal forest (de Wet *et al*. 2010). The northern coastal forest runs along the Indian Ocean. There is a variety of vegetation ranging from alpine grasses, hardy savanna bushveld and afromontane forests, providing refugia for many animal species (Eeley *et al*. 1999; de Wet *et al.* 2010). There are two mountain ranges found in this province, the Drakensberg mountain range and the Lebombo mountain range. Due to the inarticulate topographical landforms, the climate varies from inland to coastal regions. The average rainfall is approximately 1000 mm per annum varying with change in altitude, with seasonal temperatures ranging between below freezing in winter seasons and 30°C and above in summer seasons (Eeley *et al*. 1999; Nel &

Somner 2012). Properties consisting of horses are fairly widespread in terms of towns and regions, however, are closely situated within each region or town.

Gauteng Province is the most urbanized of the nine provinces in South Africa, thus has limited vegetation types. A region of the province consists of grassland situated at a higher altitude, lending to the name 'Highveld', where northern regions are made up of savanna bushveld (Grobler *et al*. 2002; Reilly *et al*. 2003). Gauteng has a subtropical climate with high humidity and annual rainfall ranging from 650 mm to 700 mm in different regions (Dyson *et al*. 2009). Temperatures range from 32°C in summer seasons and a minimum of approximately 3°C in winter seasons. Horses and equestrian properties in Gauteng are very closely located throughout the province and are situated on rural community roads, main roads and highways.

The Eastern Cape Province consists of the grassland and coastal forest biomes (Avery 1991; Horak *et al*. 1992). The coastal forest vegetation within this biome is located along the southern Indian Ocean. Seasonal temperatures vary more in the inland grassland, ranging from -4°C in the colder seasons to 29°C in the warmer seasons, whereas the coastal region experiences temperatures ranging from 11°C in the winter season to 26°C in the summer season. Eastern Cape Province receives a smaller quantity of precipitation per year of 450 mm (Mandleni & Adim 2011). Proximity of equestrian properties is extensively spread throughout the different regions of the Eastern Cape Province.

The Limpopo Province is commonly known as the Waterberg due to the presence of a mixture of bushveld and dry deciduous forests. It is situated in the northeastern corner of South Africa and boarders three of the eight provinces, that being Gauteng, Mpumalanga and North West (Aneck-Hahn *et al.* 2007). The average temperature for the summer season in the Limpopo Province is 27°C, however can reach temperatures of up to 46°C in regions such as Phalaborwa, and decrease to an average of 20°C in winter with an average annual precipitation of approximately 1200 mm per annum (Tshiala 2011). There are larger equine populations in major towns in close proximity of one another, and then further expand in distance towards the smaller towns within the Limpopo Province.

The Mpumalanga Province is one of the most diverse provinces in terms of vegetation in South Africa, consisting of three biomes; savannah, grassland and forest biomes. Each of these biomes consists of their own vegetation types and microclimates (Schmidt *et al*. 2002). The main vegetation types include grassland forests located along the escarpment, Highveld grasslands, savannah and bushveld (Schmidt *et al*. 2002). The average rainfall varies within the province from 610 mm in the bushveld and savannah to 850 mm in the forest biome. Temperatures reach a maximum of between 30°C and 40°C throughout the province, with a general average of 26°C. In winter, temperatures decrease, to between 1°C and 8°C varying throughout the province. Seasonal temperatures differ monthly through a range of between 0.2°C and 0.3°C, thus temperatures in different regions of the province experience great variation (Kruger & Shongwe 2004). Similar to Limpopo, the equestrian estates are condensed within the major towns of Mpumalanga and develop further distances and lesser number of properties toward the smaller towns within this province.

Northern Cape Province is the biggest province in South Africa, occupying one third of the total land mass. The Nama-Karoo biome dominates the majority of this province, in which vegetation is sparse and consists of hardy grassland and shrub land types. Vegetation adapts to the harsh environmental conditions in cases of drought and intense sunlight exposure and in some regions, woody species have been seen to dominate over typical grass species, such as *Eragrostis Lehmanniana* (Moore *et al.* 1985; Anonymous 2003). The average precipitation for the Northern Cape is very low, varying from 50 mm to 400 mm across the province; however, the majority of regions vary between 300 mm and 400 mm (Berry & Crowe 1985). Summer temperatures average at approximately 34°C to 40°C but in particular regions can reach a maximum temperature of roughly 48°C (Anonymous 2003). The Northern Cape Province provides a harsher environment for horses to occur, thus more of the indigenous and other hardier breeds are located in this province. There are a large number of horses situated in the rural towns, especially those of a poorer nature, however, actual equestrian properties are extremely widespread and occur within a larger approximation from one another.

The Free State Province consists of a Grassland biome, in which the major vegetation types are grassland and woody grassland, comprising of sourveld grasses (Brand *et al*. 2011). Annual precipitation is experienced in summer seasons with averages between 500 mm and 600 mm, however can increase to 1200 mm towards the Drakensberg region (Brand *et al.* 2011). Winter seasons experience an average temperature of approximately 7°C, however varies in different regions and can reach a minimum of -9°C, where as the average summer temperatures range from 32°C to 38°C (Avenant & Cavallini 2007). Due to the farming nature of this province, properties containing horses are closely situated in terms of neighbourhoods, however, due to the quantifiable sizes of the properties, the actual distances between horses on different properties is quite extensive.

The North West Province is predominantly made up of the Savannah biome with the remaining portion belonging to the Grassland biome (Cilliers *et al*. 1999). The prevalent vegetation types in this province are the bushveld, grassland, thornveld and shrub bushveld. The latter constitute the drier more arid regions of this province. Precipitation is experienced in summer with an annual precipitation ranging between 300 mm to 700 mm. In the regions closer to the Gauteng Province, the average annual precipitation is around 600 mm (Cilliers *et al*. 1999). Summer season temperatures range between 22°C and 34°C, with winter temperatures decreasing to a range of between 2°C to 20°C with few regions reaching minimum temperatures of below 0°C (Cilliers *et al*. 1999). Equestrian properties are located in small concentrated areas with larger sized distances separating the conglomerates of concentrated areas of properties throughout the province.

2.5 Sample Collection

Blood samples were collected from individual horses at particular yards, studs or private properties within the nine provinces (six provinces for this study, three provinces already collected), using 4 ml tubes. The horses sampled in the different properties were selected by their owners (10 horses per property were sampled) at random or in properties with 10 or less horses, all horses were sampled. Whole blood was acquired through jugular venipuncture, performed by either a veterinarian or

veterinary nurse. The blood tubes were then transported to the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria. Further procedures were conducted in a BSL 2+ laboratory in the DVTD. Blood was centrifuged at low speed to separate the serum, and serum samples were then alloquoted into 2 ml cryotubes (Lasec Laboratory Service Provider) and inactivated at 56°C for one hour. Unique codes were allocated to the different samples, associating them with individual horses from whom the samples were drawn and thereafter stored at -20°C throughout the duration of the study.

2.6 Ethics Approval

Ethics approval was obtained from the Animal Ethics Committee of the University of Pretoria, South Africa (Appendix I). A Section 20 approval was obtained from the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (Appendix III).

2.7 Serological Analysis

Serum samples collected were subjected to two serological tests, a SNT and an ELISA. The SNT was used as an initial screening test for serum antibodies to WNV. A selected number of seropositive and negative samples from the SNT's were then subjected to a capture IgG sandwich ELISA for comparison. The results of which were used to determine the overall seroprevalence of WNV in South Africa. The amount of agreement between the two tests was determined using a Kappa test (Cantor 1996).

2.7.1 Serum neutralization test

African green monkey kidney cells (Vero cells) were cultured and passaged for continual use throughout the SNT in 96-well flat bottom plates (AEC-Amersham (PTY) LTD), according to the Standard Operating Procedure (SOP) of the BSL 2 SANAS accredited Virology Laboratory in the DVTD. To prepare the antigen for the SNT, WNV (strain H442) was cultured on Vero cells and the virus was separated from the cells by centrifugation (479 g for 2 minutes) with a benchtop centrifuge. The accumulated virus

supernatant was then stored at -80°C before use. The titre of WNV used in each SNT was determined using the $TCID_{50}$ method of Käber (Irwin & Cheeseman 1939). The virus was used at a concentration of $100TCID_{50}$ in the SNT.

Serum samples were diluted 1:5 using phosphate-buffered saline containing calcium and magnesium (PBS +) (Biochrom Scientific Group). A volume of 100 µl of minimum essential medium (Biochrom Scientific Group) containing 5% gamma-radiated foetal calf serum (Bio West, Celtic) and 1 ml of Genta 50 Phenix antibiotics (50 mg/1 ml) (Virbac) was added to each well of the 96-well flat bottom plates. An additional 100 µl of the diluted serum samples (1:5) was placed in the first row. The diluted serum and 5% minimum essential medium were mixed well and then diluted at a 1:2 dilution series throughout the remaining five columns according to the SOP. A volume of 100 µl of diluted WNV virus, at a concentration of $100TCID_{50}$, was then added to all serum sample containing wells and a further 200 µl into specified wells without serum used as positive controls.

Cultured Vero cells were used in order to observe whether there was prevention of viral growth and hence presence of neutralizing antibodies within the individual serum samples. The plates were then incubated (Labex Smart Cell) at a temperature of 37°C in an incubator with a 5% CO_2 atmospheric composition for five days. The cytopathic effect within each well was observed over the five-day period and seropositive or seronegative samples were identified on Day 5 of the SNT. The titre was determined according to the last dilution where cytopathic effect was evident. Positive samples were deduced with a serum dilution of 1:10 or more, as set out in the SOP.

2.7.2 Capture IgG sandwich ELISA

An IgG capture ELISA was developed to capture existing IgG antibodies within the individual serum samples, indicating seropositive cases. A confirmed seropositive and seronegative sample determined with the SNT was used as controls throughout all ELISA's. Two checkerboard titrations were initially used to determine the optimum dilution concentrations of both the WNV core antibody to be used in the coating buffer and the dilution concentration of the sera.

A coating buffer made up of a dilution of 1:1600 of WNV Core Primary Rabbit Antibody in PBS (Novus Biologicals, Whitehead Scientific) was used initially to coat the plates. The coating buffer was dispensed at a volume of 50 µl in each well of a Maxisorp 96-well flat bottom plate (Nunc) and was set at 4°C overnight. A 10% blocking buffer composed of Iistant skimmed milk powder (Pick 'n Pay Supermarket) and PBS was used both in the blocking buffer stage and as a diluent for the remaining reagent composition stages throughout the ELISA. A volume of 200 µl of blocking buffer was added to each well and incubated for 30 minutes in the ES-20 Orbital shaker-incubator (Biosan) and shaken at 150 rpm. Antigen was cultured on Vero cells in the same manner as the SNT's described in Section 2.7.1. 50 µl of the WNV antigen was set in all wells of the 96-well plate and then incubated for an hour at 37°C, where after, the plate was manually washed three times using wash buffer composed of PBS, Millipore water and Tween20. The serum samples were prepared using a dilution of 1:10 and placed in specified wells in vertical duplicates, before being incubated for an hour at 37°C, allowing for any present IgG antibodies to bind to the antigen epitopes. Another wash step was conducted before adding 50 µl of the goat anti-equine secondary antibody HRP (Novus Biologicals, Whitehead Scientific) conjugate at a dilution of 1:10 000 to each well. The plate was again washed with wash buffer three times before the addition of 100 µl of the substrate, O-Phenylenediamine dihydro-chloride (Sigma-Aldrich). After 15 minutes incubation time, 50 µl of sulfuric acid stop solution was added to each well. A checkerboard was initially conducted to determine the concentrations for both serum and WNV core antibody dilutions. The checkerboard results and the plate setup are indicated in Appendix IV.

The optical densities (OD) of each sample were determined using the Gen5 program (BioTek). The plate was shaken for 10 seconds before reading at an absorbance wavelength of 650 nm to provide the background reading and then read again at 490 nm to provide the OD values of the sera samples. The background reading values were then subtracted from the OD values before the mean was calculated for each sample. As this ELISA was still to be validated, negative samples were deduced at a value of less than three standard deviations of the positive control mean.

2.8 Vector Analysis

Mosquitoes were caught in two provinces; Mpumalanga and Gauteng. Specific mosquito traps were handmade using solid white material and mosquito netting material (Figures 2.1, 2.2 & 2.3).

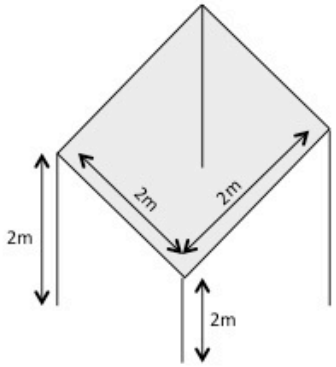

Figure 2.1. Mosquito trap pole structure, each pole measuring 2m in height. The grey region represents the material used to cover the trap. All sides of the trap measure 2m each.

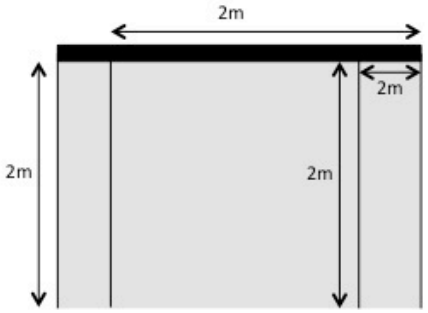

Figure 2.2. A 3D representation of the material structure used to cover the four poles. The solid black region represents the solid white material and the grey region illustrates the mosquito netting material, both of which were 2 m by 2 m.

Figure 2.3. Image of the mosquito trap after being set up at a specified site in Mpumalanga Province

The traps had an overall dimension of 2 m^2 of material covering four individual metal poles (Figure 2.1). The individual poles were placed in each of the holes within the solid white material to form the roof of the trap (Figure 2.2) and the mosquito netting covered all four sides from the roof of the trap to the floor (Figure 2.3). Ropes were attached to the top of each pole to stabilize the pole to the material and to the ground with the use of metal tent pegs to maintain the structure of the trap. Before trapping, the lower portion of the trap was lifted approximately 30 cm off the ground and secured with material ties at about 5 pm. The lower portions were then grounded at 5 am every morning in order to ensure no insects escaped from the trap.

A mixture of brewer's yeast (Anchor Instant Yeast, Pick 'n Pay Supermarket) and Selati brown sugar (Pick 'n Pay Supermarket) were formulated to provide a source of carbon dioxide as an attractant for the mosquitoes. This mixture was placed in a large container and positioned in the centre of the trap (Abdon-Liwanag & Tansengco 2015). The mosquitoes were collected using a perspex pipe (34 cm) attached to a translucent rubber tube (62 cm). Mosquito netting was placed at the conjunction of the perspex and the rubber tube to prevent the inhalation of individual mosquitoes. Individuals were then vacuumed manually into the perspex tube and then placed in a polystyrene cup covered in mosquito netting to prevent individuals from escaping. Once all

mosquitoes were collected, the polystyrene cups were placed in a freezer, resulting in mortality of the collected mosquitoes and remained there for storage purposes. The collected mosquitoes were then transported to the DVTD.

2.8.1 Vector Identification

Individual mosquitoes were identified using a light microscope and guidelines outlined by Jupp (1996). The mosquitoes were separated into specific species per the two provinces and individuals of each species were pooled into groups of 50 or less individuals for further analysis.

2.8.2 Nested SYBR green real-time RT-PCR

Pooled mosquitoes were first homogenized using PBS+, centrifuged at 7656 g one minute, after which the resultant supernatant was used to extract viral RNA using Trizol® (Invitrogen, Thermo Fisher Scientific). RNA was stored at -20°C until used. A positive control was created through spiking of a pool of mosquitoes (Species 1) from Gauteng with 10µl of WNV (strain H442), whereas water was used as a negative control. The positive control indicated a melting point of 84.34°C. Both positive and negative control samples undertook the extraction procedure along with the pooled groups of mosquitoes. The total RNA concentration for each pooled group was determined using a spectrophotometer at a wavelength of 260 nm. The RNA concentrations for the separate pooled groups of mosquitoes ranged between 9,771ng/µl and 625 938 ng/µl. A one-step cDNA, PCR was performed using the one-step Power SYBR® Green "RT-module (Express Superscript mix)" that was included in the Power SYBR® Green RT-PCR reagent kit (Inquaba Biotec). A volume of 5 µl of the extracted RNA was denatured at a temperature of 95°C for 5 minutes followed by cooling to 4°C before 2µl of the extracted template was added to the master mix for each of the pooled mosquito groups. The master mix contained; 10 µl of Power SYBR Green Master Mix, 1 µl of 10 µM of primers FU1 (Kuno *et al.* 1998) and WN9317 (Zaayman *et al.* 2009) (Table 2.1), 2 µl Express Superscript mix (RT-module) (for the cDNA synthesis step) and 4 µl nuclease free water. Both PCR rounds were conducted using a StepOne Real-time Plus thermocycler (Applied Biosystems) and the

corresponding StepOne Software version 2.3 (Applied Biosystems) program. An additional incubation step was added to conduct the RT (reverse transcriptase) step prior to commencement of the PCR cycles. The temperature profile for the first PCR included a hold stage of 50°C for 15 minutes for cDNA synthesis, followed by a second hold stage of 95°C denaturation for 10 minutes; 40 cycles of 95°C annealing for 30 seconds and an extension of 50°C for 30 seconds. A melt curve analysis was set between 55°C and 95°C.

A volume of 1 µl of the PCR templates from the first PCR round was then used in a nested PCR. The Master Mix for the nested PCR consisted of 10 µl Power SYBR green Master Mix, 1 µl of 10 µM of primers FS778 (Scaramozzino *et al.* 2001; Zaayman *et al*. 2009) and CFD2 (Kuno *et al.* 1998; Scaramozzino *et al.* 2001; Zaayman *et al.* 2009) (Table 2.1) and 7 µl nuclease free water. The cycle program of the nested PCR was as follows: an initial hold stage of 95°C for 10 minutes, followed by a cycling stage at 95°C for 30 seconds and 60°C for 30 seconds for 45 cycles. The melt curve was set between 55°C and 95°C. The melt curve illustrated peak melting temperature per pooled sample and thus based on the known melting temperature for WNV (Kuno *et al*. 1998; Zaayman *et al*. 2009).

Table 2.1. Nucleotide sequences and locations of primers used in the SYBR green nested real time RT-PCR

Primer	Orientation	Concentration (µM)	Melting Temperature (°C)	Sequence (5'-3')
FU1	Forward	10	59.2	TACAACATGGGAAAGAGAGAGAA
WN9317	Reverse	10	58.4	TCGTGATGCGTGTGTCC
FS778	Forward	10	63.5	AARGGHAGYMCDGCHATHTGGT
CFD2	Reverse	10	74.05	GTGTCCCAGCCGGCGGTGTCATCAGC

2.9 Questionnaires

To determine possible risk factors associated with the prevalence of WNV, questionnaires were compiled and were completed by owners of the horses sampled in this study. One questionnaire was created for this study (Appendix I), comprising of two sections; the first section consisting of information on the medical history of each horse, and the second section pertaining to management factors, climatological factors, spatial and temporal factors, and population factors, such as; such as, age, sex and

breed. Seropositivity as deduced from the SNT results was then compared with the acquired information to determine any associations with the various risk factors.

2.10 Data Analysis Questionnaires

2.10.1 Spatial Analysis

Maps were created in Epi-Info to illustrate the areas in which the blood samples were collected, along with an overall collection map for South Africa.

2.10.2 Statistical Analysis

The distribution of collected samples was plotted on the South African map using Epi Info 7 (Centre for Disease Control). The plotted map used GPS coordinates of each location of the properties from which samples were collected. The latitude and longitude coordinates were recorded using degrees (d.d°) with as many decimal places as possible to increase precision. Latitudinal values were all positive values, and the longitudinal values were all negative values.

2.10.2.1 Apparent Prevalence

Tested samples that indicated a titre of 1:10 or more in the SNT assays were considered positive for WNV. Thus, the apparent prevalence for each province was calculated as a division of positive cases to the number of horses within a specified province, as follows:

AP = WNV positive cases in a province/Number of horses sampled in a province

2.10.2.2 Prevalence Estimate

The 95% confidence interval (CI) for each province, and overall for South Africa, for the prevalence estimate was determined using Equation 1 and Equation 2 (Thrusfield 2005):

Equation 1

$$V = P^2(\Sigma n^2) - 2P(\Sigma nm) + (\Sigma m^2)$$

Where:

V = Between-cluster variance

P = Proportion of seropositive horses

n = Total individual horses in each cluster

m = Number of seropositive individual horses in each cluster

The between-cluster variance, calculated in Equation 1, was then used in Equation 2 to determine the lower CI along with the upper CI for the prevalence estimate.

Equation 2

$$\hat{P} + 1.96 \left\{ \frac{C}{T} \sqrt{\frac{V}{C(C-1)}} \right\};\ \hat{P} - 1.96 \left\{ \frac{C}{T} \sqrt{\frac{V}{C(C-1)}} \right\}$$

Where:

P = Proportion of seropositive horses

C = Number of clusters (properties)

T = Total number of individual horses

2.10.2.3 Univariable Analysis

The data collected from the completed questionnaires were recorded in an Excel spreadsheet (Microsoft Excel 2010), which was then exported to Epi Info 7 for the initial univariable analysis. The Chi-squared test was used to determine associations between individual risk factors and seropositive horses. The risk fators that indicated an association to the outcome variable ($p<0.15$) were then included in further multivariable analysis using NCSS 11 statistical software, LLC.

2.10.2.4 Multivariable Analysis

All the variables from the univariable analysis with a $p<0.15$ (Chi-squared) were included in the multivariate logistic regression models, used to determine the association of individual variables (risk factors) on the dependent variable (seroprevalence). A subset selection was used to determine independent variables that are significant or illustrate a prediction of the dependent variable. The statistical programme NCSS version 11 software, designed for statistical analysis forscientific studies and evaluation of data with the use of plot procedures, spreadsheets and databases, was used to determine the multivariable analysis. Automated algorithms (regression equations) in NCSS 11 software add or remove separate variables at different steps in the run. These algorithms were used in hierarchical modeling, of which forward selection with switching was used. Variables were added to the model starting at the maximum number of variables to determine the independent variables that showed the largest values for log likelihood. The included variables were switched individually at each step in order to increase the value of log likelihood for each variable. The significance of the variables to the dependent variable was based on the Wald test. The remaining variables were considered significant if the resultant P_{wald} values were less than 0.05 ($P_{wald}<0.05$). Multi-test corrections and interactions between the variables were excluded due to the software used did not cater for these factors.

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CHAPTER 3

RESULTS

3.1 Sample size

The number of horses collected per province in this study ranged from 51 to 288 horses. A total number of 639 serum samples were obtained from a previous study; 288 were collected in Gauteng, 172 in KwaZulu-Natal and 179 in the Western Cape provinces (Figure 3.1). A further 559 serum samples were collected in this study; 51 from the Northern Cape, 85 from the Eastern Cape, 110 from the Free State, 84 from North West, 135 from Mpumalanga and 94 from Limpopo provinces. The total number of samples used in this study was 1198 (Figure 3.1).

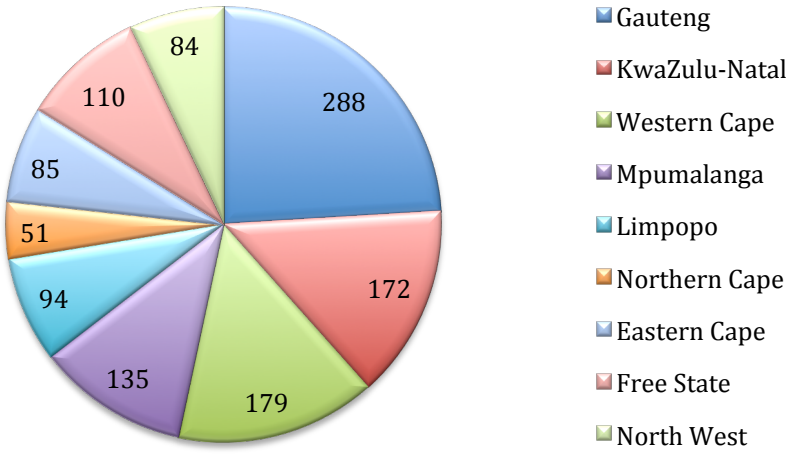

Figure 3.1 Number of samples collected per province. Separate colours, shown in the legend, indicate the separate provinces

The required number of horses per province was not obtained due to the inability to include 245 horses per province. This was a result of climatic factors, i.e the drought in the Northern Cape where the horses were relocated, or not enough owners willing to take part in the study, thus even though an original sample size was calculated, it had to be altered to take number of properties as a sample size instead of the number of horses for realistic measures. So due to the geographic nature of this study and the large distribution of horses throughout the country, properties containing horses were

considered for cluster sampling. The horses within each sampled property were considered a sub-sample from each cluster. In order to determine the exact sample size, taking clustering into consideration, the following formula from Thrusfield (2005) was used:

$$T_s = \frac{1.96^2 P_{exp}(1 - P_{exp})}{gd^2 - 1.96^2 V_c}$$

T_s = total number of animals to be sampled
g =number of clusters (properties) to be sampled
P_{exp} = expected prevalence
d = Desired absolute precision
V_c = between-cluster variance

The clusters were the number of properties, which were fixed at 112 (n=112) because there were 112 properties included in this study, thus using the above equation, the total number of horses per province (T_s) was calculated. An estimated expected prevalence of 20%, with a desired absolute precision (d) of 10% and a between-cluster variance of 1% was used. The total number of horses per province, as calculated, was 64. The total number of horses per property was calculated with the division of the number of horses to be sampled per province by the number of properties (112), giving a total of 0.57 ≈ 1 horse per property. Although this was the calculation, due to the high proportion of total variance, the average number of horses per cluster was kept to 10 horses per property, provided there were more than 10 horses located on the property.

3.2 Study Area

The samples from the previous study were collected through three large equine hospitals, which included; Baker and McVeigh Equine Hospital consisting of two branches, one in Summerveld (KwaZulu-Natal) and Milnerton (Western Cape), Onderstepoort Veterinary Academic Hospital (equine clinic) in Pretoria, Gauteng, and the Drakenstein Veterinary Clinic, Western Cape. These samples originated from; nine

regions in the Western Cape, three regions in KwaZulu-Natal and six regions in Gauteng Province. This batch of blood samples were added to those collected in 2016 from the remaining six provinces, including; Northern Cape (five regions), Eastern Cape (five regions), Limpopo (eight regions), Mpumalanga (nine regions), Free State (seven regions) and North West (six regions) provinces. These collection sites were coordinated through equine societies and breeding studs in the different provinces. The overall distribution of samples throughout all nine provinces is illustrated in Figure 3.2, where the blue dots represented the samples collected in 2016 and the red dots illustrate the samples contributed from a previous study.

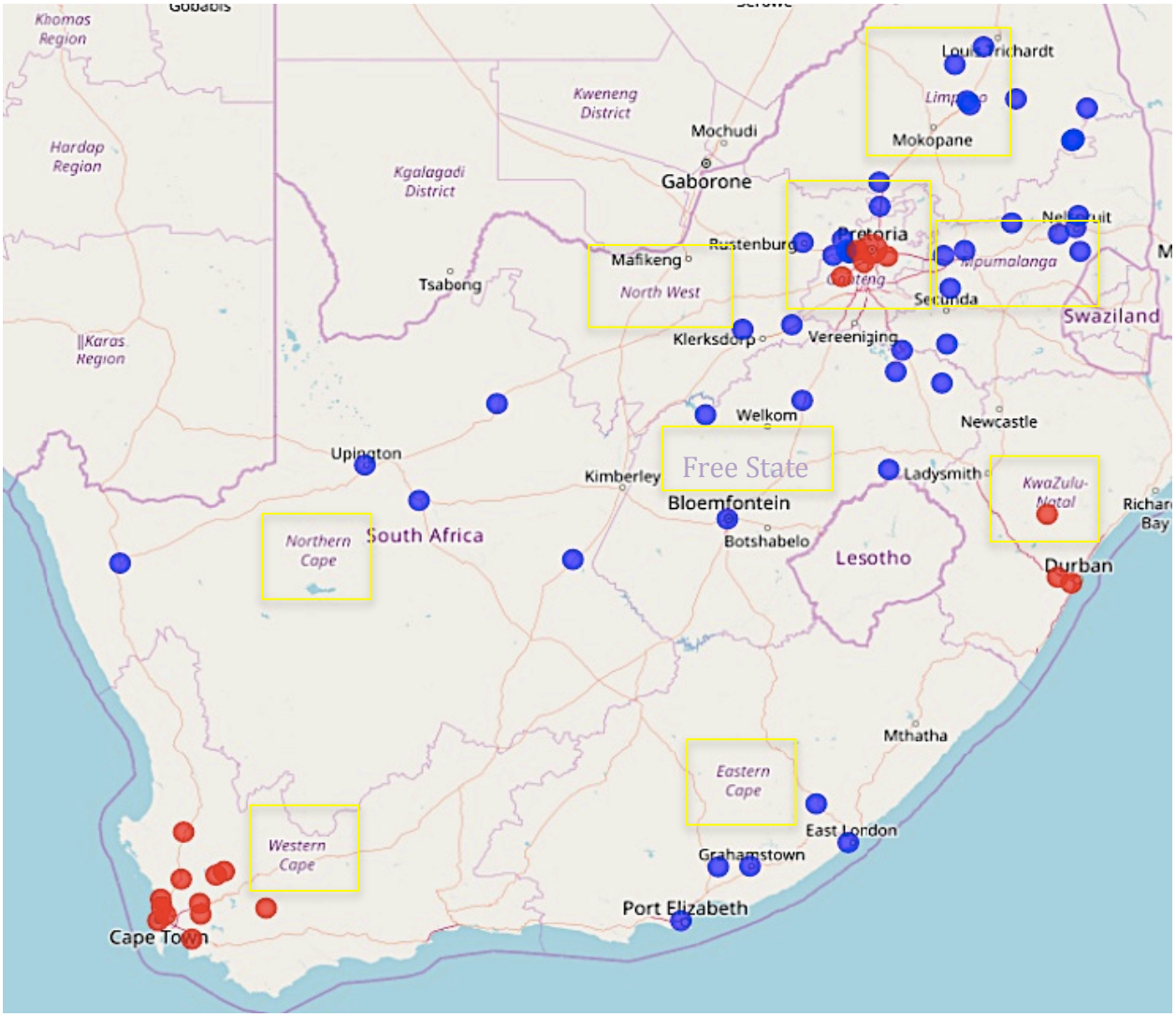

Figure 3.2 The overall spatial distributions for the blood samples collected throughout South Africa. The blue dots represent the samples collected over 2015/2016 in this study and the red dots indicate the samples obtained from a previous study

3.3. Seroprevalence of West Nile virus in South Africa and the different provinces

3.3.1 South Africa

A total of 1198 samples were collected in this study from 112 properties in 61 regions representing the 9 provinces of South Africa. A total of 704 or 59% (95% CI 54-63%) were seropositive for WNV, thus accurately falling within the cluster interval of between 54% and 63% as calculated.

Using the SNT, the majority of seropositive samples had a titre of 80 (23.5%) and a titre of 320 (21%) was observed in 20.1% of samples. 19% had a titre of 160 and 20.1% of the seropositive samples had a titre of 40. Only 2% of samples had a titre of 10 and 14.4% had a titre of 20.

3.3.2 Gauteng Province

From the 1198 collected samples, 288 were from Gauteng Province. After serological testing, the results indicated that 177 of the 288 samples or 61% (95% CI 61-62%) were seropositive for WNV in this Province. A prevalence estimate for the Gauteng Province with results for clustering was between 61% and 62% with a total of 15 properties sampled from this Province.

Table 3.1 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region in the Gauteng Province, including the apparent prevalence, with a prevalence estimate of 61-62% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
City of Tshwane	9	279	172	62
Kyalami	1	4	2	50
Madibeng	2	2	1	50
Mogale	2	2	2	100
Tygerpoort	1	1	0	0

3.3.3 KwaZulu-Natal Province

There were 172 samples collected in KwaZulu-Natal, of which, 93 samples were seropositive or 54% (95% CI 43-65%). Blood samples were collected from 5 properties in the KwaZulu-Natal Province, of these, 1 property was located in the Summerveld region, whilst 2 properties were situated in the Umhlathuze region and the remaining 2 properties were situated in the Umvoti region (Table 3.2).

Table 3.2 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region of KwaZulu-Natal, including the apparent prevalence, with a prevalence estimate of 43-65% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Summerveld	1	78	37	47
Umhlathuze	2	34	24	71
Umvoti	2	60	32	53

3.3.4 Western Cape Province

In the Western Cape Province, 179 horses within 20 properties were sampled of which 116 or 64.8% (95% CI 51-79%) were seropositive for WNV. Eight properties were located in the Milnerton region and the remaining 11 regions all consisted of one property each (Table 3.3).

Table 3.3 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region within the Western Cape Province, including the apparent prevalence, with a prevalence estimate of 51-79% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Big Bay	1	1	0	0
Cape Town	2	2	2	100
Ceres	1	14	9	64
Gordons Bay	1	1	1	100
L'Ormarins	1	40	27	68
Malmesbury	1	2	2	100
Milnerton	8	21	5	24
Paarl	1	16	6	38
Piketberg	1	35	27	77
Robertson	1	35	29	83
Van Riebeeck	1	1	1	100
Wolseley	1	11	7	64

3.3.5 Northern Cape Province

A total of 51 samples were collected for the Northern Cape Province, of which 29 or 57% (95% CI 48-66%) were seropositive for WNV. Samples were obtained from 5 regions throughout the Province and each region consisted of 1 property each (Table 3.4).

Table 3.4 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region in the Northern Cape Province, including the apparent prevalence, with a prevalence estimate of 48-66% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Groblershoop	1	13	6	46
Hopetown	1	8	6	75
Kathu	1	5	3	60
Springbok	1	11	6	55
Upington	1	14	8	57

3.3.6 Eastern Cape Province

In the Eastern Cape Province, there were an overall of 41 or 48% (95% CI 43-54%) seropositive horses from a total of 85 samples collected. These samples were obtained from 11 properties through 5 regions of the province. The region of Port Elizabeth had the most properties (4), followed by East London with a total of 3 properties and then Grahamstown with 2 properties. The regions Stutterheim and Alicedale both consisted of 1 property each (Table 3.5).

Table 3.5 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region in the Eastern Cape Province, including the apparent prevalence, with a prevalence estimate of 43-54% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Alicedale	1	2	1	50
East London	3	30	14	47
Grahamstown	2	11	7	64
Port Elizabeth	4	31	15	48
Stutterheim	1	11	4	36

3.3.7 Free State Province

A total of 110 samples were collected in the Free State Province, of which 80 or 72.7% (95% CI 64-81%) were seropositive for WNV. Samples were collected from 7 regions within the province with a total of 13 properties (Table 3.6). Five of the 13 properties were situated in Bloemfontein, whilst 3 of the properties were located in the Frankfort region. The remaining 5 regions each consisted of 1 property from which samples were collected (Table 3.6).

Table 3.6 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region in the Free State Province, including the apparent prevalence, with a prevalence estimate of 64-81% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Bloemfontein	5	43	32	74
Clarens	1	8	7	88
Frankfort	3	24	19	79
Hoopstad	1	5	5	100
Kroonstad	1	10	6	60
Villiers	1	10	4	40
Vrede	1	10	7	70

3.3.8 North West Province

A total of 36 out of 84 samples collected or 42.8% (95% CI 34-52%) were positive for WNV in the North West Province. Samples were collected from 12 properties located in 7 regions in the province. Both the Hartebeespoort and Skeerpoort regions consisted of 3 properties each (Table 3.7), the Potchefstroom region contained 2 properties and the Brits, Broederstroom, Hartbeesfontein and Rustenburg regions all comprised of 1 property each (Table 3.7).

Table 3.7 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region the North West Province, including the apparent prevalence, with a prevalence estimate of 34-52% (95% confidence interval) taking clustering into consideration

Distict	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Brits	1	9	4	44
Broederstroom	1	10	4	40
Hartbeesfontein	1	7	3	43
Hartebeespoort	3	14	8	57
Potchefstroom	2	15	7	47
Rustenburg	1	11	2	18
Skeerpoort	3	18	8	44

3.3.9 Mpumalanga Province

An overall of 76 samples or 56% (95% CI 40-73%) out of 135 collected samples were seropositive for WNV. Sixteen (16) properties were collected in 9 regions within the Mpumalanga Province. Of those properties 4 were situated in Nelspruit, 3 properties were located in White River and both the Dullstroom and Kaapsehoop regions had 2 properties each. The Barberton, Homedean, Kriel, Middelburg and Witbank regions consisted of 1 property each (Table 3.8).

Table 3.8 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region in Mpumalanga Province, including the apparent prevalence, with a prevalence estimate of 40-73% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Barberton	1	8	6	75
Dullstroom	2	15	11	73
Homedean	1	10	10	100
Kaapsehoop	2	44	16	36
Kriel	1	5	3	60
Middelburg	1	11	8	73
Nelspruit	4	20	12	60
White River	3	15	7	47
Witbank	1	7	3	43

3.3.10 Limpopo Province

A total of 94 samples were collected in Limpopo, of which 56 or 59.5% (95% CI 45-74%) were seropositive for WNV. Samples were collected from 13 properties throughout 8 regions of the Limpopo Province. The Hoedspruit region consisted of 3 properties, the Polokwane, Phalaborwa and Bela Bela regions comprised of 2 properties each and the Dendron, Louis Trichardt, Pienaarsrivier and Tzaneen regions resided of 1 property each (Table 3.9).

Table 3.9 The seroprevalence of WNV samples in comparison to the number of properties sampled for each region within the Limpopo Province, including the apparent prevalence, with a prevalence estimate of 45-74% (95% confidence interval) taking clustering into consideration

District	No. of properties	No. of horses	Seropositive horses	Apparent prevalence (%)
Bela Bela	2	20	10	50
Dendron	1	5	4	80
Hoedspruit	3	20	13	65
Louis Trichardt	1	3	1	33
Phalaborwa	2	18	15	83
Pienaarsrivier	1	9	4	44
Polokwane	2	16	6	38
Tzaneen	1	3	3	100

3.4 Sandwich IgG Capture ELISA Results

A sandwich IgG-capture ELISA was developed and the results acquired from the SNT's were then used as a comparison with those obtained from the ELISA.

A checkerboard trial was conducted to determine the optimum serum and WNV core antibody dilutions before performing using the collected serum samples. The resultant optical density (OD) values suggested that a dilution of 1:10 for serum samples was optimal to achieve the greatest possible OD values for each sample (Figure 3.3). The WNV core antibody dilutions of 1:1600 and 1:2000 indicated the two largest OD values of 0.812 and 0.930, respectively (Figure 3.3). Therefore, the 1:1600 WNV core antibody dilution was used in further trials.

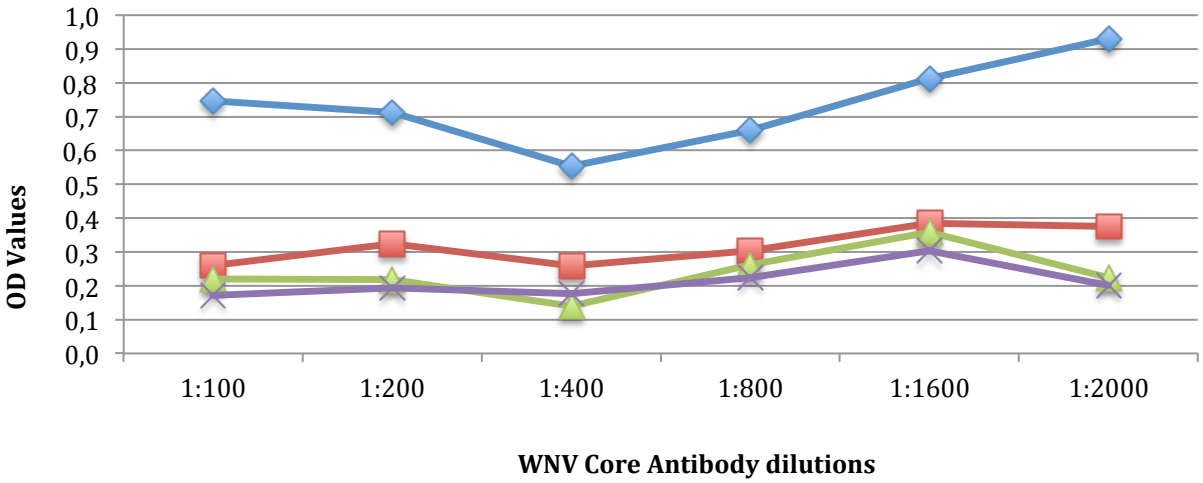

Figure 3.3 Line regressions illustrating the OD values obtained through the checkerboard run for various serum and WNV core antibody dilutions. The blue line ( ) indicates the 1:10 serum dilution, the red line ( ) represents the 1:20 serum dilution, the green line ( ) represents the 1:40 serum dilution and the purple line ( ) indicates the 1:80 serum dilution

In order to evaluate the ELISA, 418 antibody positive and negative serum samples, previously tested with SNT were tested using ELISA and 257 or 61% (95% CI 44-79%) were seropositive for WNV using the ELISA (Table 3.10). The results per province are outlined in Table 3.10. Using a Kappa test, there was a moderate agreement between the ELISA and SNT (Kappa = 0.5).

Table 3.10 Number of horses tested, total seropositive horses, total seronegative horses and the percentage of outliers for each province and overall for South Africa

Province	No. of horses	Seropositive horses (SNT)	Seropositive horses (ELISA)	Apparent Prevalence (%)	Outliers (%)
Gauteng	36	177	17	47	47.2
KwaZulu-Natal	38	93	9	24	36.8
Western Cape	63	116	37	59	27
Northern Cape	51	29	40	78	43.1
Eastern Cape	59	41	40	68	28.8
Free State	34	80	24	71	23.5
North West	34	36	20	59	41.2
Mpumalanga	57	76	45	79	35.1
Limpopo	46	56	25	54	28.3
South Africa	418	704	257	61	35.2

The Mpumalanga Province indicated the highest percentage of seropositive horses (79%; 95% CI 44-79%). KwaZulu-Natal Province indicated the lowest seroprevalence of 24% (95% CI 44-79%). The seroprevalence for WNV across South Africa was 61% (95% CI 44-79%) (Table 3.10).

Outliers were defined as the ELISA results that differed to the results from the SNT for the collected samples. The percentage for the outliers across all nine provinces ranged between 23.5% and 47.2%. There were fewer numbers of samples subjected to the ELISA in comparison to the SNT for the majority of the nine provinces.

3.5 Mosquito analysis

A total of 550 mosquitoes were collected from both the Gauteng and Mpumalanga Provinces, 250 (45.5%) were collected in Gauteng Province and 300 (54.5%) were collected in Mpumalanga Province. There were 2 species identified for Gauteng, including; *Aedes vexans* and *Culex pipiens*, and a futher 3 species identified in Mpumalanga; *Aedes aegypti, Anopheles freeborni* and *Culex quinquefasciatus*.

Overall, 10 pools with a total of 439 mosquitoes were tested, 4 of which were pooled mosquitoes collected in Gauteng Province, where each pool consisted of 50 mosquitoes. Fifty mosquitoes were used to provide a great enough number of individuals for analysis whilst providing enough pools to be tested. The remaining 6 pools each consisted of 50 mosquitoes with the exception of one pool, which contained 39 mosquitoes, were collected in Mpumalanga Province. The spiked positive pooled samples peaked at a temperature of 84.34°C, indicating a positive result for these samples, however, the majority of trial samples of the pooled species all showed varying peaks between 65°C and 70°C, except for one sample, which peaked at 80°C (Figure 3.4). All of these samples peaked below the positive peak of 84.34°C thus all the pooled mosquito samples presented a negative result for WNV.

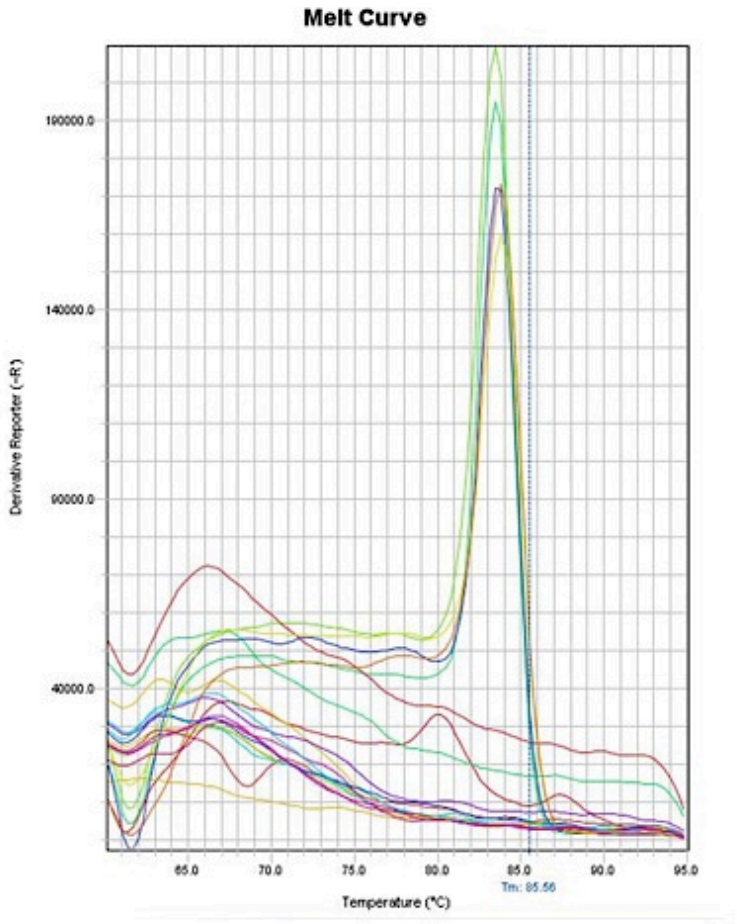

Figure 3.4 Melt curve analysis for WNV in 10 groups of pooled mosquitoes, including 4 spiked positive pools, a negative sample and a blank.

3.6 Univariable analysis of risk factors associated with WNV in the different provinces and South Africa

3.6.1 Gauteng Province

3.6.1.1 Demographic factors

The univariate analysis showed an association between seropositive horses and age (χ^2 = 46.09, df = 31, p = 0.04); breed (χ^2 = 13.23, df = 6, p = 0.04) and discipline (χ^2 = 16.21, df = 8, p = 0.04) (Table 3.11). Hence, these variables were included in the multivariable analysis.

Gender was not associated with seropositivity ($\chi^2 = 2.02$, df = 2, p = 0.36) in the Gauteng Province (Table 3.11).

3.6.1.2 Geographic and environmental factors

The samples from the Gauteng Province were obtained from a previous study and so specific variables were not recorded in a manner concurrent to this study, such as annual rainfall and seasonal temperatures. These variables were not included in the univariable analysis for the Gauteng Province. The Pasture Percentage, which is the average period of time horses spent in the paddock per day, was found to be associated with seropositive horses ($\chi^2 = 14.91$, df = 7, p = 0.04). The movement of horses into and out of towns and provinces was not associated with seroprevalence of WNV in Gauteng (Table 3.11).

Table 3.11 Seroprevalence of WNV for the Gauteng Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	df	$\chi 2$	P-value
Age[a]						
0-3	7	2	28.57	31	46.09	0.04
4-8	69	36	52.17			
9-16	136	91	66.91			
>17	69	43	62.32			
Breed[a]						
Arabian	23	15	65.22	6	13.23	0.04
Nooitgedacht	65	51	78.46			
Other	64	36	56.25			
Pony	42	26	61.90			
Thoroughbred	58	31	53.45			
Warmblood	30	14	46.67			
Discipline[a]						
Breeding	17	11	64.71	8	16.21	0.04
D	1	0	0			
D + S + SJ	1	1	100			
Equestrian events	109	61	55.96			
General pleasure	61	32	52.46			
Other	52	42	80.77			
SJ	1	0	0			
S	1	1	100			
Work horse	41	26	63.41			
Gender						
Gelding	146	84	57.53	2	2.02	0.36
Mare	129	85	65.89			
Stalion	8	5	62.5			
Pasture Percentage (%)[a]						
10-50	113	58	51.33	7	14.91	0.04
60-100	171	116	67.84			
Travelled						
Not travelled	202	125	61.88	2	2.81	0.24
Province	20	15	75			
Town	71	39	54.93			

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; D: Dressage; S: Showing; SJ: Show Jumping

3.6.1.3 Management factors

The neighboring properties housing pigs ($\chi^2 = 6.7$, $p = 0.01$), as well as direct contact with animals ($\chi^2 = 8.8$, $p < 0.01$), including; dogs ($\chi^2 = 7.83$, $p < 0.01$), other ($\chi^2 = 8.2$, $p < 0.01$), pigs ($\chi^2 = 6$, $p = 0.01$) and small ruminants ($\chi^2 = 4.8$, $p = 0.03$) were all associated with seroprevalence of WNV (Table 3.14). Horses that were fed concentrates ($\chi^2 = 10$, $p < 0.01$), were stabled at night ($\chi^2 = 0.2$, $p < 0.01$) and had travelled recently ($\chi^2 = 8.8$, $p < 0.01$) were all significantly associated with seroprevalence and were thus included in the multivariable analysis. Properties that had rodent problems ($\chi^2 = 9.9$, $p < 0.01$) and the occurrence of rodents in the feed storage areas ($\chi^2 = 9.3$, $p < 0.01$), as well as, standing pools of water ($\chi^2 = 3.1$, $p = 0.08$) and bore hole ($\chi^2 = 4.9$, $p = 0.03$), municipal ($\chi^2 = 6.1$, $p = 0.01$) and river water sources ($\chi^2 = 8.2$, $p < 0.01$) were all associated with the seroprevalence of WNV in Gauteng and subjected to further multivariable analysis. The remaining variables that were not associated with seroprevalence are included in Table 3.12.

Table 3.12 Seroprevalence of WNV for the Gauteng Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	293	276	94	0.53	3.81	0.5	0.48
Agricultural activities							
Other	280	276	99	0.22	11.56	0.2	0.64
Cattle	178	152	85	0.5	2.69	0.1	0.74
Animals of neighboring properties							
Other	177	136	77	0.81	3.31	1.9	0.17
Pigs[a]	178	90	51	1.21	4.12	6.7	0.01
Small ruminants	178	1	1	-	-	1.6	0.21
Wild animals	178	40	22	0.36	1.51	0.7	0.4
Concrete floors	235	212	90	0.62	3.5	0.8	0.37
Contact with species							
Cattle	284	1	0	-	-	0.6	0.43
Dog[a]	284	203	71	0.25	0.79	7.83	<0.01
Other[a]	284	105	37	0.3	0.8	8.2	<0.01
Pigs[a]	284	59	21	0.27	0.87	6	0.01
Small ruminants[a]	284	61	21	0.3	0.94	4.8	0.03
Wild animals	284	1	0	-	-	0.6	0.43
Contact with other animals[a]	284	207	73	0.23	0.75	8.8	<0.01
Contact with other horses	128	127	99	-	-	0.6	0.44
Drainage system	284	241	85	0.76	2.8	1.3	0.26
Fed concentrates[a]	284	232	82	0.15	0.66	10	<0.01
Horses have travelled[a]	284	91	32	0.53	1.48	0.2	<0.01
Neighboring properties with animals	284	182	64	0.55	1.49	0.2	0.7
Rodent problem[a]	285	227	80	0.17	0.68	9.9	<0.01
Rodents in feed storage[a]	283	229	81	0.17	0.7	9.3	<0.01
Rodents on property	284	283	100	-	-	1.6	0.21
Horses stabled[a]	284	228	80	0.18	0.72	8.8	<0.01
Standing water[a]	284	95	33	0.95	2.67	3.1	0.08
Water source							
Bore hole[a]	284	124	44	0.36	0.94	4.9	0.03
Dam water	284	1	0	-	-	0.6	0.43
Municipal[a]	284	60	21	1.16	4.2	6.1	0.01
River[a]	284	149	52	1.24	3.28	8.2	<0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.1.4 Historical health records of individual horses

Two of the three WNV symptom related variables showed a significant association to seroprevalence. Both fever ($\chi^2 = 2.22$, p = 0.14) and kidney or liver disease ($\chi^2 = 5.31$, p = 0.02) was included in the multivariable analysis. The symptom ocular disease ($\chi^2 = 1.74$, p = 0.19) was not associated with the seroprevalence of WNV in horses in Gauteng (Table 3.13).

Table 3.13 Seroprevalence of WNV for the Gauteng Province concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever[a]	266	10	3	0.11	1.41	2.22	0.14
Kidney or liver disease[a]	278	6	2	0.01	1.03	5.31	0.02
Ocular disease	284	35	12	0.77	3.65	1.74	0.19

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.2 KwaZulu-Natal Province

3.6.2.1 Demographic factors

Age was not significantly associated with seroprevalence of WNV ($\chi^2 = 18.5$, df = 23, p = 0.7), nor was discipline ($\chi^2 = 6.2$, df = 4, p = 0.2) (Table 3.14). Breed ($\chi^2 = 13.7$, df = 4, p < 0.01) including; Arabian, Other, Pony, Thoroughbred and Warmblood, as well as gender ($\chi^2 = 3.8$, df = 2, p = 0.15) were associated with seropositive horses and was included in the logistic regression.

3.6.2.2 Geographic and environmental factors

The samples from the KwaZulu-Natal Province were obtained from a previous study and so specific variables were not recorded in a manner concurrent to this study, such as annual rainfall and seasonal temperatures. These variables were not included in the univariable analysis for KwaZulu-Natal. The singular geographic variable was the

recent travel of horses ($\chi^2 = 0.32$, $p = 0.6$), which was not significant for seroprevalence of WNV.

Table 3.14 Seroprevalence of WNV for the KwaZulu-Natal Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age							
0-3	8	5	63				
4-8	57	21	37				
9-16	66	41	62	df=23		18.5	0.7
>17	28	20	71				
Breed[a]							
Arabian	158	8	5				
Other	158	12	8				
Pony	158	10	6	df=4		13.7	<0.01
Thoroughbred	158	53	34				
Warmblood	158	4	3				
Discipline							
Breeding	158	3	2				
Equestrian event	158	44	28				
General pleasure	158	19	12	df=4		6.2	0.2
Racing	158	6	4				
Work horse	158	15	9				
Gender[a]							
Gelding	159	44	28				
Mare	159	43	27	df=2		3.8	0.15
Stallion	159	0	0				
Travelled							
Not travelled	148	118	80	0.35	1.79	0.32	0.6
Town	148	30	20				

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.2.3 Management factors

There was a significant association between the agricultural activity 'Other' in the area (χ^2 = 2.8, p = 0.09) and seroprevalence of WNV. Similarly, direct contact with other species, such as; cattle (χ^2 = 2.05, p = 0.15), other species (χ^2 = 2.6, p = 0.11), pigs (χ^2 = 2.6, p = 0.11) and small ruminants (χ^2 = 4.45, p = 0.04), as well as neighboring properties containing animals (χ^2 = 2.6, p = 0.11) were all associated with seropositive horses and subjected to further multivariable analysis (Table 3.15). The remaining management factors all had a P-value > 0.15, and hence did not have any significant association with seroprevalence (Table 3.15).

Table 3.15 Seroprevalence of WNV for the KwaZulu-Natal Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	172	155	90	0.65	4.9	1.26	0.26
Agricultural activities							
Forestry	172	25	15	0.27	1.5	1.19	0.27
Other[a]	172	130	76	0.9	3.67	2.8	0.09
Animals of neighboring properties							
Cattle	172	71	41	0.6	2.15	0.25	0.62
Other	172	70	41	0.37	1.27	1.44	0.23
Small ruminants	172	6	3	0.17	4.3	0.04	0.84
Wild animals	172	8	5	0.11	2.1	0.93	0.34
Concrete floors	172	128	74	0.33	1.35	1.3	0.26
Contact with species							
Cattle[a]	172	71	41	0.85	2.9	2.05	0.15
Dog	172	105	61	0.61	2.1	0.15	0.7
Other[a]	172	23	13	0.83	5.5	2.6	0.11
Pigs[a]	172	23	13	0.83	5.5	2.6	0.11
Small ruminants[a]	172	15	9	1.01	13.8	4.45	0.04
Contact with other horses	172	40	23	0.45	1.87	0.05	0.82
Contact with other animals	172	105	61	0.61	2.1	0.15	0.7
Fed concentrates	172	153	89	0.18	1.4	1.77	0.18
Horses have travelled	148	30	20	0.56	2.86	0.32	0.57
Neighboring properties with animals[a]	172	149	87	0.18	1.2	2.6	0.11
Rodent problem	172	88	51	0.69	2.29	0.55	0.46
Rodents in feed storage	172	96	56	0.67	2.23	0.42	0.52
Rodents on property	172	119	69	0.63	2.29	0.3	0.58
Horses stabled	172	153	89	0.18	1.4	1.77	0.18
Standing water	148	45	30	0.39	1.6	0.48	0.49
Water source							
Bore hole	172	99	58	0.63	2.1	0.2	0.65
Municipal	172	80	47	0.49	1.62	0.15	0.7
River	172	17	10	0.2	1.55	1.3	0.26

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.2.4 Historical health records of individual horses

Fever was the only significant symptom related variable to the seroprevalence of WNV (χ^2 = 2.53, p = 0.11). Kidney or liver disease (χ^2 = 0.83, p = 0.26) was not associated with seropositive horses, along with ocular disease (χ^2 = 0.04, p = 0.85), which also had no significant association (Table 3.16).

Table 3.16 Seroprevalence of WNV for the KwaZulu-Natal Province concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever[a]	157	3	2	-	-	2.53	0.11
Kidney or liver disease	159	1	0.6	-	-	0.83	0.26
Ocular disease	159	4	2.5	0.11	5.99	0.04	0.85

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.3 Western Cape Province

3.6.3.1 Demographic factors

Breed was not significantly associated with the seroprevalence of WNV in Western Cape (χ^2 = 2.7, df = 2, p = 0.25). Conversely, there was a significant association between the age (χ^2 = 42.75, df = 20, p < 0.01), discipline (χ^2 = 12.3, df = 3, p < 0.01), gender (χ^2 = 7.8, df = 1, p < 0.01) and pasture percentage (χ^2 = 21.1, df = 7, p < 0.01), and the seropositive horses. These variables had a P-value < 0.15 and were included in further multivariable analyses.

3.6.3.2 Geographic and environmental factors

The samples from the Western Cape Province were obtained from a previous study and so specific variables were not recorded in a manner concurrent to this study, such as annual rainfall and seasonal temperatures. These variables were not included in the univariable analysis for Western Cape. The singular geographic variable was the recent travel of horses (χ^2 = 0.6, df = 1, p = 0.44), which was not associated with seroprevalence of WNV (Table 3.17).

Table 3.17 Seroprevalence of WNV for the Western Cape Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	df	χ2	P-value
Age[a]						
4-8	56	26	46	20	42.75	<0.01
9-16	88	72	82			
>17	24	15	63			
Breed						
Other	6	4	67	2	2.7	0.25
Thoroughbred	165	109	66			
Warmblood	8	3	38			
Discipline[a]						
Breeding	151	105	70	3	12.3	<0.01
Equestrian events	13	6	46			
General pleasure	5	3	60			
Racing (retired)	10	2	20			
Gender[a]						
Gelding	25	10	40	1	7.8	<0.01
Mare	154	106	69			
Pasture Percentage[a]						
10-50 (%)	22	7	32	7	21.1	<0.01
60-100 (%)	157	109	69			
Travelled						
Not travelled	173	113	65	1	0.6	0.44
Town	6	3	50			

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence

3.6.3.3 Management factors

Particular agricultural activities in the area, such as; crops ($\chi^2 = 5.1$, $p = 0.02$) and 'Other' ($\chi^2 = 2.5$, $p = 0.11$) were associated with the seropositive horses. Likewise, neighboring properties with animals ($\chi^2 = 3.04$, $p = 0.08$), direct contact with dogs ($\chi^2 = 8.7$, $p < 0.01$) and other animal species ($\chi^2 = 4.2$, $p = 0.04$) were also associated with seroprevalence. Other management factors, such as; the presence of a drainage system ($\chi^2 = 8.9$, $p < 0.01$), horses fed concentrates ($\chi^2 = 6.2$, $p = 0.01$), horses stabled at night ($\chi^2 = 6.2$, $p = 0.01$) and standing pools of water ($\chi^2 = 2.5$, $p = 0.12$) were also associated with seroprevalence of WNV. Additionally, there was an association between seropositive horses and rodent problems ($\chi^2 = 4.3$, $p = 0.04$) and presence of rodents in the feed storage area ($\chi^2 = 4.3$, $p = 0.04$) of a property. Lastly, both municipal ($\chi^2 = 13.7$, $p < 0.01$) and river water sources ($\chi^2 = 6.2$, $p = 0.01$) were significant to the seroprevalence, and was subjected, along with the other significant management factors to the multivariable analysis. The remaining variables had P-values > 0.15 and were thus not included in the multivariable analyses (Table 3.18).

Table 3.18 Seroprevalence of WNV for the Western Cape Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	179	163	91	0.7	5.5	1.7	0.19
Agricultural activities[a]							
Crops	179	100	56	1.1	3.8	5.1	0.02
Other	179	63	35	0.32	1.1	2.5	0.11
Animals of neighboring properties							
Cattle	179	106	59	0.75	2.6	1.1	0.3
Other	179	104	58	0.5	1.6	0.2	0.66
Pigs	179	26	15	0.25	1.4	1.6	0.2
Small ruminants	179	14	8	0.3	3.05	<0.01	0.97
Contact with species							
Cattle	179	91	51	0.73	2.5	0.9	0.34
Dog[a]	179	18	10	0.08	0.65	8.7	<0.01
Other[a]	179	7	4	0.04	1.1	4.2	0.04
Small ruminants	179	50	28	0.6	2.4	0.3	0.6
Contact with other horses	179	2	1	0.03	8.8	0.2	0.7
Contact with other animals	179	110	61	0.37	1.3	1.1	0.29
Drainage system[a]	179	146	82	1.4	6.8	8.9	<0.01
Fed concentrates[a]	179	144	80	0.12	0.8	6.2	0.01
Horses have travelled	179	6	3	0.1	2.7	0.6	0.43
Neighboring properties with animals[a]	179	134	75	0.24	1.1	3.04	0.08
Rodent problem[a]	179	166	93	1.01	10.3	4.3	0.04
Rodents in feed storage[a]	179	166	93	1.01	10.3	4.3	0.04
Horses stabled[a]	179	144	80	0.12	0.8	6.2	0.01
Standing water[a]	179	124	69	0.9	3.2	2.5	0.12
Water source							
Bore hole	179	105	59	0.65	2.3	0.4	0.5
Dam water	179	25	14	0.4	2.3	<0.01	0.93
Municipal[a]	179	25	14	0.08	0.5	13.7	<0.01
River[a]	179	35	20	1.2	8.1	6.2	0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.3.4 Historical health records of individual horses

Ocular disease was significantly associated with the seroprevalence of WNV (χ^2 = 2.8, p = 0.09), whereas, fever did not show a significant association to the seropositive horses (χ^2 = 1.3, p = 0.25) and was subjected to multivariable analysis.

Table 3.19 Seroprevalence of WNV for the Western Cape Province concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever	179	3	2	0.02	3	1.3	0.25
Ocular disease[a]	179	4	2	0.02	1.7	2.8	0.09

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.4 Northern Cape Province

3.6.4.1 Demographic factors

There was no significant association between the age groups (χ^2 = 13.4, df = 15, p = 0.6) and breeds (χ^2 = 0.9, df = 4, p = 0.9) with the seroprevalence of WNV in the Northern Cape Province (Table 3.20). Of the 6 individual disciplines within the group of disciplines (χ^2 = 7.1, df = 5, p = 0.2), none were associated with the seropositive horses. Gender (χ^2 = 0.6, df = 2, p = 0.7) also was not associated with seroprevalence. None of the demographic factors had P-values of >0.15, and so were not included in the logistic regression.

3.6.4.2 Geographic and environmental factors

The annual rainfall was separated into either 100-200 mm/annum or 201-300 mm/annum and seasonal temperatures were categorized on the 4 seasons; Autumn, Spring, Summer and Winter. Of the seasonal temperatures, none were significant for seroprevalence since they all had P-values > 0.15 (Table 3.20). Annual rainfall (χ^2 = 1.72, df = 4, p = 0.79) also did not show any significant association to seroprevalence. There

was also no significance between the recent movements (travelling) of horses and the seropositive horses ($\chi^2 < 0.01$, p = 0.98) (Table 3.20).

Table 3.20 Seroprevalence of WNV for the Northern Cape Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age							
0-3	1	1	100				
4-8	18	9	50	df=15		13.4	0.6
9-16	30	17	57				
>17	2	2	100				
Breed							
Arab	51	10	20				
Crossbreed	51	14	27				
Fresian	51	1	2	df=4		0.9	0.9
Nooitgedacht	51	3	6				
Thoroughbred	51	1	2				
Discipline							
Breeding	51	3	6				
E	51	6	12				
General pleasure riding	51	13	25	df=5		7.1	0.2
Not in work	51	2	4				
Retired	51	3	6				
S + D + SJ	51	2	4				
Gender							
Gelding	51	13	25				
Mare	51	13	25	df=2		0.6	0.7
Stallion	51	3	6				
Travelled							
Not travelled	51	37	73	0.28	3.4	<0.01	0.98
Town	51	14	27				
Annual rainfall (mm/annum)							
100-200	46	26	57	df=4		1.72	0.79
201-300	5	3	60				
Seasonal Temperatures							
Autumn	51	29	57	df=2		0.9	0.63
Spring	51	29	57	df=3		1.4	0.7
Summer	51	29	57	df=4		1.7	0.8
Winter	51	29	57	df=3		0.9	0.8

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance S: Showing; SJ: Show Jumping

3.6.4.3 Management factors

The Chi-square results for the management factors showed the P-values of all the variables to be >0.15, thus none of the variables were significantly associated with seroprevalence of WNV (Table 3.21).

Table 3.21 Seroprevalence of WNV for the Northern Cape Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities							
Crops	51	27	53	0.2	1.98	0.6	0.44
Fodder plantation	51	14	27	0.3	3.5	<0.01	0.98
Livestock	51	27	53	0.2	1.98	0.6	0.44
Wildlife	51	14	27	0.3	3.5	<0.01	0.98
Animals of neighboring properties							
Cattle	51	43	84	0.07	2.1	1.3	0.26
Small ruminants	51	27	53	0.2	1.98	0.6	0.44
Wild animals	51	14	27	0.3	3.5	<0.01	0.98
Biting insects							
Culicoides	51	22	43	0.5	5.1	0.7	0.4
Flies	51	30	59	0.3	3	<0.01	0.97
Ticks	51	43	84	0.07	2.1	1.3	0.26
Concrete floors	51	33	65	0.5	4.9	0.5	0.5
Contact with species							
Cattle	51	18	35	0.2	2.1	0.5	0.5
Dog	51	35	69	0.3	3.4	<0.01	0.95
Other	51	8	16	0.5	14.4	1.3	0.26
Small ruminants	51	38	75	0.13	1.9	1.1	0.3
Wild animals	51	19	37	0.34	3.37	0.01	0.9
Contact with other horses	51	33	65	0.5	4.9	0.5	0.5
Contact with wild birds	51	43	84	0.07	2.1	1.3	0.3
Control measures	51	8	16	0.5	14.4	1.3	0.3
Fed Concentrates	51	43	84	0.3	6.3	0.2	0.7
Horses wear protective items	51	8	16	0.5	14.4	1.3	0.3
Horses have travelled	51	14	27	0.3	3.5	<0.01	0.98
Mosquito problem	51	35	69	0.3	3.4	<0.01	0.95
Mosquitoes bite horse	51	8	16	0.5	14.4	1.3	0.3
Mosquitoes per season							
Spring	51	21	41	0.33	3.1	<0.01	0.97

Neighboring properties with animals	51	43	84	0.07	2.1	1.3	0.26
Rodent problem	51	22	43	0.5	5.1	0.7	0.4
Rodents in feed storage	51	33	65	0.5	4.9	0.5	0.46
Rodents on property	51	46	90	0.1	5.7	0.02	0.88
Roaming chickens	51	24	47	0.2	1.8	0.9	0.35
Horses stabled	51	11	22	0.56	10.4	1.4	0.23
Standing water	51	22	43	0.5	5.1	0.7	0.4
Water source							
Dam water	51	5	10	0.2	7.6	0.02	0.88
Municipal	51	22	43	0.5	5.1	0.7	0.4

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.4.4 Historical health records of individual horses

There was an association between seropositive horses and fever ($\chi^2 = 2.1$, p = 0.15), as well as swelling of the supraorbital fossa ($\chi^2 = 2.4$, p = 0.12). Both ocular disease ($\chi^2 = 1.8$, p = 0.18), stiffness in the hindquarter ($\chi^2 = 0.7$, p = 0.4) and swollen joints ($\chi^2 = 0.12$, p = 0.7) were not significantly correlated with seroprevalence (Table 3.22).

Table 3.22 Seroprevalence of WNV for the Northern Cape Province concurring to symptom variables, including results of the univariable analysis

Variable	**No. of horses**	**Seropositive horses**	**AP (%)**	**95% CI**		**χ2**	**P-value**
				Lower	Upper		
Fever[a]	51	12	24	0.7	12.1	2.1	0.15
Ocular disease	51	4	8	0.02	2.3	1.8	0.18
Hindquater stiffness	51	7	14	0.4	12	0.7	0.4
Supraorbital fossa swelling[a]	51	3	6	-	-	2.4	0.12
Swollen joints	51	8	16	0.3	6.2	0.12	0.7

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.5 Eastern Cape Province

3.6.5.1 Demographic factors

The demographic factors; age (χ^2 = 22.5, df = 22, p = 0.4), breed (χ^2 = 8.3, df = 7, p = 0.3), discipline (χ^2 = 11, df = 8, p = 0.45) and gender (χ^2 = 2.3, df = 2, p = 0.7) all had P-values >0.15, therefore were not significantly associated with seroprevalence of WNV (Table 3.23).

3.6.5.2 Geographic and environmental factors

There was no association shown between the seroprevalence of WNV and annual rainfall (χ^2 = 0.27, p = 0.6), seasonal temperature (χ^2 = 1.7, p = 0.2; χ^2 = 0.27, p = 0.6) and the management of horses in the field, where horses are either in paddocks alone or with other horses (shared) (χ^2 = 0.9 p = 0.33) in the Eastern Cape Province.

Table 3.23 Seroprevalence of WNV for the Eastern Cape Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age							
0-3	6	2	33				
4-8	22	13	59				
9-16	39	19	49	df=22		22.5	0.4
>17	17	7	41				
Breed							
Appaloosa	85	1	1				
Arabian	85	0	0				
Boerperd	85	0	0				
Crossbreed	85	19	22				
Nooitgedacht	85	3	4	df=7		8.3	0.3
Thoroughbred	85	10	12				
Warmblood	85	7	8				
Welsh	85	1	1				
Discipline							
Breeding	85	5	6				
D + S	85	0	0				
General pleasure riding	85	22	26				
Not in work	85	2	2				
Polo	85	3	4	df=8		11	0.45
Retired	85	4	5				
SJ	85	1	1				
S	85	2	2				
S +D + SJ	85	2	2				
Gender							
Gelding	85	21	25				
Mare	85	19	22	df=2		2.3	0.7
Stallion	85	1	1				
Annual rainfall (mm/annum)							
600	30	20	67	0.32	7	0.27	0.6
650	30	10	33				
Seasonal Temperatures (°C)							
Spring	30	14	47	0.6	14.3	1.7	0.2
Winter	30	14	47	0.32	7	0.27	0.6
Autumn	30	14	47	0.6	14.3	1.7	0.2
Field management							
Alone	85	0	0	-	-	0.9	0.33
Shared	85	41	48				

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; S: Showing; SJ: Show Jumping

3.6.5.3 Management factors

The singular significant association to the seroprevalence of WNV was direct contact with other horses (χ^2 = 3.8 p = 0.05). The remaining factors all had P-values <0.15 (Table 3.24), hence were not associated with seropositive horses nor included in the multivariable analysis (Table 3.24).

Table 3.24 Seroprevalence of WNV for the Eastern Cape Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	85	26	31	0.4	2.4	<0.01	0.98
Agricultural activities							
Crops	85	7	8	0.6	7.6	1.2	0.27
Fodder plantation	85	1	1	0.07	17.8	<0.01	0.96
Livestock	85	5	6	0.2	1.8	1.1	0.3
Animals of neighboring properties							
Cattle	85	32	38	0.6	4	0.6	0.4
Chickens	85	22	26	0.5	3	0.3	0.6
Other	85	12	14	0.4	2.8	0.04	0.84
Pigs	85	13	15	0.5	3.6	0.5	0.5
Small ruminants	85	9	11	0.23	1.6	1	0.3
Wild animals	85	18	21	0.4	2	0.12	0.7
Biting insects							
Flies	85	25	29	0.3	1.8	0.5	0.5
Ticks	85	19	22	0.3	1.7	0.6	0.4
Concrete floors	85	33	39	0.6	4.7	1.1	0.28
Contact with species							
Cattle	85	18	21	0.3	1.5	0.96	0.3
Dog	85	38	45	0.6	10	1.5	0.2
Other	85	8	9	0.7	8.8	1.9	0.17
Pigs	85	12	14	0.5	3.7	0.5	0.5
Small ruminants	85	5	6	0.2	1.7	1	0.3
Wild animals	85	22	26	0.5	2.7	0.11	0.7
Contact with other horses[a]	85	29	34	1	6	3.8	0.05
Control measures	85	5	6	0.2	1.7	1	0.3
Drainage system	85	7	8	0.3	2.4	0.16	0.7
Fed concentrates	85	31	36	0.2	1.5	1.6	0.2
Horses wear protective items	85	12	14	0.4	2.2	0.07	0.8
Horses have travelled	85	4	5	0.6	1.4	0.24	0.6
Mosquito problem	85	22	26	0.3	2	0.25	0.6
Mosquitoes bite horse	85	15	18	0.24	1.4	1.6	0.2
Mosquitoes per season							
Spring	85	23	27	0.4	2.1	0.08	0.8
Neighboring properties with animals	85	38	45	0.6	10	1.5	0.2
Rodent problem	85	33	39	0.4	3.1	0.01	0.9
Rodents in feed storage	85	33	39	0.4	3.1	0.01	0.9

Roaming chickens	85	27	32	0.6	3.2	0.4	0.5
Horses stabled	85	25	29	0.3	1.8	0.5	0.5
Standing water	85	25	29	0.3	1.8	0.5	0.5
Water source							
Bore hole	85	34	40	0.7	5.8	1.8	0.2
Dam water	85	26	31	0.4	2.4	<0.01	0.98
Municipal	85	15	18	0.5	2.7	0.06	0.8
Other	85	3	4	0.1	1.7	1.5	0.2
River	85	4	5	0.2	2.6	0.3	0.6

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.5.4 Historical health records of individual horses

The symptom related factors; kidney or liver disease ($\chi^2 = 1.1$, $p = 0.3$), ocular disease ($\chi^2 < 0.01$, $p = 0.98$), stiffness in the hindquarter ($\chi^2 < 0.01$, $p = 0.96$), supraorbital fossa swelling ($\chi^2 < 0.01$, $p = 0.96$) and swollen joints ($\chi^2 = 0.05$, $p = 0.47$), all had no significant association with seroprevalence of WNV. However, fever was associated with seroprevalence ($\chi^2 = 3.3$, $p = 0.07$), and consequently was subjected to the multivariable analysis (Table 3.25).

Table 3.25 Seroprevalence of WNV for the Eastern Cape Province concurring to symptom variables, including results of the univariable analysis

Variable	**No. of horses**	**Seropositive horses**	**AP (%)**	**95% CI**		**χ2**	**P-value**
				Lower	Upper		
Fever[a]	85	11	13	0.9	9.1	3.3	0.07
Kidney or liver disease	85	1	1	-	-	1.1	0.3
Ocular disease	85	12	14	0.4	2.5	<0.01	0.98
Hindquater stiffness	85	1	1	0.07	17.8	<0.01	0.96
Supraorbital fossa swelling	85	1	1	0.07	17.8	<0.01	0.96
Swollen joints	85	8	9	0.5	4.9	0.5	0.47

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.6 Free State Province

3.6.6.1 Demographic factors

Age (χ^2 = 43.7, df = 28, p = 0.03), breed (χ^2 = 21.3, df = 9, p = 0.13) and gender (χ^2 = 7.2, df = 2, p = 0.03) were significantly associated with seroprevalence of WNV and were included in the logistic regression. Discipline (χ^2 = 10.6, df = 8, p = 0.6) was not associated with seropositive horses for the Free State Province (Table 3.26).

3.6.6.2 Geographic and environmental factors

The annual rainfall for this province ranged between 468 mm/annum and 764 mm/annum, however was not significantly associated with seroprevalence (χ^2 = 7.6, df = 6, p = 0.3) (Table 3.28). Of all the seasonal temperatures, the winter range (χ^2 = 6.9, df = 4, p = 0.13) was the only significant association to seroprevalence of WNV, the remaining seasons; Autumn (χ^2 = 1.7, df = 3, p = 0.6), Spring (χ^2 = 1.5, df = 3, p = 0.7), Summer (χ^2 = 6.3, df = 5, p = 0.3), were not associated with seroprevalence. Both the factors; recent movement of horses (travelled) (χ^2 = 0.17, p = 0.7) and field management (χ^2 = 0.17, p = 0.7) were not associated with seroprevalence of WNV (Table 3.26).

Table 3.26 Seroprevalence of WNV for the Free State Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age[a]							
0-3	5	1	20				
4-8	37	20	54				
9-16	48	41	85	df=28		43.7	0.03
>17	19	17	89				
Breed[a]							
Anglo Arab	110	1	1				
Arab	110	4	4				
Boerperd	110	4	4				
Connemara	110	7	6				
Crossbreed	110	34	31				
Fresian	110	1	1	df=9		21.3	0.13
Nooitgedacht	110	10	9				
Thoroughbred	110	7	6				
Warmblood	110	13	12				
Welsh Pony	110	0	0				
Discipline							
Breeding	110	12	11				
SJ	110	1	1				
D	110	0	0				
D + SJ + EV	110	3	3				
E	110	7	6				
General pleasure riding	110	33	30	df=8		10.6	0.6
Not in work	110	2	2				
Retired	110	12	11				
S	110	9	8				
Gender[a]							
Gelding	110	40	36				
Mare	110	34	31	df=2		7.2	0.03
Stallion	110	5	5				
Annual rainfall (mm/annum)							
468	110	6	5				
517	110	4	4				
524	110	7	6				
548	110	32	29	df=6		7.6	0.3
600	110	19	17				
700	110	4	4				
764	110	7	6				

Seasonal Temperatures							
Autumn	110	79	72	df=3		1.7	0.6
Spring	110	79	72	df=3		1.5	0.7
Summer	110	79	72	df=5		6.3	0.3
Winter[a]	110	79	72	df=4		6.9	0.13
Travelled							
Not travelled	110	75	68	0.07	5.8	0.17	0.7
Province	110	4	4				
Field management							
Alone	110	4	4	0.17	14.9	0.17	0.7
Shared	110	75	68				

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance; EV: Eventing; S: Showing; SJ: Show Jumping

3.6.6.3 Management factors

The livestock agricultural activities in the area (χ^2 = 2.3, p = 0.12), neighboring properties containing animals (χ^2 = 2.5, p = 0.1) and neighboring properties with cattle (χ^2 = 4.5, p = 0.03), small ruminants (χ^2 = 2.2, p = 0.14) and wild animals (χ^2 = 2.5, p = 0.1) showed a significant association with seroprevalence. Likewise, the biting insects 'Ticks' (χ^2 = 3.4, p = 0.07), rodents present in feed storage areas (χ^2 = 2.3, p = 0.13), horses stabled at night (χ^2 = 2.6, p = 0.1), horses that wear protective items (χ^2 = 3, p = 0.08) and the presence of a drainage system (χ^2 = 2, p = 0.15) were also associated with seropositive horses (Table 3.27). The remaining management factors had P-values >0.15, and were not associated with the seroprevalence of WNV (Table 3.27).

Table 3.27 Seroprevalence of WNV for the Free State Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities							
Crops	110	71	65	0.03	2.4	1.4	0.2
Fodder plantation	110	53	48	0.3	1.8	0.5	0.5
Livestock[a]	110	58	53	0.13	1.3	2.3	0.12
Other	110	5	5	0.1	1.8	1.3	0.3
Animals of neighboring properties							
Cattle[a]	110	53	48	0.1	0.1	4.5	0.03
Chickens	110	9	8	0.2	2.2	0.4	0.5
Other	110	17	15	0.35	2.6	0.01	0.9
Pigs	110	5	5	0.11	1.8	1.3	0.3
Small ruminants[a]	110	36	33	0.2	1.2	2.2	0.14
Wild animals[a]	110	21	19	0.2	1.2	2.5	0.1
Biting insects							
Culicoides	110	51	46	0.3	1.8	0.4	0.5
Flies	110	20	18	0.4	3.1	0.09	0.8
Ticks[a]	110	46	42	0.9	5.2	3.4	0.07
Concrete floors	110	58	53	0.3	2.1	0.2	0.7
Contact with other animal species							
Cattle	110	26	24	0.3	1.8	0.3	0.6
Dog	110	72	65	0.14	3.6	0.17	0.7
Other	110	24	22	0.3	1.6	0.7	0.4
Pigs	110	8	7	0.4	28.2	1.4	0.2
Small ruminants	110	32	29	0.2	1.3	1.9	0.2
Wild animals	110	16	15	0.3	1.9	0.4	0.5
Contact with other horses	110	21	19	0.5	4.2	0.6	0.4
Contact with other animals	110	76	69	0.08	8.4	0.02	0.9
Control measures	110	25	23	0.4	2.4	<0.01	0.95
Drainage system[a]	110	29	26	0.2	1.3	2	0.15
Fed concentrates	110	66	60	0.4	3.6	0.13	0.7
Horses wear protective items[a]	110	12	11	0.7	43.2	3	0.08
Horses have travelled	110	4	4	0.2	14.9	0.2	0.7
Mosquito problem	110	53	48	0.5	2.7	0.07	0.8
Mosquitoes bite horse	110	68	62	0.09	2	1.2	0.3
Mosquitoes per season							
Spring	110	26	24	0.6	3.6	0.5	0.5
Autumn	110	5	5	0.1	1.8	1.3	0.3
Winter	110	5	5	0.1	1.8	1.3	0.3
Neighboring properties with animals[a]	110	61	55	0.1	1.3	2.5	0.1

Other insects bite horse	110	75	68	0.07	5.8	0.2	0.7
Rodent problem	110	40	36	0.6	3.3	0.7	0.4
Rodents in feed storage[a]	110	55	50	0.15	1.3	2.3	0.13
Rodents on property	110	75	68	0.07	5.8	0.17	0.7
Roaming chickens	110	46	42	0.3	1.6	0.8	0.4
Horses stabled[a]	110	44	40	0.9	4.6	2.6	0.1
Standing water	110	44	40	0.34	1.9	0.3	0.6
Water source							
Bore hole	110	75	68	0.07	5.8	0.17	0.7
Dam water	110	26	24	0.5	3	0.15	0.7
Municipal	110	33	30	0.7	4.3	1.5	0.2
River	110	10	9	0.2	2.4	0.2	0.6

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.6.4 Historical health records of individual horses

From the horse samples collected in the Free State, only supraorbital swelling was associated with seroprevalence of WNV (χ^2 = 2.1, p = 0.15). The remaining factors included; fever (χ^2 = 1.5, p = 0.2), ocular disease (χ^2 = 1.8, p = 0.2), stiffness in the hindquarter (χ^2 = 1.6, p = 0.2) and swollen joints (χ^2 = 0.6, p = 0.4), were shown not to be associated with seroprevalence, and so were not incorporated in the multivariable analysis (Table 3.28).

Table 3.28 Seroprevalence of WNV for the Free State Province concurring to symptom variables, including results of the univariable analysis

Variable	**No. of horses**	**Seropositive horses**	**AP (%)**	**95% CI**		**χ2**	**P-value**
				Lower	Upper		
Fever	110	12	11	0.5	12.3	1.5	0.2
Ocular disease	110	9	8	0.5	31.8	1.8	0.2
Hindquater stiffness	110	4	4	-	-	1.6	0.2
Supraorbital fossa swelling[a]	110	5	5	-	-	2.1	0.15
Swollen joints	110	9	8	0.4	9.2	0.6	0.4

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.7 North West Province

3.6.7.1 Demographic factors

All the demographic factors, including; age (χ^2 = 20.1, df = 18, p = 0.3), breed (χ^2 = 8.3, df = 6, p = 0.2), discipline (χ^2 = 6.4, df = 8, p = 0.6) and gender (χ^2 = 1.9, df = 2, p = 0.4), presented P-values > 0.15, thus were not significantly associated with seroprevalence of WNV (Table 3.29). These factors were excluded from the multivariable analysis.

3.6.7.2 Geographic and environmental factors

Annual rainfall ranged between 500mm/annum and 850mm/annum for the North West Province, and was not associated to seroprevalence of WNV (χ^2 = 4.1, df = 5, p = 0.5) (Table 3.29). Likewise, the four seasonal temperature ranges; Autumn (χ^2 = 1.2, df = 3, p = 0.8), Spring (χ^2 = 3.9, df = 5, p = 0.6), Summer (χ^2 = 3.5, df = 3, p = 0.3) and Winter (χ^2 = 2.7, df = 4, p = 0.6), were also not significantly associated with seropositive horses. Field management (sharing or alone) (χ^2 = 0.5, p = 0.5) had a P-value >0.15, and was not associated with seroprevalence of WNV (Table 3.29).

Table 3.29 Seroprevalence of WNV for the North West Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age							
0-3	1	0	0				
4-8	26	13	50				
9-16	47	17	36	df=18		20.1	0.3
>17	10	6	60				
Breed							
Appaloosa	84	2	2				
Arab	84	2	2				
Crossbreed	84	17	20				
Nooitgedacht	84	1	1	df=6		8.3	0.2
Thoroughbred	84	9	11				
Warmblood	84	5	6				
Welsh pony	84	0	0				
Discipline							
Breeding	84	6	7				
D	84	1	1				
E	84	3	4				
General pleasure riding	84	18	21				
Retired	84	3	4	df=8		6.4	0.6
SJ	84	3	4				
S	84	0	0				
S + D	84	2	2				
S + SJ	84	0	0				
Gender							
Gelding	84	16	19				
Mare	84	19	23	df=2		1.9	0.4
Stallion	84	1	1				
Annual rainfall (mm/annum)							
500	84	7	8				
510	84	2	2				
540	84	12	14				
550	84	8	10	df=5		4.1	0.5
650	84	3	4				
850	84	4	5				
Seasonal Temperatures (°C)							
Autumn	84	36	43	df=3		1.2	0.8
Spring	84	36	43	df=5		3.9	0.6
Summer	84	36	43	df=3		3.5	0.3

Winter	84	36	43	df=4		2.7	0.6
Field management							
Alone	84	1	1	0.04	4.3	0.5	0.5
Shared	84	35	42				

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance; S: Showing; SJ: Show Jumping

3.6.7.3 Management factors

The agricultural activity 'Other' (χ^2 = 2.5, p = 0.11) occurring in that area, as well as 'Other' biting insects (χ^2 = 2.3, p = 0.12) were both associated with seroprevalence for WNV (Table 3.30). Likewise, direct contact with small ruminants (χ^2 = 3.1, p = 0.08) was also significantly associated with seroprevalence, as was annual frost (χ^2 = 3.9, p = 0.05). The residual variables displayed P-values > 0.15, therefore did not have significant associations and were excluded from the multivariable analyses (Table 3.30).

Table 3.30 Seroprevalence of WNV for the North West Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	84	31	37	0.3	3.7	<0.01	0.9
Agricultural activities							
Crops	84	6	7	0.2	1.6	1.3	0.3
Fodder plantation	84	27	32	0.2	1.5	1.4	0.2
Livestock	84	12	14	0.4	2.3	0.04	0.8
Other[a]	84	5	6	0.7	20.3	2.5	0.11
Wildlife	84	18	21	0.5	2.6	0.04	0.9
Animals of neighboring properties							
Cattle	84	13	15	0.8	1.9	0.3	0.6
Chickens	84	3	4	0.1	1.6	1.8	0.2
Other	84	10	12	0.5	3.5	0.3	0.6
Pigs	84	1	1	-	-	1.3	0.2
Small ruminants	84	22	26	0.3	1.9	0.3	0.6
Wild animals	84	11	13	0.4	2.7	0.02	0.9
Annual frost[a]	84	34	40	0.9	21.9	3.9	0.05
Biting insects							
Culicoides	84	30	36	0.3	3.2	0	1
Flies	84	22	26	0.4	2.3	0.02	0.9
Other[a]	84	6	7	0.7	12.9	2.3	0.12
Ticks	84	19	23	0.4	2.1	0.1	0.8
Concrete floors	84	31	37	0.3	3.7	<0.01	0.9
Contact with species							
Cattle	84	11	13	0.3	2.2	0.07	0.8
Dog	84	34	40	0.3	8.9	0.2	0.6
Other	84	13	15	0.5	3.1	0.21	0.6
Pigs	84	3	4	0.1	1.6	1.8	0.18
Small ruminants[a]	84	2	2	0.05	1.3	3.1	0.08
Wild animals	84	10	12	0.4	2.7	<0.01	0.9
Contact with other horses	84	20	24	0.6	3.5	0.8	0.4
Contact with other animals	84	36	43	-	-	0.8	0.4
Contact with wild birds	84	28	33	0.4	2.9	<0.01	0.9
Control measures	84	22	26	0.5	2.7	0.07	0.8
Drainage system	84	2	2	0.4	3.7	0.1	0.7
Horses wear protective items	84	5	6	0.2	2.7	0.12	0.7
Horses have travelled	84	3	4	0.13	2.2	0.8	0.4
Mosquito problem	84	24	29	0.4	2.5	0	1
Mosquitoes bite horse	84	19	23	0.4	2.5	0.02	0.9
Mosquitoes per season							

Spring	84	15	18	0.4	2.2	0.04	0.8
Summer	84	29	35	0.3	2.9	<0.01	0.9
Neighboring properties with animals	84	31	37	0.25	3.2	0.04	0.9
Other insects bite horse	84	30	36	0.4	3.6	0.06	0.8
Rodent problem	84	28	33	0.5	3.6	0.3	0.6
Rodents in feed storage	84	35	42	0.3	29.8	1.1	0.3
Rodents on property	84	35	42	0.3	29.8	1.1	0.3
Roaming chickens	84	16	19	0.5	2.9	0.2	0.7
Horses stabled	84	25	30	0.7	4	1.1	0.3
Standing water	84	15	18	0.5	2.6	0.04	0.8
Water source							
Bore hole	84	27	32	0.2	2	0.5	0.5
Dam water	84	7	8	0.3	2.7	0.02	0.9
Municipal	84	20	24	0.7	3.8	1.1	0.3
River	84	7	8	0.3	3.1	<0.01	0.9

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.7.4 Historical health records of individual horses

There was a significant association with fever (χ^2 = 9.3, p < 0.01), swelling of the supraorbital fossa (χ^2 = 5.4, p = 0.02) and swollen joints (χ^2 = 5.3, p = 0.02) and seroprevalence of WNV in the North West Province (Table 3.33). However, ocular disease (χ^2 = 0.4, p = 0.6) and stiffness in the hindquarter (χ^2 = 0.2, p = 0.6) both were not associated with seropositive horses (Table 3.31).

Table 3.31 Seroprevalence of WNV for the North West Province concurring to symptom variables, including results of the univariable analysis

Variable	**No. of horses**	**Seropositive horses**	**AP (%)**	**95% CI**		**χ2**	**P-value**
				Lower	Upper		
Fever[a]	84	15	18	1.7	14.8	9.3	<0.01
Ocular disease	84	7	8	0.4	4.5	0.4	0.6
Hindquater stiffness	84	5	6	0.4	5.2	0.2	0.6
Supraorbital fossa swelling[a]	84	11	13	1.2	12.1	5.4	0.02
Swollen joints[a]	84	14	17	1.2	8.8	5.3	0.02

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.8 Mpumalanga Province

3.6.8.1 Demographic factors

The discipline group incorporated multiple disciplines, as well as wild horses, retired horses, horses that are not in work (too young or due to injury) and horses used for breeding, which were associated with the seroprevalence of WNV ($\chi^2 = 19.2$, df = 11, p = 0.06). There were 13 different breeds of horses included in the breed category, including; Appaloosa, Arabs, Boerperd, Crossbreeds, Fresians, Miniature pony, Nooitgedacht, Noriker, Quarter horse, Thoroughbred, Warmblood, Warmblood cross Thoroughbred and Welsh ponies, which were associated with seropositive horses ($\chi^2 = 18.6$, df = 12, p = 0.1) (Table 3.32). Gender ($\chi^2 = 4.5$, df = 2, p = 0.2) and age group ($\chi^2 = 27.8$, df = 22, p = 0.2) were not significantly associated with seroprevalence and so were omitted from the multivariable analysis (Table 3.32).

3.6.8.2 Geographic and environmental factors

Pasture percentage ranged between 70% and 100% and was significantly associated with seroprevalence ($\chi^2 = 11.6$, df = 3, p < 0.01), along with field management ($\chi^2 = 2.5$, df = 1, p = 0.11), travelling of horses ($\chi^2 = 5.8$, df = 2, p = 0.06) and annual rainfall (ranging between 550 mm/annum and 1000 mm/annum) ($\chi^2 = 20.2$, df = 8, p = 0.01). Of the seasonal temperatures, Spring ($\chi^2 = 14.9$, df = 8, p = 0.06), Summer ($\chi^2 = 14.7$, df = 7, p = 0.04) and Winter ($\chi^2 = 17.1$, df = 6, p < 0.01) were all associated with seroprevalence and were involved in the logistic regression, whereas Autumn ($\chi^2 = 2.8$, df = 4, p = 0.6) was not significantly associated with seroprevalence (Table 3.32).

Table 3.32 Seroprevalence of WNV for the Mpumalanga Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	df	χ2	P-value
Age						
0-3	134	1	1	22	27.8	0.2
4-8	134	50	37			
9-16	134	68	51			
>17	134	15	11			
Breed[a]						
Appaloosa	135	6	4	12	18.6	0.1
Arab	135	13	10			
Boerperd	135	5	4			
Crossbreed	135	37	27			
Fresian	135	0	0			
Miniature pony	135	0	0			
Nooitegedacht	135	1	1			
Noriker	135	0	0			
Quarter Horse	135	1	1			
Thoroughbred	135	7	5			
Warmblood	135	5	4			
Warmblood x TB	135	0	0			
Welsh pony	135	1	1			
Discipline[a]						
Breeding	135	19	14	11	19.2	0.06
D	135	6	4			
D + SJ	135	4	3			
D + SJ + EV	135	2	1			
D + S	135	1	1			
D + S + SJ	135	2	1			
E	135	13	10			
General pleasure riding	135	75	56			
Not in work	135	7	5			
Retired	135	4	3			
S	135	1	1			
Wild	135	1	1			
Gender						
Gelding	135	43	32	2	4.5	0.2
Mare	135	29	21			
Stallion	135	4	3			

Pasture percentage (%)[a]						
70	135	8	6	3	11.6	<0.01
80	135	20	15			
90	135	27	20			
100	135	21	16			
Field management[a]						
Alone	135	10	7	1	2.5	0.11
Shared	135	66	49			
Travelled[a]						
Not travelled	135	71	53	2	5.8	0.06
Province	135	4	3			
Town	135	1	1			
Annual rainfall (mm/annum)[a]						
550	135	10	7	8	20.2	0.01
570	135	8	6			
660	135	4	3			
670	135	12	9			
680	135	13	10			
750	135	7	5			
800	135	3	2			
850	135	3	2			
1000	135	16	12			
Seasonal Temperatures						
Autumn	135	76	56	4	2.8	0.6
Spring[a]	135	76	56	8	14.9	0.06
Summer[a]	135	76	56	7	14.7	0.04
Winter[a]	135	76	56	6	17.1	<0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance; EV: Eventing; S: Showing; SJ: Show Jumping

3.6.8.3 Management factors

The following agricultural activities were all significantly associated with WNV in the Mpumalanga Province; fodder plantation ($\chi^2 = 11.7$, $p < 0.01$), forestry ($\chi^2 = 9.7$, $p < 0.01$), livestock ($\chi^2 = 9.8$, $p < 0.01$) and wildlife ($\chi^2 = 3.2$, $p = 0.08$). Neighboring properties housing chickens ($\chi^2 = 8.3$, $p < 0.01$), small ruminants ($\chi^2 = 2.9$, $p = 0.08$) and wild animals ($\chi^2 = 5.3$, $p = 0.02$) were associated with seropositive horses. There was also a positive association with *Culicoides* ($\chi^2 = 2.6$, $p = 0.1$), ticks ($\chi^2 = 4.3$, $p = 0.04$), other

biting insects (χ^2 = 6.9, p = 0.01), direct contact with horses (χ^2 = 2.6, p = 0.1), other species (χ^2 = 0.5, p < 0.01), small ruminants (χ^2 = 4.4, p = 0.04) and wild animals (χ^2 = 5.2, p = 0.02) and seroprevalence of WNV. The presence of a drainage system (χ^2 = 5.3, p = 0.02), horses that were fed concentrates (χ^2 = 6.1, p = 0.01), standing pools of water (χ^2 = 3.2, p = 0.07) and river water sources (χ^2 = 6.9, p < 0.01) were also associated with seroprevalence. The pest management factors including the presence of a rodent problem (χ^2 = 4.4, p = 0.04), rodents occurring in the feed storage area (χ^2 = 2.8, p = 0.09) and the presence of roaming chickens (χ^2 = 2.2, p = 0.14) were associated with seroprevalence. Equally, vector factors, such as; an occurrence of a mosquito problem (χ^2 = 4.7, p = 0.03), the occurrence of mosquitoes biting horses (χ^2 = 5, p = 0.03) and the presence of mosquitoes per season (χ^2 = 8.4, p < 0.01) were also significantly correlated with the seroprevalence of WNV. Lastly, the time horses were placed in their stables from the paddocks was also associated with seropositive horses (χ^2 = 7, df = 4, p = 0.14). The remaining management factors all had P-values > 0.15, thus were not associated with seroprevalence, and so were not included in the multivariable analysis (Table 3.33).

Table 3.33 Seroprevalence of WNV for the Mpumalanga Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	135	73	54	0.4	8.2	0.5	0.5
Agricultural activities							
Crops	135	16	12	0.9	6.5	0.9	6.5
Fodder plantation[a]	135	29	21	1.8	11.4	11.7	<0.01
Forestry[a]	135	31	23	0.2	0.7	9.7	<0.01
Livestock[a]	135	32	24	1.6	8	9.8	<0.01
Other	135	17	13	0.7	3.9	1.1	0.3
Wildlife[a]	135	11	8	0.8	11.9	3.2	0.08
Animals on neighboring properties							
Cattle	135	54	40	0.4	1.7	0.5	0.5
Chickens[a]	135	27	20	1.5	8.5	8.3	<0.01
Pigs	135	3	2	0.12	2.6	0.5	0.5
Small ruminants[a]	135	27	20	0.9	4.2	2.9	0.08
Wild animals[a]	135	34	25	1.1	5	5.3	0.02
Annual frost	135	66	49	0.3	2.2	0.3	0.6
Biting insects							
Culicoides[a]	135	64	47	0.1	1.3	2.6	0.1
Flies	135	19	14	0.7	3.8	1.3	0.3
Other[a]	135	23	17	0.2	0.8	6.9	0.01
Ticks[a]	135	38	28	1.1	4.3	4.3	0.04
Concrete floors	135	34	25	0.8	3.2	1.6	0.2
Contact with species							
Cattle	135	46	34	0.3	1.3	1.7	0.2
Dog	135	69	51	0.13	2.1	0.8	0.4
Other[a]	135	50	37	0.13	0.7	0.5	<0.01
Pigs	135	3	2	0.12	2.6	0.5	0.5
Small ruminants[a]	135	25	19	1.1	5.5	4.4	0.04
Wild animals[a]	135	53	39	0.15	0.9	5.2	0.02
Contact with other horses[a]	135	51	38	0.2	1.2	2.6	0.1
Control measures	135	28	21	0.6	2.5	0.3	0.6
Drainage system[a]	135	25	19	0.2	0.9	5.3	0.02
Fed concentrates[a]	135	59	44	0.08	0.8	6.1	0.01
Horses wear protective items	135	28	21	0.6	2.7	0.6	0.4
Horses have travelled	135	5	4	0.25	3.8	<0.01	0.96
Mosquito problem[a]	135	33	24	1.1	4.7	4.7	0.03
Mosquitoes bite horse[a]	135	43	32	1.1	4.4	5	0.03
Mosquitoes per season[a]							

Spring	135	10	7	-	-	8.4	<0.01
Neighboring properties with animals	135	67	50	0.5	3.6	0.3	0.6
Other insects bite horse	135	68	50	0.1	1.8	1.3	0.3
Rodent problem[a]	135	25	19	1.1	5.5	4.4	0.04
Rodents in feed storage[a]	135	34	25	0.9	3.8	2.8	0.09
Rodents on property	135	58	43	0.3	1.7	0.5	0.5
Roaming chickens[a]	135	27	20	0.8	3.8	2.2	0.14
Horses stabled	135	34	25	0.7	2.9	1.2	0.3
Standing water[a]	135	63	47	0.1	1.1	3.2	0.07
Time into stable[a]	135	76	56	df=4		7	0.14
Water source							
Bore hole	135	73	54	0.5	9.8	1.2	0.3
Dam water	135	46	34	0.4	1.7	0.2	0.6
Municipal	135	39	29	0.3	1.3	1.8	0.2
Other	135	3	2	0.12	2.6	0.5	0.5
River[a]	135	23	17	0.2	0.8	6.9	<0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.8.4 Historical health records of individual horses

The singular symptom related variable, swollen joints ($\chi^2 = 2.5$, $p = 0.1$), was the only factor associated with the seroprevalence of WNV and was included in the multivariable analysis. The residual symptom related variables; fever ($\chi^2 = 0.3$, $p = 0.6$), kidney or liver disease ($\chi^2 = 1.6$, $p = 0.2$), ocular disease ($\chi^2 = 0.4$, $p = 0.5$), stiffness in the hindquarter ($\chi^2 = 0.09$, $p = 0.8$) and supraorbital fossa swelling ($\chi^2 < 0.01$, $p = 0.9$) were not significantly associated with seropositive horses (Table 3.34).

Table 3.34 Seroprevalence of WNV for the Mpumalanga Province concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever	135	17	13	0.5	2.9	0.3	0.6
Kidney or liver disease	135	2	1	-	-	1.6	0.2
Ocular disease	135	11	8	0.3	1.8	0.4	0.5
Hindquater stiffness	135	9	7	0.3	2.4	0.09	0.8
Supraorbital fossa swelling	135	8	6	0.3	3.2	<0.01	0.9
Swollen joints[a]	135	8	6	0.7	16.4	2.5	0.1

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.9 Limpopo Province

3.6.9.1 Demographic factors

Age (χ^2 = 27.3, df = 20, p = 0.13) and discipline (χ^2 = 21.3, df = 9, p = 0.01) were both significantly associated with the seroprevalence of WNV in the Limpopo Province and were included in the logistic regression. The breed (χ^2 = 4, df = 8, p = 0.9) and gender (χ^2 = 0.3, df = 2, p = 0.9) categories were not significantly associated with seroprevalence (Table 3.35).

3.6.9.2 Geographic and environmental factors

Annual rainfall for the Limpopo Province ranged between 420 mm/annum and 880 mm/annum and was significantly associated with the seroprevalence of WNV (χ^2 = 11.8, df = 6, p = 0.07). The seasonal temperatures; Autumn (χ^2 = 12.9, df = 6, p = 0.04), Spring (χ^2 = 11.8, df = 6, p = 0.07), Summer (χ^2 = 9.4, df = 5, p = 0.1) and Winter (χ^2 = 11.6, df = 7, p = 0.1), all were associated with seropositive horses, as was field management (χ^2 = 3.1, p = 0.08), thus all three variables were subjected to the multivariable analysis (Table 3.35).

Table 3.35 Seroprevalence of WNV for the Limpopo Province concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Age[a]							
0-3	3	0	0				
4-8	19	13	68				
9-16	48	25	52	df=20		27.3	0.13
>17	23	18	78				
Breed							
Appaloosa	94	2	2				
Arab	94	4	4				
Boerperd	94	5	5				
Crossbreed	94	12	13				
Fresian	94	1	1	df=8		4	0.9
Miniature	94	1	1				
Nooitgedacht	94	3	3				
Thoroughbred	94	21	22				
Warmblood	94	7	7				
Discipline[a]							
Breeding	94	7	7				
D + SJ	94	6	6				
E	94	3	3				
General pleasure riding	94	21	22				
Not in work	94	4	4				
Retired	94	9	10	df=9		21.3	0.01
SJ	94	5	5				
S + D	94	0	0				
S + D + SJ	94	0	0				
S + SJ	94	1	1				
Gender							
Gelding	94	29	31				
Mare	94	24	26	df=2		0.3	0.9
Stallion	94	3	3				
Annual rainfall (mm/annum)[a]							
420	94	4	4				
450	94	28	30				
480	94	10	11				
490	94	6	6	df=6		11.8	0.07
495	94	1	1				
560	94	4	4				
880	94	3	3				

Seasonal Temperatures[a]							
Autumn	94	56	60		df=6	12.9	0.04
Spring	94	56	60		df=6	11.8	0.07
Summer	94	56	60		df=5	9.4	0.1
Winter	94	56	60		df=7	11.6	0.1
Field management[a]							
Alone	94	14	15				
Shared	94	42	45	0.9	9.4	3.1	0.08

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance; S: Showing; SJ: Show Jumping

3.6.9.3 Management factors

The agricultural activity of crops was the only agricultural activity in the Limpopo Province that was significantly associated with seroprevalence of WNV ($\chi^2 = 2.4$, $p = 0.1$). Likewise, direct contact with cattle ($\chi^2 = 3.2$, $p = 0.07$), the presence of other species of animals on neighboring properties ($\chi^2 = 5.9$, $p = 0.01$), *Culicoides* biting insects ($\chi^2 = 2.8$, $p = 0.09$) and the presence of a rodent problem ($\chi^2 = 3.1$, $p = 0.08$) were associated with seroprevalence. Lastly, horses that were fed concentrates ($\chi^2 = 4.1$, $p = 0.04$), standing pools of water ($\chi^2 = 2.1$, $p = 0.15$) and dam ($\chi^2 = 3.9$, $p = 0.05$) and municipal ($\chi^2 = 5.6$, $p = 0.02$) water sources were also associated with seropositive horses and involved in the multivariable analysis. The residual management factors all had a P-value > 0.15, thus were not significantly associated with seroprevalence (Table 3.36).

Table 3.36 Seroprevalence of WNV for the Limpopo Province concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area	94	53	56	0.2	7.9	0.2	0.6
Agricultural activities							
Crops[a]	94	15	16	0.2	1.2	2.4	0.1
Fodder plantation	94	5	5	0.3	5.1	0.03	0.9
Livestock	94	27	29	0.3	1.6	0.9	0.3
Wildlife	94	39	41	0.7	3.9	1.4	0.2
Animals of neighboring properties							
Cattle	94	20	21	0.4	2.2	0.01	0.9
Chickens	94	11	12	0.3	2.5	0.03	0.9
Other[a]	94	8	9	-	-	5.9	0.01
Pigs	94	4	4	0.1	2	1	0.3
Small ruminants	94	11	12	0.3	2.5	0.03	0.9
Wild animals	94	39	41	0.7	3.9	1.4	0.2
Biting insects							
Culicoides[a]	94	24	26	0.2	1.1	2.8	0.09
Flies	94	33	35	0.5	2.4	0.01	0.9
Other	94	10	11	0.2	1.6	1	0.3
Ticks	94	23	24	0.4	2	0.1	0.7
Concrete floors	94	30	32	0.4	1.9	0.2	0.7
Contact with species							
Cattle[a]	94	15	16	0.2	1.1	3.2	0.07
Dog	94	29	31	0.5	2.7	0.2	0.7
Pigs	94	4	4	0.1	2	0.9	0.3
Small ruminants	94	8	9	0.3	2.8	0.04	0.8
Wild animals	94	35	37	0.5	2.8	0.2	0.7
Contact with other horses	94	22	23	0.7	3.8	1	0.3
Contact with other animals	94	50	53	0.6	6.1	1.1	0.3
Contact with wild birds	94	50	53	0.6	6.1	1.1	0.3
Control measures	94	37	39	0.6	3.3	0.7	0.4
Drainage system	94	18	19	0.7	4.6	1.4	0.2
Fed concentrates[a]	94	53	56	1	16.6	4.1	0.04
Horses wear protective items	94	20	21	0.5	2.5	0.02	0.9
Horses have travelled	94	9	10	0.4	4.1	0.2	0.7
Mosquito problem	94	25	27	0.3	1.5	1	0.3
Mosquitoes bite horse	94	9	10	0.3	2.1	0.4	0.5

Mosquitoes per season							
Autumn	94	7	7	0.4	6.9	0.5	0.5
Spring	94	11	12	0.3	2.5	0.03	0.9
Neighboring properties with animals	94	43	46	0.5	3.4	0.4	0.5
Other insects bite horse	94	49	52	0.1	2.5	0.5	0.5
Rodent problem[a]	94	23	24	0.9	5.6	3.1	0.08
Rodents in feed storage	94	29	31	0.5	2.5	0.03	0.9
Rodents on property	94	45	48	0.4	3	0.03	0.9
Roaming chickens	94	14	15	0.6	5.1	1.2	0.3
Horses stabled	94	37	39	0.6	3.3	0.7	0.4
Standing water[a]	94	8	9	0.2	1.3	2.1	0.15
Water source							
Bore hole	94	44	47	0.4	2.7	<0.01	0.97
Dam water[a]	94	4	4	0.08	1	3.9	0.05
Municipal[a]	94	45	48	1.2	7.5	5.6	0.02
River	94	8	9	0.5	7.9	0.9	0.3

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.9.4 Historical health records of individual horses

Ocular disease ($\chi^2 = 3.9$, $p = 0.05$) and swelling of the supraorbital fossa ($\chi^2 = 2.1$, $p = 0.15$) were both significantly associated with seroprevalence of WNV. However, the symptom related variables, including; fever ($\chi^2 = 0.4$, $p = 0.5$), kidney or liver disease ($\chi^2 = 1.4$, $p = 0.2$), stiffness in the hindquarter ($\chi^2 = 1.9$, $p = 0.2$) and swollen joints ($\chi^2 = 1.6$, $p = 0.2$) were not associated with seroprevalence, thus excluded from the logistic regression (Table 3.37).

Table 3.37 Seroprevalence of WNV for the Limpopo Province concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever	94	15	16	0.5	3.7	0.4	0.5
Kidney or liver disease	94	2	2	-	-	1.4	0.2
Ocular disease[a]	94	11	12	0.9	21.1	3.9	0.05
Hindquater stiffness	94	8	9	0.6	15	1.9	0.2
Supraorbital fossa swelling[a]	94	3	3	-	-	2.1	0.15
Swollen joints	94	15	16	0.7	5.6	1.6	0.2

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.10 South Africa

3.6.10.1 Demographic factors

Gender was the singular demographic factor that was not associated for WNV for South Africa (χ^2 = 10.1, df = 2, p = 0.3). Conversely, the categories; age (χ^2 = 57.8, df = 36, p = 0.01), breed (χ^2 = 50.5, df = 15, p = 0.01) and discipline (χ^2 = 63, df = 16, p < 0.01) were all significantly associated with seroprevalence and were included in the multivariable analysis (Table 3.38).

3.6.10.2 Geographic and environmental factors

The annual rainfall across South Africa ranged between 106 mm/annum and 1000 mm/annum and pasture percentage ranged between 0% and 100% (Table 3.38). Annual rainfall (χ^2 = 54.5, df = 31, p < 0.01), pasture percentage (χ^2 = 19.6, df = 10, p = 0.03), as well as seasonal temperature for Autumn (χ^2 = 23.6, df = 15, p = 0.07), Spring (χ^2 = 35.1, df = 16, p < 0.01) and Summer (χ^2 = 26, df = 12, p = 0.01) were all significantly associated with seroprevalence of WNV. Likewise, the sharing or lack thereof of paddock space between horses (field management) (χ^2 = 3.6, df = 1, p = 0.06) was also associated with seroprevalence, but recent movement of horses (travelled) (χ^2 = 3.5, df = 2, p = 1.2) was not associated with seropositive horses (Table 3.38).

Table 3.38 Seroprevalence of WNV for South Africa concurring to demographic, geographic and environmental variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	df	χ2	P-value
Age[a]						
0-3	31	11	35	36	57.8	0.01
4-8	352	173	49			
9-16	570	367	64			
>17	207	138	67			
Breed[a]						
Anglo Arab	1180	1	0	15	50.5	0.01
Appaloosa	1180	11	1			
Arabian	1180	56	5			
Boerperd	1180	14	1			
Connemara	1180	7	1			
Crossbreed	1180	169	14			
Fresian	1180	3	0			
Irish Sport Horse	1180	1	0			
Miniature	1180	1	0			
Nooitegedacht	1180	72	6			
Noriker	1180	0	0			
Other	1180	52	4			
Quarter Horse	1180	1	0			
Thoroughbred	1180	248	21			
Warmblood	1180	56	5			
Welsh	1180	2	0			
Discipline[a]						
Breeding	1180	166	14	16	63	<0.01
D	1180	4	0			
D + SJ	1180	9	1			
D + SJ + EV	1180	4	0			
D + S	1180	3	0			
D + S + SJ	1180	8	1			
E	1180	31	3			
General pleasure riding	1180	196	17			
Not in work	1180	14	1			
Other	1180	153	13			
Polo	1180	3	0			
Racing (Retired)	1180	8	1			
Retired	1180	32	3			
SJ	1180	10	1			
S	1180	12	1			

Wild	1180	0	0			
Work horse	1180	41	3			
Gender						
Gelding	1180	300	25			
Mare	1180	372	32	2	10.1	0.3
Stallion	1180	22	2			
Field management[a]						
Alone	563	29	5	1	3.6	0.06
Shared	563	290	52			
Pasture percentage (%)[a]						
0	1194	27	2			
10	1194	1	0			
20	1194	13	1			
40	1194	71	6			
50	1194	28	2			
60	1194	93	8	10	19.6	0.03
70	1194	35	3			
80	1194	101	8			
90	1194	128	11			
95	1194	22	2			
100	1194	181	15			
Travelled						
Not travelled	1179	586	50			
Province	1179	29	2	2	3.5	1.2
Town	1179	79	7			
Annual rainfall (mm/annum)[a]						
106	504	6	1			
150	504	8	2			
199	504	6	1			
200	504	6	1			
240	504	3	1			
420	504	4	1			
450	504	28	6			
468	504	6	1	31	54.5	<0.01
480	504	10	2			
490	504	6	1			
495	504	1	0			
500	504	7	1			
510	504	2	0			
517	504	4	1			
524	504	7	1			
540	504	12	2			

548	504	32	6			
550	504	18	4			
560	504	4	1			
570	504	8	2			
600	504	29	6			
650	504	7	1			
660	504	4	1			
670	504	12	2			
680	504	13	3			
700	504	4	1			
750	504	7	1			
764	504	7	1			
800	504	3	1			
850	504	7	1			
880	504	3	1			
1000	504	16	3			
Seasonal Temperatures[a]						
Autumn	504	290	58	15	23.6	0.07
Spring	504	290	58	16	35.1	<0.01
Summer	504	290	58	12	26	0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval; D: Dressage; E: Endurance; EV: Eventing; S: Showing; SJ: Show Jumping

3.6.10.3 Management factors

The overall presence of agricultural activities ($\chi^2 = 6.3$, $p = 0.01$) in South Africa and the specific agricultural activities of crops ($\chi^2 = 6.2$, $p = 0.01$), forestry ($\chi^2 = 9.6$, $p < 0.01$) and other activities ($\chi^2 = 2.6$, $p = 0.11$) were significantly associated with the seroprevalence of WNV. The occurrence of neighboring properties containing animals ($\chi^2 = 4.2$, $p = 0.04$), contact with other animals overall ($\chi^2 = 4.7$, $p = 0.03$) and specific species, including; dogs ($\chi^2 = 7$, $p < 0.01$), other species ($\chi^2 = 4.3$, $p = 0.04$), pigs ($\chi^2 = 2.5$, $p = 0.11$) and wild animals ($\chi^2 = 2.6$, $p = 0.11$) were all associated with seroprevalence. Two of the water sources; river ($\chi^2 = 2.2$, $p = 0.12$) and other sources ($\chi^2 = 3.9$, $p = 0.05$), as well as the presence of drainage systems ($\chi^2 = 2.6$, $p = 0.11$) and presence of biting insects, including *Culicoides* ($\chi^2 = 5.4$, $p = 0.02$) and other biting insects ($\chi^2 = 3.8$, $p = 0.05$) were also significantly associated with the seroprevalence of WNV (Table 3.39). Lastly, the time at which horses were brought in from the paddocks and placed in the stables was

also significantly associated with seroprevalence (χ^2 = 19.2, df = 11, p = 0.06). These management factors were subjected to further multivariable analysis. All the variables that had a P-value > 0.15 were not associated with seroprevalence and therefore, were excluded from the multivariable analyses (Table 3.39).

Table 3.39 Seroprevalence of WNV for South Africa concurring to management variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Agricultural activities of area[a]	1203	655	54	1.1	2.5	6.3	0.01
Agricultural activities							
Crops[a]	1203	201	17	1.1	1.8	6.2	0.01
Fodder plantation	1203	123	10	0.8	1.5	0.3	0.6
Forestry[a]	1203	42	3	0.3	0.8	9.6	<0.01
Livestock	1203	148	12	0.8	1.4	0.3	0.6
Other[a]	1203	338	28	1	1.5	2.6	0.11
Wildlife	1203	76	6	0.7	1.4	<0.01	0.96
Annual Frost	535	213	40	0.8	1.6	0.2	0.6
Animals of neighboring properties							
Cattle	1087	400	37	0.8	1.3	0.1	0.7
Chickens	1084	72	7	0.7	1.5	<0.01	0.97
Other	1083	233	22	0.9	1.5	0.8	0.4
Pigs	1087	103	9	0.9	1.7	1.1	0.3
Small ruminants	1085	131	12	0.6	1.1	1.6	0.2
Wild animals	1084	156	14	0.7	1.3	0.06	0.8
Biting insects							
Culicoides[a]	559	224	40	0.4	0.9	5.4	0.02
Flies	559	136	24	0.6	1.2	0.6	0.4
Other[a]	559	39	7	0.4	1	3.8	0.05
Ticks	559	168	30	0.8	1.6	0.8	0.4
Concrete floors	1145	510	45	0.9	1.5	0.7	0.4
Contact with species							
Cattle	1194	231	19	0.7	1.1	1	0.3
Dog[a]	1194	440	37	0.6	0.9	7	<0.01
Other[a]	1194	172	14	0.6	1	4.3	0.04
Pigs[a]	1194	74	6	0.5	1.1	2.5	0.11
Small ruminants	1194	168	14	0.8	1.3	<0.01	0.97
Wild animals[a]	1194	148	12	0.6	1.1	2.6	0.11
Contact with other horses	1038	264	25	0.8	1.3	<0.01	0.95
Contact with other animals[a]	1194	550	46	0.5	1	4.7	0.03

Contact with wild birds	563	299	53	0.7	2.5	0.8	0.4
Control measures	559	123	22	0.8	1.5	0.2	0.7
Drainage system[a]	1172	423	36	1	1.5	2.6	0.11
Fed concentrates	1193	569	48	0.3	0.7	17.4	3.1
Horses wear protective items	559	83	15	0.8	1.7	0.7	0.4
Horses have travelled	1170	108	9	0.7	1.3	0.06	0.8
Mosquito problem	559	177	32	0.8	1.5	0.4	0.6
Mosquitoes bite horse	559	160	29	0.9	1.7	1.6	0.2
Mosquitoes per season							
Autumn	559	12	2	0.5	3.4	0.3	0.6
Spring	559	97	17	0.7	1.5	0.05	0.8
Summer	559	310	55	0.6	4.7	1.1	0.3
Winter	559	5	1	0.3	3.6	<0.01	0.9
Neighboring properties with animals[a]	1194	532	45	0.6	1	4.2	0.04
Other insects bite horse	559	292	52	0.4	1.6	0.3	0.6
Rodent problem	1195	453	38	0.9	1.5	1.7	0.2
Rodents in feed storage	1193	502	42	0.8	1.3	0.07	0.8
Rodents on property	1194	636	53	0.8	1.8	0.8	0.4
Roaming chickens	563	144	26	0.9	1.7	1	0.3
Horses stabled	1193	470	39	0.7	1.2	0.8	0.4
Standing water	1170	342	29	0.8	1.3	0.2	0.6
Time into stable[a]	559	317	57	df=11		19.2	0.06
Water source							
Bore hole	1194	474	40	0.7	1.2	0.3	0.6
Dam water	1194	129	11	0.6	1.1	1.3	0.3
Municipal	1194	261	22	0.8	1.3	0.02	0.9
Other[a]	1194	6	1	0.1	1	3.9	0.05
River[a]	1194	191	16	0.9	1.6	2.2	0.12

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.6.10.4 Historical health records of individual horses

The symptom related variables that were significantly associated with seroprevalence of WNV in South Africa were fever ($\chi^2 = 5$, $p = 0.03$), supraorbital fossa swelling ($\chi^2 = 4.6$, $p = 0.03$) and swollen joints ($\chi^2 = 6.1$, $p = 0.01$). Kidney or liver disease ($\chi^2 < 0.01$, $p = 0.97$), ocular disease ($\chi^2 = 0.5$, $p = 0.5$) and stiffness in the hindquarter ($\chi^2 = 1.5$, $p = 0.2$) were not associated with seroprevalence (Table 3.40).

Table 3.40 Seroprevalence of WNV for South Africa concurring to symptom variables, including results of the univariable analysis

Variable	No. of horses	Seropositive horses	AP (%)	95% CI		χ2	P-value
				Lower	Upper		
Fever[a]	1011	87	9	1.1	2.3	5	0.03
Kidney or liver disease	1167	7	1	0.3	3.1	<0.01	0.97
Ocular disease	1020	79	8	0.8	1.7	0.5	0.5
Hindquater stiffness	563	33	6	0.8	2.6	1.5	0.2
Supraorbital fossa swelling[a]	563	33	6	1.1	4	4.6	0.03
Swollen joints[a]	563	61	11	1.1	3	6.1	0.01

[a]Variable significant (P<0.15) and thus, subjected to the multiple logistic regression.

AP: Apparent Prevalence; CI: Confidence Interval

3.7 Multivariable analysis of risk factors associated with WNV in the different provinces

3.7.1 Gauteng Province

Of the 20 risk factors subjected to the multivariable logistic regression analysis, only direct contact with dogs (P_{Wald} = 0.01, OR = 0.47) and a river water source (P_{Wald} = 0.01, OR = 1.92) were associated with the seroprevalence of WNV in Gauteng (Table 3.41).

Table 3.41 Multivariable analysis for WNV risk factors in the Gauteng Province

Variables	B	SE	OR	95% CI		P_{Wald}
				Lower	Upper	
Contact with species						
Dog	-0.75	0.29	0.47	-1.32	-0.17	0.01
Water Source						
River	0.65	0.25	1.92	0.16	1.14	0.01

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.2 KwaZulu-Natal Province

Breed, gender, other agricultural activities, direct contact with cattle, other species, pigs and small ruminants, neighboring properties with animals and fever were all included in the multivariable logistic regression. The multivariable analysis results for KwaZulu-Natal showed an association with Thoroughbred (P_{Wald} = 0.05, OR = 0.12) and Warmblood (P_{Wald} = 0.03, OR = 0.07) breeds and direct contact with small ruminants (P_{Wald} = 0.05, OR = 3.75) and seroprevalence of WNV in this province (Table 3.42). Breed and direct contact with other animal species were separately analyzed in order to minimize any bias resulting from collinearity between the variables.

Table 3.42 Multivariable analysis for WNV risk factors in the KwaZulu-Natal Province

Variables	B	SE	OR	95% CI		Pwald
				Lower	Upper	
Breed						
Thoroughbred	-2.13	1.08	0.12	-	-	0.05
Warmblood	-2.64	1.23	0.07	-	-	0.03
Contact with species						
Small ruminants	1.32	0.66	3.75	0.02	2.63	0.05

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.3 Western Cape Province

There were 18 risk factors included in the multivariable analysis for the Western Cape Province. Of these risk factors, 'other' agricultural activities (P_{Wald} = 0.02, OR = 0.44), direct contact with other species (P_{Wald} = 0.03, OR = 0.16), retired racehorses (P_{Wald} = 0.01, OR = 0.11), gender (P_{Wald} = 0.01, OR = 3.31), 90% pasture percentage (P_{Wald} = 0.01, OR = 4.71), 100% pasture percentage (P_{Wald} < 0.01, OR = 8.86) and standing water (P_{Wald} = 0.04, OR = 2.06) were significantly associated with seroprevalence of WNV (Table 3.43). The discipline, gender and pasture percentage groups were analyzed independently to the agricultural activities and contact with species groups to circumvent biasness from collinearity between risk factors.

Table 3.43 Multivariable analysis for WNV risk factors in the Western Cape Province

Variables	B	SE	OR	95% CI		Pwald
				Lower	Upper	
Agricultural activities						
Other	-0.82	0.35	0.44	-1.51	-0.13	0.02
Contact with species						
Other	-1.86	0.87	0.16	-3.58	-0.15	0.03
Discipline						
Racing (retired)	-2.21	0.81	0.11	-	-	0.01
Gender						
Mare	1.2	0.44	3.31	-	-	<0.01
Pasture percentage (%)						
90	1.55	0.57	4.71	-	-	0.01
100	2.18	0.68	8.86	-	-	<0.01
Standing water	0.72	0.36	2.06	0.02	1.43	0.04

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.4 Northern Cape Province

The only risk factors included in the multivariable logistic regression were the symptom related factors fever and swelling of the supraorbital fossa. However, none of these risk factors were significantly associated with seroprevalence of WNV in the Northern Cape Province.

3.7.5 Eastern Cape Province

Direct contact with other horses and fever were the only risk factors subjected to the multivariable analysis, and of these, direct contact with other horses (P_{Wald} = 0.05, OR = 2.42) was associated with seroprevalence of WNV in the Eastern Cape Province (Table 3.44).

Table 3.44 Multivariable analysis for WNV risk factors in the Eastern Cape Province

Variables	B	SE	OR	95% CI		P_{Wald}
				Lower	Upper	
Contact with species						
Horses	0.88	0.46	2.42	-0.01	1.78	0.05

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.6 Free State Province

Fifteen risk factors were subjected to the multivariable logistic regression analysis. Evaluation of the multivariable results demonstrated that livestock agricultural activity (P_{Wald} = 0.03, OR = 0.26), the discipline of general pleasure riding (P_{Wald} = 0.04, OR = 3.33), the gender mares (P_{Wald} = 0.01, OR = 0.28) and neighboring properties housing small ruminants (P_{Wald} = 0.03, OR = 0.36) were associated with seroprevalence of WNV in the Free State Province (Table 3.45). The discipline and gender groups were analyzed separately to livestock agricultural activity and small ruminants on neighboring properties to decrease any probability of bias between these variables due to collinearity.

Table 3.45 Multivariable analysis for WNV risk factors in the Free State Province

Variables	B	SE	OR	95% CI		Pwald
				Lower	Upper	
Agricultural activities						
Livestock	-1.34	0.63	0.26	-2.58	-0.10	0.03
Discipline						
General pleasure riding	1.20	0.58	3.33	-	-	0.04
Gender						
Mare	-1.26	0.49	0.28	-	-	0.01
Neighboring animals						
Small ruminants	-1.01	0.47	0.36	-1.92	-0.09	0.03

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.7 North West Province

Other agricultural activities, annual frost, other biting insects, direct contact with small ruminants and symptom related factors; fever and swollen joints were all included in the multivariable analysis. Of which, annual frost (P_{Wald} = 0.06, OR = 4.47) and fever (P_{Wald} < 0.01, OR = 5) were the only significantly associated risk factors to seroprevalence of WNV for the North West Province (Table 3.46). Annual frost was analyzed separately to fever due to probable bias as a result of collinearity.

Table 3.46 Multivariable analysis for WNV risk factors in the North West Province

Variables	B	SE	OR	95%CI		Pwald
				Lower	Upper	
Annual frost	1.5	0.81	4.47	-0.09	3.09	0.06
Fever	1.6	0.6	5	0.53	2.69	<0.01

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.8 Mpumalanga Province

There were 34 risk factors shown significant to seroprevalence with the Chi-squared test, which were included in the multivariable logistic regression. The multivariable analysis results indicated that of these 34 factors, the fodder plantation agricultural activity (P_{Wald} < 0.01, OR = 4.86), the general pleasure riding discipline (P_{Wald} = 0.04, OR = 0.31), the presence of chickens on neighboring properties (P_{Wald} < 0.01, OR = 3.77) and horses brought into stables from their paddocks at 2pm (P_{Wald} = 0.04, OR = 3.92) were significantly associated with seroprevalence. Likewise, seasonal temperatures in spring of 18°C (P_{Wald} = 0.01, OR = 5.69) and 24°C (P_{Wald} = 0.01, OR = 4.9) and in winter of 10°C (P_{Wald} = 0.02, OR = 0.21) were also significant for seroprevalence of WNV in the Mpumalanga Province (Table 3.47). The discipline and seasonal temperature groups were separately analyzed to minimize bias between the variables as a result of collinearity.

Table 3.47 Multivariable analysis for WNV risk factors in the Mpumalanga Province

Variables	B	SE	OR	95% CI		Pwald
				Lower	Upper	
Agricultural activities						
Fodder plantation	1.58	0.48	4.86	0.64	2.52	<0.01
Discipline						
General pleasure riding	-1.16	0.57	0.31	-	-	0.04
Neighbouring animals						
Chickens	1.33	0.47	3.77	0.41	2.24	<0.01
Seasonal Temperature						
Spring: 18°C	1.74	0.65	5.69	-	-	0.01
Spring: 24°C	1.59	0.61	4.9	-	-	0.01
Winter: 10°C	-1.57	0.66	0.21	-	-	0.02
Time into stable: 2pm	1.37	0.68	3.92	-	-	0.04

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.9 Limpopo Province

There were 19 risk factors incorporated into the multivariable analysis. The results indicated that annual frost (P_{Wald} < 0.01, OR = 0.22), horses not currently in work (P_{Wald} = 0.05, OR = 0.19) and the presence of a rodent problem (P_{Wald} = 0.02, OR = 3.29) were all significant for seropositive horses. Seasonal temperatures, including; summer of 36°C (P_{Wald} = 0.04, OR = 6.67) and 38°C (P_{Wald} = 0.02, OR = 5.33), spring of 32°C (P_{Wald} < 0.01, OR = 10), winter of 28°C (P_{Wald} = 0.01, OR = 8.33) and autumn of 29°C (P_{Wald} = 0.04, OR = 11.67) and 35°C (P_{Wald} = 0.01, OR = 8.33) were also all significantly associated with seroprevalence of WNV in the Limpopo Province (Table 3.48). Discipline and seasonal temperatures were separately analyzed so as to decrease bias due to collinearity between the variables.

Table 3.48 Multivariable analysis for WNV risk factors in the Limpopo Province

Variables	**B**	**SE**	**OR**	**95% CI**		**Pwald**
				Lower	Upper	
Annual frost	-1.51	0.48	0.22	-2.45	-0.57	<0.01
Discipline						
Not in work	-1.66	0.85	0.19	-	-	0.05
Rodent problem	1.19	0.52	3.29	0.17	2.21	0.02
Seasonal Temperature						
Summer: 36°C	1.90	0.94	6.67	-	-	0.04
Summer: 38°C	1.67	0.73	5.33	-	-	0.02
Spring: 32°C	2.30	0.81	10	-	-	<0.01
Winter: 28°C	2.12	0.82	8.33	-	-	0.01
Autumn: 29°C	2.46	1.19	11.67	-	-	0.04
Autumn: 35°C	2.12	0.82	8.33	-	-	0.01

B: Regression Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

3.7.10 South Africa

There were 28 factors subjected to the multivariable logistic regression. From the overall 28 factors, the forestry (P_{Wald} = 0.01, OR = 0.5) and other agricultural activities (P_{Wald} = 0.01, OR = 2.06), direct contact with dogs (P_{Wald} = 0.01, OR = 1.67), fever (P_{Wald} = 0.03, OR = 1.56), pasture percentage of 50% (P_{Wald} = 0.04, OR = 2.51) and 100% (P_{Wald} = 0.02, OR = 1.98) and the time that horses are brought into their stables from the paddocks being 5pm (P_{Wald} = 0.01, OR = 2.2) were all significantly associated with seroprevalence. Likewise, the following disciplines were also associated with seropositive horses; dressage (P_{Wald} = 0.03, OR = 0.25), equestrian event (P_{Wald} = 0.02, OR = 0.65), not in work (P_{Wald} = 0.01, OR = 0.37), other (P_{Wald} = 0.02, OR = 2.39), retired from racing and show jumping (P_{Wald} = 0.02, OR = 0.35). Lastly, seasonal temperatures including; spring at 18°C (P_{Wald} = 0.01, OR = 5.69), 23°C (P_{Wald} = 0.02, OR = 3.79), 24°C (P_{Wald} = 0.02, OR = 2.8), 25°C (P_{Wald} = 0.01, OR = 2.67), 27°C (P_{Wald} < 0.01, OR = 3.87) and 32°C (P_{Wald} < 0.01, OR = 10.5), as well as winter at 10°C (P_{Wald} = 0.02, OR = 0.21) and 19°C (P_{Wald} = 0.03, OR = 0.24), were all significantly associated with the seroprevalence of WNV (Table 3.49). The risk factors were separated into 3 groups, the first consisting of; agricultural activities and contact with other species, the second group entailed of; discipline, pasture percentage, seasonal temperature and time into stable and the third group comprised of the fever variable. These 3 groups were analyzed separately in order to avoid bias between the samples due to collinearity.

Table 3.49 Multivariable analysis for WNV risk factors in South Africa

Variables	B	SE	OR	95% CI		Pwald
				Lower	Upper	
Agricultural activities						
Forestry	-0.69	0.27	0.5	-1.22	-0.15	0.01
Other	0.72	0.27	2.06	0.19	1.25	0.01
Contact with animal species						
Dog	0.51	0.25	1.67	0.01	1.01	0.05
Discipline						
Dressage	-1.37	0.61	0.25	-	-	0.03
Equestrian event	-0.44	0.19	0.65	-	-	0.02
Not in work	-0.99	0.38	0.37	-	-	0.01
Other	0.87	0.37	2.39	-	-	0.02
Racing (retired)	-1.19	0.46	0.3	-	-	0.01
Show jumping	-1.05	0.47	0.35	-	-	0.02
Fever	0.44	0.2	1.56	0.05	0.83	0.03
Pasture percentage (%)						
50	0.92	0.44	2.51	-	-	0.04
100	0.68	0.3	1.98	-	-	0.02
Seasonal Temperature						
Spring: 18°C	1.74	0.65	5.69	-	-	0.01
Spring: 23°C	1.33	0.58	3.79	-	-	0.02
Spring: 24°C	1.03	0.45	2.8	-	-	0.02
Spring: 25°C	0.98	0.38	2.67	-	-	0.01
Spring: 27°C	1.35	0.42	3.87	-	-	<0.01
Spring: 32°C	2.35	0.7	10.5	-	-	<0.01
Winter: 10°C	-1.57	0.66	0.21	-	-	0.02
Winter: 19°C	-1.42	0.67	0.24	-	-	0.03
Time into stable: 5pm	0.79	0.3	2.2	-	-	0.01

B: Regession Coefficient; SE: Standard Error; OR: Odd Ratio; CI: Confidence Interval

CHAPTER 4

DISCUSSION

There have been many outbreaks of WNV in horses worldwide, ranging from South America, North America, Asia, the Middle East, Europe and Africa (Romi *et al*. 2004; Murgue *et al*. 2001; Ostlund *et al*. 2001). Previously, there have been notable outbreaks in humans in South Africa, but no outbreaks have been recorded for horses in South Africa (Peterson & Roehig 2001). Horses affected by WNV in South Africa normally exhibit neurological signs (90% of cases), where 40% of positive cases result in mortality (Venter *et al.* 2009).

The two WNV lineages are both apparent in Africa; lineage 1 is mostly seen to result in encephalitis in infected human cases, whereas lineage 2 resides predominantly in the enzootic cycle infecting horses rather than humans (Peterson & Roehig 2001; Lanciotti *et al*. 1999). Of the WNV lineages, lineage 2 is endemic to southern Africa (Venter *et al*. 2009).

The original sample size calculated using individual horses was changed to calculate sample size using the number of properties instead. The number of individuals per province ranged from 50 to 294 horses. As a result of the major drought experienced in South Africa throughout 2015 and 2016, horses were moved out of certain drier provinces to provinces with more grazing, which was seen predominantly in the Northern Cape Province where horses were moved to Gauteng, North West and Kwa-Zulu Natal to prevent mortality. Therefore the sample size collected for this province was significantly less than that of the other eight provinces.

In order to determine the seroprevalence of WNV in the nine provinces of South Africa, 1198 samples were collected in this study from asymptomatic horses and tested using the SNT and ELISA. The results were analyzed firstly per province and then for South

Africa as a whole. Risk factors associated with the presence of the virus were determined by conducting a questionnaire. The results from the SNT were used to determine the seroprevalence and associated risk factors of WNV in South Africa and were utilized as a standard to test a capture antibody ELISA.

Many of the tested samples in the ELISA correlated with the SNT results; however, there were a percentage of outliers (being the seropositive samples from the SNT that showed negative in the ELISA and visa versa), ranging between 23.5% and 47.2%, which varied from the SNT results. Samples that were negative in the SNT and positive in the ELISA and visa versa (outliers), had OD values very close to the 'negative boundary' (OD value used as a cut-off point for negative and positive samples). The Kappa test indicated that there was a moderate agreement between the ELISA and SNT results (Kappa = 0.5), thus the ELISA was moderately reliable when compared with the SNT. Since the SNT has been validated and used in the Virology Laboratory of the DVTD, the main limitation in results between these tests obtained in this study might be because the IgG capture ELISA has not been thoroughly validated, this was beyond the scope of this study.

In a previous study by Venter *et al*. (2009), an inhouse IgM capture ELISA was used to determine more recent WNV infections in horses opposed to results obtained through HI assays. The results from this study illustrated a positive correlation between the outcomes of both the ELISA and the HI assay. Loroño-Pino *et al*. (2003) also made use of HI assays and ELISA's to compare results, which proved effective in correlation between assays.

In this study, the seroprevalence for WNV was the highest in the Free State Province (73%). Risk factors associated with the presence of the virus include livestock agricultural activities, the presence of small ruminants on neighbouring properties, horses used for general riding pleasure (trail riding) and gender (due to a greater number of a specific gender on a farm at any point, such as a greater number of mares on a stud farm) as associated risk factors.

The Western Cape Province had the next highest seroprevalence (68.2%). Associated risk factors included other agricultural activities, including but not limited to vineyards, berry farms and olive farms; direct contact with other species, being of a wide variety of both smaller and larger species as a result of the fynbos, grassland and forest ecosystems in the various regions of the Western Cape. The remaining associated risk factors were mares, the racing discipline (retires horses), which is the more predominant discipline in the Western Cape and the pasture percentage of both 90% and 100%, indicating that horses that spent a great portion of the day in the fields were more susceptible to WNV infection. Standing water serves as an ideal breeding ground for mosquito species (Okogun *et al*. 2003), the primary vector of WNV, which was also a risk factor pertaining to seroprevalence in the Western Cape Province.

The Mpumalanga Province had a high seroprevalence for WNV (63%), with fodder plantations being the primary agricultural activity associated with seroprevalence for this region. General pleasure riding, including trial riding and hacking competitive horses, bringing horses into the stables from the fields at 2 pm in the afternoons and neighboring properties housing chickens, which include chicken farms, were also associated risk factors for this Province. Associated ecological risk factors for seroprevalence in Mpumalanga Province were also related to varying temperatures during the spring and winter seasons, a large contributing factor to mosquito vector replication (Okogun *et al*. 2003).

The seroprevalence for WNV in the Limpopo Province was 61.6% with temperature in all four seasons an associated ecological risk factor. These temperatures occur in the higher ranges of between 28°C to 38°C. The higher temperature range leads to a greater replication and population growth rate in mosquito populations (Lindblade *et al*. 2000). The presence of annual frost was also a risk factor for this region, like temperature, influences mosquito vector population growth rate (Lindblade *et al*. 2000). Horses that were not in work, including young horses that are not yet under saddle or injured horses, and an occurring rodent problem on the properties were also associated risk factors for seroprevalence in the Limpopo Province.

The samples collected in the Northern Cape Province were below the required 64 horses per province. This was due to the El Niño experienced in the time period of 2015 to 2016, wherein the drought had detrimental effects on grazing and water sources in these regions (Kruger 1999), many horses were moved to surrounding provinces in order to avoid mortality. Seroprevalence of WNV in the Northern Cape Province was 58.6%, showing a higher seroprevalence than anticipated for the lower number of samples collected for this Province. There were no associated risk factors for seroprevalence of WNV in the Northern Cape Province. However, seroprevalence of WNV in horses in this Province could be a result of a denser association between horses due to harsh environmental conditions; creating a beneficial environment for vectors and as a result, an increase in transmission of disease. Horses were not moved back into the Province from the different locations they were initially moved to, during the duration of this study.

The KwaZulu-Natal Province had a seroprevalence of 57% for WNV. One of the associated risk factors for this province was direct contact between sampled horses and small ruminants, thus the small ruminants (i.e: Sheep and goats) were located on the same properties as the sampled horses. This could be as a result of generalized association of horses being located in the same vicinity as small ruminants, or could be a practice of mixed farming in this province. However, ruminants have shown to be poor sentinals of WNV in Northwest Senegal and instead, dogs were an important sentinel in the surveillance of WNV in Northwest Senegal (Davoust *et al*. 2016). The variations in Senegal and South Africa could be as a result of differing climatic and environmental factors and association with horses and agricultural activities within the two countries. The second associated risk factor was both Thoroughbred horses and Warmblood horses. The KwaZulu-Natal Province is also highly involved in the racing industry in South Africa and as Thoroughbreds are the only breeds of horses used in this discipline, they were highly associated with seroprevalence of WNV. Likewise, KwaZulu-Natal is one of the leading provinces for the breeding and production of competition horses, where Warmbloods are the predominant breed of horses used for competition. The lower seroprevalence of WNV in this Province opposed to that of other provinces (e.g the Northern Cape Province) could be a result of various differing environmental conditions and biomes in the Province. The

regulation of vaccinating competition horses and racehorses is unknown in this Province, however the vaccination of horses could also play a role in a lower seroprevalence of WNV amongst the equine population in this province overall, however none of the samples tested were known to have been vaccinated.

The Gauteng Province showed a seroprevalence of 52.4% for WNV. Horses that had direct contact with dogs on the same properties as they resided was one of the associated risk factors for this province. Blackburn *et al.* (1989) conducted a serological study on the pathogenicity of WNV in dogs in South Africa, in which it was found that dogs are able to develop low titres of antibodies to WNV. Although dogs are not of major importance in the epidemiology of WNV, they can play a small role in transmission and maintenance of the virus (Blackburn *et al*. 1989). Another risk factor was the presence of rivers as a water source for horses. Rivers, being a water source for breeding mosquitoes, is a contributing factor for the maintenance of the vector of the virus (Okogun *et al*. 2003).

The Eastern Cape Province had a seroprevalence of 49% for WNV. The only risk factor associated with seroprevalence was horses having direct contact with other horses, whether on the same property, neighboring properties or at competitions. This could be as a result of horses coming into contact with other asymptomatic infected horses in different regions of the Province.

The North West Province had the lowest seroprevalence (42%) for WNV. Annual frost was one of the risk factors associated with seroprevalence in this Province. Annual frost is a contributing factor to mosquito vector replication and a causative agent affecting the population of mosquitoes in this region. Fever was the second risk factor for the North West Province associated with WNV. Fever is one of many documented clinical signs of WNV infection in horses, along with; ataxia, partial or complete paralysis, partial blindness, grinding of teeth and seizures, to name a few (Benenson 1995; Venter *et al*. 2010). Five of the horses in this province were vaccinated for WNV with the Proteq WNV vaccine; however, this did not have an influence on the results, as two of the known vaccinated cases were seronegative in the SNT.

The seroprevalence for South Africa ranged between 54% and 63% for all 1198 horses, where the majority of seropositive cases had titres higher than 10, for instance; 80 (23.5%) and 320 (21%), using the SNT. There is a crucial need for more knowledge when addressing the spatio-temporal aspects of WNV and how these aspects correlate with the epidemiology of WNV in South Africa. Forestry as well as other agricultural activities, including vinyards, olive oil and sunflower oil farms and fruit and vegetable farms, along with horses having direct contact with dogs on the same properties as they reside, were a few of the risk factors associated with WNV seroprevalence across the country.

Horses used in the majority of disciplines were included in this study; dressage, show jumping, competitive equestrian events and other events, along with horses currently not in work and retired racing horses. Horses mostly used for competition purposes are exposed to many different environments, animal species and other horses, allowing for a greater probability of infection. The most predominant gender associated with seroprevalence was mares (females) and the associated breeds with seroprevalence as Thoroughbreds and Warmbloods. Horses that had a 50% and 100% pasture percentage, indicating that they were in fields for either half-day (stabled at night) or full day (not stabled at all) were associated with seroprevalence. Horses brought into their stables from the fields at 5 pm in the afternoon showed an association of lower risk, where horses not stabled at all had a higher risk of contracting the virus. Horses in these circumstances (not stabled) had a greater exposure rate to mosquitoes as opposed to horses stabled. It is known for vectors to become more abundant at both dusk and dawn time periods since these are the cooler time periods of the day (Ndoen *et al*. 2011), and therefore, if horses are outside their stables at these time intervals, it is probable that they have a higher exposure to vectors of WNV. Thus, the above associated risk factors indicated that avoidance of outdoor living conditions and earlier time schedules for stabling will decrease the probability of WNV infection in horses.

The habitats of different regions was a major contributing risk factor for exposure to the virus, so owners should take initiative to manage environmental factors per property to decrease the probability of infection. Control measures can include insecticides and protective wear for horses, eliminating standing pools of water or

other present water sources, limit movement between the spring and winter seasons and necessary movement overall, decreasing exposure to other potential carrier species and horses. The most predominant ecological risk factor was environmental temperature experienced in both spring and winter seasons, with temperatures ranging between 10°C (winter) and 32°C (spring). Warmer temperatures favour the replication of vectors and therefore horses are at a lower exposure risk in the winter seasons and exposed to a higher risk in the spring due to higher environmental temperature. Horses that had experienced a fever between 2015 and 2016 were positively associated with seropositive horses indicating a correlation between fever and seroprevalence of WNV in horses. However, the presence of a fever could also be due to any other infection.

The temporary movement of horses between different habitats for competition and general pleasure riding or trails has also shown to prove as a potential exposure risk for horses. Individual horses that come into direct contact with other animal species, such as dogs, small ruminants and other species (i.e. ducks) demonstrated to be a potential exposure risk. Spillover from livestock and domestic animals (horses) to humans increases in rural locations, hence resulting in greater virulence as a result of rapid reproduction of viraemic mosquitoes (Weaver 2005). As a result, a larger number of humans and horses residing in agricultural holdings are at a greater risk of contracting the disease (Weaver 2005; Weaver & Reisen 2010). Rats are an example of an amplifying host for WNV and the presence of rats also have an association with seroprevalence in horses (Dobler 2010). Wild birds were not significant in this study, which could be a result of the drought and lack of water sources on properties. However, it is known that wild birds have an important role in the transmission cycle and are asymptomatic reservoir hosts (Venter *et al*. 2009; McLean *et al*. 2001).

The mosquito species collected in both Gauteng and Mpumalanga provinces were all negative for WNV, this could mainly be a result of too few mosquitoes caught (439) and tested. Hubálek and Halouzka (1999) caught and tested a large number (in order to detect the virus) and variety of field caught mosquito species for WNV in Europe. Species positive for WNV from their study were also identified in this sudy and include *Culex pipiens*, *Aedes vexans, Aedes aegypti* and *Culex quinquefasciatus*. Although no

transmission studies were carried out, these mosquito species have been shown to carry WNV. *Anopheles freeborni* also identified in this study has not yet been proven as a vector for WNV (Hubálek & Halouzka 1999). Goddard *et al*. (2002) also tested 10 different mosquito species for the presence of WNV in California, which included *Culex, Aedes* and *Ochlerotatus* species, results of which indicated that *Culex* species were the primary vector in the transmission of WNV. Results from these studies indicate that mosquito species collected in this study are capable of transmitting WNV.

The risk factors associated with seroprevalence of WNV were area specific, thus indicating that habitats for potential vectors to inhabit were crucial in the transmission and exposure rate of WNV in all nine provinces and across South Africa indicating the varying biomes and ecosystems wherein horses reside. Seasonal temperatures and particular water sources or presence of standing pools of water has an impact on the potential for vectors to replicate in the different habitats (Lindblade *et al*. 2000; Reisen *et al*. 2005).

There are currently two vaccines available in South Africa; Proteq West Nile vaccine (Lakato) and Duvaxyn (Zoetis), however vaccination of horses is not compulsory in South Africa. In this study only 5 from the 1198 horses sampled were vaccinated for WNV using the Proteq West Nile vaccine and were included in the study. There is a clear lack of awareness programmes among horse owners; therefore owners are not vaccinating their horses against WNV. This might be due to a lack of knowledge about the disease. Fever was significantly associated with seroprevalence of WNV in this study, however, the majority of the seropositive horses did not show any clinical signs. This correlates with results shown in a previous study, wherein infection of WNV was not connected to neurological diseas in horses (Guthrie *et al*. 2003). Thus, a great margin of infected cases are not being reported as a result of an absence of clinical signs or a lack of knowledge among owners and equine personel pertaining to the related clinical symptoms of WNV in horses.

In conclusion, there was a high seroprevalence (54%-63%) of WNV amongst horses in South Africa. The seroprevalence results from this study corresponds to that of another study performed by Guthrie *et al*. (2003), wherein the prevalence for WNV was found

to be 75% in Thoroughbred mares. This indicates that there is a high exposure rate of the virus amongst horse populations from all nine provinces. The second aim of the study indicated an absence of the virus in field caught mosquitoes. This however is not an indication that WNV is not present in mosquitoes in South Africa. The questionnaires were efficient in determining the absence of knowledge and awareness for virus and prevention thereof amongst equine owners in South Africa. The questionnaires also provided an effective means of determining the various risk factors associated with the prevalence of WNV in all nine provinces as well as South Africa as a whole. A weakness of the study was the occurrence of low numbers for certain risk factors.

This study will be able to bring further awareness of WNV in horses in South Africa and provide essential awareness for the prevention of infection amongst horses. A defiency of this study is the small number of mosquitoes that have been collected and tested which could provide a more informative result of the prevalence of WNV in mosquitoes. A larger number of blood samples could have been tested with the ELISA for a better comparison with the SNT results. Future work, which will be essential for the presence of WNV in horses should include research on effective preventative methods, such as the effect of vaccinating horses on the epidemiology of WNV, the immune response to vaccination and raise awareness of the importance of vaccinating horses among the owners. Research in the role of wildlife in the transmission cycle of WNV, as well as in domesticated animal species (e.g. birds, rodents or dogs) would provide a greater insight into the epidemiology of WNV in South Africa.

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APPENDICES

Appendix I: Animal Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Seroprevalence and occurrence of flaviviruses in equine populations in South Africa**
PROJECT NUMBER	**V080-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. RE Jeal**

STUDENT NUMBER (where applicable)	**UP_29008698**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	***Equus caballus* (Horse)**	
NUMBER OF ANIMALS	**1800**	
Approval period to use animals for research/testing purposes		**October 2015 – October 2016**
SUPERVISOR	**Prof. EH Venter**	

KINDLY NOTE:

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

APPROVED	Date 9 September 2015
CHAIRMAN: UP Animal Ethics Committee	Signature 

Appendix II: Questionnaire survey forms

ANIMAL OWNER CONSENT FORM

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Owner's Name: _____

Address: _____

Phone No: _____

Patient's Name: _____

Species: Equine

Breed: _____

Age: _____Year(s)/Month(s)

Sex: Male/Female/Gelding

I am the owner or the representative of the owner of the animal described above, and I have the authority to execute this consent.

I hereby give the University of Pretoria staff, or representatives, consent and authority to collect blood samples from the animal for the reason explained to me. The nature of this procedure has been explained to me, and I understand what will be done.

I have also been informed that there can be certain risks associated with this procedure. I further understand that during the course of the procedure, unforeseen conditions may crop up that may necessitate the performance of additional procedures.

I understand that support personnel will be used as deemed necessary by the veterinarian.

Please consider the following:

The University of Pretoria staff or students involved in this project can contact me for further information regarding this horse: **YES** **NO**

The best means of contact for this would be

Telephone (H)/(W)/(C): _____

Email (H)/(W):_____

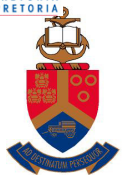
Other: _____

I would like to receive feedback on the results: **YES** **NO**

I give consent to be involved in a short questionnaire survey regarding my horse **YES** **NO**

Signed: _____

Date: _____



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YUNIBESITHI YA PRETORIA

Risk factor questionnaire Case No……...

Date : …… Name of owner : …….. Address:……

Province : ……. Postal Code : …… Tel:.....

1. Name of the horse…….....

2. Gender:

Mare		Filly	
Gelding		Colt	
Stallion			

3. Breed: ……

4. Age:.....

5. Usage/Discipline:

Dressage		Racing	
Show Jumping		Polo/Polocrosse	
Eventing/Cross Country		General Pleasure Riding/Hacking/Riding School	
Showing		Breeding	
Endurance Riding		Retired/Not in work	

6. How many adult horses are on the property?.....

7. Are they managed as a group or separately?

Group/Shared	
Separate/Alone	

8. How is your horse stabled and fed concentrates?

Complete pasture access with little or no stable feeding	
Complete stable feeding with little or no pasture access	
Combination of pasture and stable feeding	
Other ……..	

a. What per cent of time does it spend on pasture?

Pasture……..% Stable……..%

9. If stabled,

a) What time does the horse go out to pasture? _.....
b) What time does the horse come in to the stable? .....
c) Do you have concrete floors in the stable?

Yes	
No	

10. Do you have a drainage system in your farm?

Yes	
No	

11. What is the water source for your horse?

River	
Dam Water	
Bore Hole Water	
Municipal Water	
Other ……	

12. Does your horse come into contact with other species of animals?

Yes	
No	

13. If yes, which ones?

Pigs		Wild Animals	
Cattle		Dogs	
Small Ruminants		Other ……	

14. Do horses from different properties come into contact with your horse?

Yes	
No	

15. Do you see rodents on your property?

Yes	
No	

16. If yes, do you consider them to be a problem?

Yes	
No	

17. If yes, do they get into feed storage areas?

Yes	
No	

18. Are there roaming chickens on the property?

Yes	
No	

19. Does your horse come into contact with wild birds (i.e; oxpecker or egret on horses back)?

Yes	
No	

20. What is the amount of annual rainfall (mm/annum) for your property?.....

21. What are the average seasonal temperatures?

Summer	
Autumn	
Winter	
Spring	

22. Does the property experience frost in the year?

Yes	
No	

23. Do you have a mosquito problem on the property?

Yes	
No	

24. Grade the severity of the mosquito problem

a) Few Mosquitoes	
b) Normal (seasonal rain)	
c) Above Normal (lots all year round)	

25. What time of the year are mosquitoes present on the property?

Spring (September – November)	
Summer (December – February)	
Autumn (March – May)	
Winter (June – August)	

26. Do mosquitoes bite your horse?

Yes	
No	

27. Are any measures used to control mosquitoes on the horse?

Blankets	
Insecticides	
Mosquito shade netting on stables	
Other ……..	

28. Do other insects bite your horse?

Yes	
No	

29. If yes, specify what insects?

……

30. Do you have standing water on the property, where mosquitoes can multiply/spread?

Yes	
No	

31. Does the horse go out to pasture with protective gear (i.e: Fly masks/fly sheets)?

……

32. Has your horse travelled outside the town/province/country in the last 12 months?

Yes	
No	

a) If yes, which one?

Town	
Province	
Country	

33. Are there other farms or properties with animals on them neighboring your farm?

Yes	
No	

34. If yes, which animals are present?

Pigs		Wild Animals	
Cattle		Chickens	
Small Ruminants		Other ……	

35. What are the main agricultural activities neighboring your farm?

Game Reserve/Wildlife		Crops	
Livestock		Fodder Plantation	
Forestry		Other ……..	

36. Has your horse ever had ocular disease (swollen eyes, watering eyes, red eyes, ulcer)?

Yes	
No	
Not Known	

37. Has your horse ever been diagnosed by your veterinarian as suffering from kidney or liver disease?

Yes	
No	
Not Known	

38. Has your horse suffered from high fever in the past one year?

Yes	
No	
Not Known	

39. Has your horse had swollen joints in the past year?

Yes	
No	
Not Known	

40. Has your horse shown any signs of stiffness in the limbs or lower back in the past year?

Yes	
No	
Not Known	

41. In the past year, has your horse experienced swelling above the eyes in the last year?

Yes	
No	
Not Known	

42. For brood mares:

a. Has the mare ever aborted?

Yes	
No	
Not Known	

b. Has the mare ever had stillbirths?

Yes	
No	
Not Known	

c. Has the mare ever experienced a phantom pregnancy?

Yes	
No	
Not Known	

d. Has the mare shown to be barren or had difficulty conceiving?

Yes	
No	
Not Known	

……..**END**……

Thank you for your participation in this study.

Appendix III: Section 20 Approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/9

Rebecca Jeal
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Email: U29008698@tuks.co.za
Tel: 084 428 2288

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "SEROPREVALENCE AND OCCURRENCE OF FLAVIVIRUSES IN EQUINE POPULATIONS IN SOUTH AFRICA."

A dispensation is hereby granted on Point 7 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Only blood samples that have been heat inactivated at 56°C for 30 minutes as described in the Section 20 permit attached may be stored.
ii) Inctivated blood samples must be stored in the access controlled BSL-2 facility at Department of Veterinary Tropical Diseases (DVTD) at the University of Pretoria.
iii) Inactivated blood samples may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 -02- 2 9

– 1 –

Appendix IV: ELISA plate setup

TRIAL	1	2	3	4	5	6	7	8	9	10	11	12
A	SPL 1	SPL 2	SPL 3	SPL 4	SPL 5	SPL 6	SPL 7	SPL 8	SPL 9	SPL 10	SPL 11	SPL 12
B	SPL 1	SPL 2	SPL 3	SPL 4	SPL 5	SPL 6	SPL 7	SPL 8	SPL 9	SPL 10	SPL 11	SPL 12
C	SPL 13	SPL 14	SPL 15	SPL 16	SPL 17	SPL 18	SPL 19	SPL 20	SPL 21	SPL 22	SPL 23	SPL 24
D	SPL 13	SPL 14	SPL 15	SPL 16	SPL 17	SPL 18	SPL 19	SPL 20	SPL 21	SPL 22	SPL 23	SPL 24
E	SPL 25	SPL 26	SPL 27	SPL 28	SPL 29	SPL 30	SPL 31	SPL 32	SPL 33	SPL 34	SPL 35	SPLCON
F	SPL 25	SPL 26	SPL 27	SPL 28	SPL 29	SPL 30	SPL 31	SPL 32	SPL 33	SPL 34	SPL 35	SPLCON
G	SPL 36	SPL 37	SPL 38	SPL 39	SPL 40	SPL 41	SPL 42	SPL 43	SPL 44	SPL 45	SPL 46	BLK
H	SPL 36	SPL 37	SPL 38	SPL 39	SPL 40	SPL 41	SPL 42	SPL 43	SPL 44	SPL 45	SPL 46	BLK

Appendix V: OD values for the serum and WNV core antibody dilutions for the checkerboard run

		WNV core antibody dilutions					
		1:100	**1:200**	**1:400**	**1:800**	**1:1600**	**1:2000**
Serum dilutions	**1:10**	0.747	0.713	0.554	0.659	0.812	0.930
	1:20	0.259	0.324	0.259	0.304	0.385	0.375
	1:40	0.220	0.218	0.140	0.263	0.358	0.225
	1:80	0.172	0.193	0.178	0.225	0.303	0.201

Appendix VI: Prevalence estimate calculations taking clustering into consideration at a 95% confidence interval. (South Africa only presented here as an example. Prevalence estimate for all nine provinces were performed in the same manner)

Prevalence estimate of WNV in South Africa

Regions	# Properties	# Samples	WNV (+)	Ap. Prev	n^2	m^2	n*m
Kaapsehoop	2	44	16	0.36	1936	256	704
White River	3	15	7	0.47	225	49	105
Barberton	1	8	6	0.75	64	36	48
Nelspruit	4	20	12	0.6	400	144	240
Dullstroom	2	15	11	0.73	225	121	165
Homedean	1	10	10	1	100	100	100
Middelburg	1	11	8	0.73	121	64	88
Kriel	1	5	3	0.6	25	9	15
Witbank	1	7	3	0.43	49	9	21
East London	3	30	14	0.47	900	196	420
PE	4	31	15	0.48	961	225	465
Stutterheim	1	11	4	0.36	121	16	44
Grahamstown	2	11	7	0.64	121	49	77
Alicedale	1	2	1	0.5	4	1	2
Hoopstad	1	5	5	1	25	25	25
Frankfort	3	24	19	0.79	576	361	456
Vrede	1	10	7	0.7	100	49	70
Kroonstad	1	10	6	0.6	100	36	60
Villiers	1	10	4	0.4	100	16	40
Clarens	1	8	7	0.88	64	49	56
Bloemfontein	5	43	32	0.74	1849	1024	1376
City of Tshwane	9	279	172	0.62	77841	29584	47988
Mogale	2	2	2	1	4	4	4
Madibeng	2	2	1	0.5	4	1	2
Tygerpoort	1	1	0	0	1	0	0
Kyalami	1	4	2	0.5	16	4	8
Summerveld	1	78	37	0.47	6084	1369	2886
Umhlathuze	2	34	24	0.71	1156	576	816
Umvoti	2	60	32	0.53	3600	1024	1920
Bela Bela	2	20	10	0.5	400	100	200
Dendron	1	5	4	0.8	25	16	20
Hoedspruit	5	20	13	0.65	400	169	260
Louis Trichardt	1	3	1	0.33	9	1	3
Phalaborwa	2	18	15	0.83	324	225	270
Pienaarsrivier	1	9	4	0.44	81	16	36
Polokwane	2	16	6	0.38	256	36	96

Tzaneen	1	3	3	1	9	9	9
Upington	1	14	8	0.57	196	64	112
Groblershoop	1	13	6	0.46	169	36	78
Springbok	1	11	6	0.55	121	36	66
Hopetown	1	8	6	0.75	64	36	48
Kathu	1	5	3	0.6	25	9	15
Brits	1	9	4	0.44	81	16	36
Broederstroom	1	10	4	0.4	100	16	40
Hartbeesfontein	1	7	3	0.43	49	9	21
Hartebeespoort	3	14	8	0.57	196	64	112
Potchefstroom	2	15	7	0.47	225	49	105
Rustenburg	1	11	2	0.18	121	4	22
Skeerpoort	3	18	8	0.44	324	64	144
Milnerton	8	21	5	0.24	441	25	105
Gordons' Bay	1	1	1	1	1	1	1
Piketberg	1	35	27	0.77	1225	729	945
L'Ormarins	1	40	27	0.68	1600	729	1080
Cape Town	2	2	2	1	4	4	4
Wolseley	1	11	7	0.64	121	49	77
Robertson	1	35	29	0.83	1225	841	1015
Big Bay	1	1	0	0	1	0	0
Ceres	1	14	9	0.64	196	81	126
Malmesbury	1	2	2	1	4	4	4
Van Riebeeckstrand	1	1	1	1	1	1	1
Paarl	1	16	6	0.38	256	36	96
TOTAL	**112**	**1198**	**704**	**36,53**	**105022**	**38872**	**63348**

$$\boldsymbol{V = Pe^2(\Sigma n^2) - 2Pe(\Sigma nm) + (\Sigma m^2)};\ \boldsymbol{Pe + 1.96\left\{\frac{c}{T}\sqrt{\frac{V}{c(c-1)}}\right\}};\ \boldsymbol{Pe - 1.96\left\{\frac{c}{T}\sqrt{\frac{V}{c(c-1)}}\right\}}$$

Pe 0.59 Range

T= 1198 0.63

C= 61 0.54

C/T= 0.05

V= 686.62

c(c-1)= 3660

Appendix VII: Multivariable logistic regression results. (South Africa only presented here as an example. Multivariable logistic regression for all nine provinces were performed in the same manner)

Logistic regression reports for South Africa

Run Summary

Item	Value	Item	Value
Dependent Variable	SNT	Rows Processed	1203
Reference Group		0 Rows Used	559
Number of Groups		2 Rows for Validation	0
Frequency Variable	None	Rows X's Missing	644
Numeric Ind. Variables		0 Rows Freq Miss. or 0	0
Categorical Ind. Variables		5 Rows Prediction Only	0
Final Log Likelihood		-371,82517 Unique Row Patterns	20
Model R^2		0,61048 Sum of Frequencies	559
Actual Convergence		4,33E-09 Likelihood Iterations	4
Target Convergence		1,00E-06 Maximum Iterations	20
Model D.F.		6 Completion Status	Normal Completion

Response Analysis

SNT Categories	Count	Unique Rows	Prior	Act vs Pred R^2	% Correctly Classified
0	242	10	0,5	0,03749	75,62
1	317	10	0,5	0,03749	36,278
Total	559	20			53,309

Subset Selection Summary

No. Terms	No. X's	Log Likelihood	R^2 Value	R^2 Change
1	1	-382,42276	0	0
2	2	-379,63168	0,16078	0,16078
3	3	-376,55524	0,338	0,17722
4	4	-374,92511	0,4319	0,0939
5	5	-373,30764	0,52508	0,09317
6	6	-371,82517	0,61048	0,0854

Coefficient Significance Tests

Independent Variable	Regression Coefficient b(i)	Standard Error Sb(i)	Wald Z-Value H0: β=0	Wald Prob Level	Odds Ratio Exp(b(i))

B0: Intercept	-0,09953	0,23662	-0,421	0,67402	0,90526
B2: (Contact_spp__Dog=1)	0,51017	0,25486	2,002	0,04531	1,66558
B3: (Biting_insect__Culicoides=1)	-0,41207	0,21696	-1,899	0,05752	0,66228
B4: (Agricultural_activities__Forestry=1)	-0,68866	0,27249	-2,527	0,01149	0,50225
B5: (Agricultural_activities__Other=1)	0,72067	0,26909	2,678	0,0074	2,05581
B1: (Water_source__Other=1)	-0,87467	0,52125	-1,678	0,09334	0,417

Coefficient Confidence Intervals

Independent Variable	Regression Coefficient b(i)	Standard Error Sb(i)	Lower 95% Confidence Limit	Upper 95% Confidence Limit	Odds Ratio Exp(b(i))
B0: Intercept	-0,09953	0,23662	-0,5633	0,36424	0,90526
B2: (Contact_spp__Dog=1)	0,51017	0,25486	0,01066	1,00969	1,66558
B3: (Biting_insect__Culicoides=1)	-0,41207	0,21696	-0,83731	0,01316	0,66228
B4: (Agricultural_activities__Forestry=1)	-0,68866	0,27249	-1,22273	-0,15459	0,50225
B5: (Agricultural_activities__Other=1)	0,72067	0,26909	0,19327	1,24807	2,05581
B1: (Water_source__Other=1)	-0,87467	0,52125	-1,8963	0,14696	0,417

Odds Ratios Report

Independent Variable	Regression Coefficient b(i)	Odds Ratio Exp(b(i))	Lower 95% Confidence Limit	Upper 95% Confidence Limit
B0: Intercept	-0,09953	0,90526	0,56933	1,43942
B2: (Contact_spp__Dog=1)	0,51017	1,66558	1,01072	2,74475
B3: (Biting_insect__Culicoides=1)	-0,41207	0,66228	0,43287	1,01325
B4: (Agricultural_activities__Forestry=1)	-0,68866	0,50225	0,29443	0,85677
B5: (Agricultural_activities__Other=1)	0,72067	2,05581	1,21321	3,48361
B1: (Water_source__Other=1)	-0,87467	0,417	0,15012	1,1583

Estimated Logistic Regression Model(s)

Model For SNT = 1

-0,0995311948664428 + 0,510174725463383*(Contact_spp__Dog=1) -0,412074210013305*(Biting_insect__Culicoides=1) - 0,688657494575269*(Agricultural_activities__Forestry=1) + 0,720669247644585*(Agricultural_activities__Other=1) - 0,87466917156112*(Water_source__Other=1)

Note that each model estimates B for a specific group, where Logit(Y) = XB. To calculate the group probabilities when there are only 2 response groups, transform the logit using Prob(Y = group) = 1/(1+Exp(-XB)) or Prob(Y ≠ group) = Exp(-XB)/(1+Exp(-XB)). For the calculation formula to use when there are more than 2 response groups, see the help documentation.

Classification Table

	Estimated		
Actual	0	1	**Total**
0	183	59	242
1	202	115	317
Total	385	174	559

Percent Correctly classified = 53,3%

Run Summary

Item	Value	Item	Value
Dependent Variable	SNT	Rows Processed	1203
Reference Group	0	Rows Used	1029
Number of Groups	2	Rows for Validation	0
Frequency Variable	None	Rows X's Missing	174
Numeric Ind. Variables	0	Rows Freq Miss. or 0	0
Categorical Ind. Variables	1	Rows Prediction Only	0
Final Log Likelihood	-697,67787	Unique Row Patterns	6
Model R^2	0,24881	Sum of Frequencies	1029
Actual Convergence	3,33E-10	Likelihood Iterations	4
Target Convergence	1,00E-06	Maximum Iterations	20
Model D.F.	3	Completion Status	Normal Completion

Response Analysis

SNT Categories	Count	Unique Rows	Prior	Act vs Pred R^2	% Correctly Classified
0	437	3	0,5	0,00739	90,16
1	592	3	0,5	0,00739	14,696
Total	1029	6			46,744

Subset Selection Summary

No. Terms	No. X's	Log Likelihood	R^2 Value	R^2 Change

1	1	-701,52994	0	0
2	3	-697,67787	0,24881	0,24881

Coefficient Significance Tests

Independent Variable	Regression Coefficient b(i)	Standard Error Sb(i)	Wald Z-Value H0: β=0	Wald Prob Level	Odds Ratio Exp(b(i))
B0: Intercept	-0,04101	0,02533	-1,619	0,10542	0,95982
B1: (Fever=1)	0,44214	0,19842	2,228	0,02586	1,55604
B2: (Fever="Not known")	-0,71455	0,48825	-1,464	0,14333	0,48941

Coefficient Confidence Intervals

Independent Variable	Regression Coefficient b(i)	Standard Error Sb(i)	Lower 95% Confidence Limit	Upper 95% Confidence Limit	Odds Ratio Exp(b(i))
B0: Intercept	-0,04101	0,02533	-0,09065	0,00863	0,95982
B1: (Fever=1)	0,44214	0,19842	0,05325	0,83103	1,55604
B2: (Fever="Not known")	-0,71455	0,48825	-1,6715	0,2424	0,48941

Odds Ratios Report

Independent Variable	Regression Coefficient b(i)	Odds Ratio Exp(b(i))	Lower 95% Confidence Limit	Upper 95% Confidence Limit
B0: Intercept	-0,04101	0,95982	0,91334	1,00867
B1: (Fever=1)	0,44214	1,55604	1,0547	2,29569
B2: (Fever="Not known")	-0,71455	0,48941	0,18797	1,2743

Estimated Logistic Regression Model(s)

Model For SNT = 1

-0,0410083519444093 + 0,442142912384405*(Fever=1) -0,714550211582727*(Fever="Not known")

Note that each model estimates B for a specific group, where Logit(Y) = XB. To calculate the group probabilities when there are only 2 response groups, transform the logit using Prob(Y = group) = 1/(1+Exp(-XB)) or Prob(Y ≠ group) = Exp(-XB)/(1+Exp(-XB)). For the calculation formula to use when there are more than 2 response groups, see the help documentation.

Classification Table

	Estimated		
Actual	0	1	**Total**

0	394	43	437
1	505	87	592
Total	899	130	1029

Percent Correctly classified = 46,7%

Coefficient Significance Tests

Independent Variable	**Regression Coefficient b(i)**	**Standard Error Sb(i)**	**Wald Z-Value H0: β=0**	**Wald Prob Level**	**Odds Ratio Exp(b(i))**
B0: Intercept	0,00618	0,0257	0,241	0,80989	1,0062
B1: (Where_to="Province")	0,51788	0,34926	1,483	0,13812	1,67847
B2: (Where_to="Town")	-0,18471	0,17881	-1,033	0,3016	0,83134
B1: (Type_of_control_measures="Blankets")	-0,85442	0,57653	-1,482	0,13834	0,42553
B2: (Type_of_control_measures="Blankets/Insecticides")	1,63049	1,0861	1,501	0,13329	5,10637
B3: (Type_of_control_measures="Insecticide")	-0,18659	0,29999	-0,622	0,53396	0,82979
B4: (Type_of_control_measures="Insecticide, shade netting")	-0,22581	0,71036	-0,318	0,75058	0,79787
B5: (Type_of_control_measures="Insecticides")	0,30482	0,4883	0,624	0,53246	1,35638
B6: (Type_of_control_measures="Insecticides, Shade netting, Fans")	0,75502	0,69853	1,081	0,27975	2,12766
B7: (Type_of_control_measures="Insecticides, blankets")	-0,73663	0,79871	-0,922	0,35639	0,47872
B8: (Type_of_control_measures="Insecticides, day sheets")	-0,44895	0,67425	-0,666	0,5055	0,6383
B9: (Type_of_control_measures="Insecticides/Chickens")	-0,85442	0,6865	-1,245	0,21328	0,42553
B10: (Type_of_control_measures="None")	-0,26254	0,26384	-0,995	0,3197	0,7691
B1: (Annual_rainfall__mm_annum_=150)	0,10536	0,81138	0,13	0,89668	1,11111
B2: (Annual_rainfall__mm_annum_=199)	0,91629	1,01653	0,901	0,36738	2,5
B3: (Annual_rainfall__mm_annum_=200)	-0,33647	0,82231	-0,409	0,68241	0,71429
B4: (Annual_rainfall__mm_annum_=240)	0,22314	1,09545	0,204	0,83859	1,25
B5: (Annual_rainfall__mm_annum_=420)	1,20397	1,27148	0,947	0,34369	3,33333
B6: (Annual_rainfall__mm_annum_=450)	0,8473	0,70879	1,195	0,23192	2,33333

B7: (Annual_rainfall__mm_annum_=468)					
	0,22314	0,88506	0,252	0,80095	1,25
B8: (Annual_rainfall__mm_annum_=480)					
	-0,18232	0,75277	-0,242	0,80863	0,83333
B9: (Annual_rainfall__mm_annum_=490)					
	-0,69315	0,79582	-0,871	0,38376	0,5
B10: (Annual_rainfall__mm_annum_=495)					
	-0,87547	1,36626	-0,641	0,52167	0,41667
B11: (Annual_rainfall__mm_annum_=500)					
	-0,31585	0,79657	-0,397	0,69172	0,72917
B12: (Annual_rainfall__mm_annum_=510)					
	-1,6864	0,98883	-1,705	0,08811	0,18519
B13: (Annual_rainfall__mm_annum_=517)					
	1,20397	1,27148	0,947	0,34369	3,33333
B14: (Annual_rainfall__mm_annum_=524)					
	0,66498	0,91807	0,724	0,46887	1,94444
B15: (Annual_rainfall__mm_annum_=540)					
	-0,40547	0,7188	-0,564	0,57269	0,66667
B16: (Annual_rainfall__mm_annum_=548)					
	0,88552	0,69916	1,267	0,20532	2,42424
B17: (Annual_rainfall__mm_annum_=550)					
	0,91629	0,76739	1,194	0,23246	2,5
B18: (Annual_rainfall__mm_annum_=560)					
	-0,40547	0,9037	-0,449	0,65367	0,66667
B19: (Annual_rainfall__mm_annum_=570)					
	0,79851	0,9083	0,879	0,37933	2,22222
B20: (Annual_rainfall__mm_annum_=600)					
	0,47692	0,68397	0,697	0,48562	1,61111
B21: (Annual_rainfall__mm_annum_=650)					
	-0,539	0,78072	-0,69	0,48995	0,58333
B22: (Annual_rainfall__mm_annum_=660)					
	1,20397	1,27148	0,947	0,34369	3,33333
B23: (Annual_rainfall__mm_annum_=670)					
	0,22314	0,75829	0,294	0,76855	1,25
B24: (Annual_rainfall__mm_annum_=680)					
	0,77319	0,80224	0,964	0,33515	2,16667
B25: (Annual_rainfall__mm_annum_=700)					
	-0,58779	0,88506	-0,664	0,50661	0,55556
B26: (Annual_rainfall__mm_annum_=750)					
	-0,02817	0,82231	-0,034	0,97267	0,97222
B27: (Annual_rainfall__mm_annum_=764)					
	1,76359	1,22863	1,435	0,15117	5,83333
B28: (Annual_rainfall__mm_annum_=800)					
	-0,47	0,97468	-0,482	0,62965	0,625
B29: (Annual_rainfall__mm_annum_=850)					
	-0,539	0,78072	-0,69	0,48995	0,58333
B30: (Annual_rainfall__mm_annum_=880)					

	10,02051	94,83792	0,106	0,91585	10000+
B31: (Annual_rainfall__mm_annum_=1000)					
	-0,74194	0,68182	-1,088	0,27652	0,47619
1: (Summer_Temperature___C_=24)					
	-10,35558	85,64323	-0,121	0,90376	0,00003
B2: (Summer_Temperature___C_=25)					
	-11,49056	85,64385	-0,134	0,89327	0,00001
B3: (Summer_Temperature___C_=26)					
	-9,95011	85,64232	-0,116	0,90751	0,00005
B4: (Summer_Temperature___C_=28)					
	-11,32891	85,64061	-0,132	0,89476	0,00001
B5: (Summer_Temperature___C_=29)					
	-10,79741	85,64085	-0,126	0,89967	0,00002
B6: (Summer_Temperature___C_=30)					
	-10,50973	85,64086	-0,123	0,90233	0,00003
B7: (Summer_Temperature___C_=31)					
	-11,49056	85,64158	-0,134	0,89327	0,00001
B8: (Summer_Temperature___C_=32)					
	-11,10279	85,64074	-0,13	0,89685	0,00002
B9: (Summer_Temperature___C_=33)					
	-10,10426	85,64434	-0,118	0,90608	0,00004
B10: (Summer_Temperature___C_=36)					
	-10,69205	85,64123	-0,125	0,90064	0,00002
B11: (Summer_Temperature___C_=37)					
	-10,50973	85,64191	-0,123	0,90233	0,00003
B12: (Summer_Temperature___C_=38)					
	-10,59674	85,6412	-0,124	0,90153	0,00002
B1: (Spring_Temperature___C_=17)					
	1,94591	1,16113	1,676	0,09376	7
B2: (Spring_Temperature___C_=18)					
	1,73827	0,65203	2,666	0,00768	5,6875
B3: (Spring_Temperature___C_=19)					
	0,27193	0,82556	0,329	0,74186	1,3125
B4: (Spring_Temperature___C_=20)					
	0,5131	0,43736	1,173	0,24073	1,67045
B5: (Spring_Temperature___C_=21)					
	1,40691	0,75789	1,856	0,0634	4,08333
B6: (Spring_Temperature___C_=22)					
	0,70272	0,49174	1,429	0,15299	2,01923
B7: (Spring_Temperature___C_=23)					
	1,33281	0,58464	2,28	0,02263	3,79167
B8: (Spring_Temperature___C_=24)					
	1,02962	0,45448	2,266	0,02348	2,8
B9: (Spring_Temperature___C_=25)					
	0,98343	0,3797	2,59	0,0096	2,67361
B10: (Spring_Temperature___C_=26)					
	0,55962	0,40888	1,369	0,1711	1,75

B11: (Spring_Temperature___C_=27)					
	1,35285	0,41792	3,237	0,00121	3,86842
B12: (Spring_Temperature___C_=28)					
	-0,94446	0,84221	-1,121	0,26212	0,38889
B13: (Spring_Temperature___C_=29)					
	0,40547	0,63854	0,635	0,52544	1,5
B14: (Spring_Temperature___C_=30)					
	1,04512	0,54785	1,908	0,05643	2,84375
B15: (Spring_Temperature___C_=31)					
	0,81093	0,59345	1,366	0,17179	2,25
B16: (Spring_Temperature___C_=32)					
	2,35138	0,69793	3,369	0,00075	10,5
B1: (Winter_Temperature___C_=10)					
	-1,57122	0,66266	-2,371	0,01774	0,20779
B2: (Winter_Temperature___C_=11)					
	0,93431	1,2181	0,767	0,44307	2,54545
B3: (Winter_Temperature___C_=12)					
	0,06104	0,66644	0,092	0,92703	1,06294
B4: (Winter_Temperature___C_=13)					
	0,37469	0,80834	0,464	0,64298	1,45455
B5: (Winter_Temperature___C_=14)					
	-0,59544	0,62805	-0,948	0,34309	0,55132
B6: (Winter_Temperature___C_=15)					
	-1,0116	0,63514	-1,593	0,11123	0,36364
B7: (Winter_Temperature___C_=16)					
	-0,72392	0,62765	-1,153	0,24875	0,48485
B8: (Winter_Temperature___C_=17)					
	-0,87184	0,65908	-1,323	0,1859	0,41818
B9: (Winter_Temperature___C_=18)					
	-1,41707	0,87039	-1,628	0,10351	0,24242
B10: (Winter_Temperature___C_=19)					
	-1,41707	0,66714	-2,124	0,03366	0,24242
B11: (Winter_Temperature___C_=20)					
	9,19124	94,83779	0,097	0,92279	9810,762
B12: (Winter_Temperature___C_=24)					
	-1,70475	1,3568	-1,256	0,20895	0,18182
B13: (Winter_Temperature___C_=27)					
	-0,31845	0,76871	-0,414	0,67867	0,72727
B14: (Winter_Temperature___C_=28)					
	-0,26439	0,71039	-0,372	0,70976	0,76768
B15: (Winter_Temperature___C_=29)					
	-1,0116	1,53	-0,661	0,5085	0,36364
B1: (Autumn_Temperature___C_=18)					
	-1,32377	1,14567	-1,155	0,2479	0,26613
B2: (Autumn_Temperature___C_=20)					
	-1,12059	1,15187	-0,973	0,33063	0,32609
B3: (Autumn_Temperature___C_=21)					

	-0,69315	1,24499	-0,557	0,5777	0,5
B4: (Autumn_Temperature___C_=22)					
	-1,32914	1,16807	-1,138	0,25516	0,26471
B5: (Autumn_Temperature___C_=23)					
	-0,74194	1,15005	-0,645	0,51884	0,47619
B6: (Autumn_Temperature___C_=24)					
	-0,94804	1,15423	-0,821	0,41144	0,3875
B7: (Autumn_Temperature___C_=25)					
	-1,0349	1,13791	-0,909	0,3631	0,35526
B8: (Autumn_Temperature___C_=26)					
	-2,23359	1,31385	-1,7	0,08912	0,10714
B9: (Autumn_Temperature___C_=27)					
	-2,02228	1,19161	-1,697	0,08968	0,13235
B10: (Autumn_Temperature___C_=28)					
	-1,33977	1,15891	-1,156	0,24766	0,2619
B11: (Autumn_Temperature___C_=29)					
	0,55962	1,54689	0,362	0,71752	1,75
B12: (Autumn_Temperature___C_=30)					
	-0,69315	1,22474	-0,566	0,57143	0,5
B13: (Autumn_Temperature___C_=32)					
	-2,07944	1,65831	-1,254	0,20986	0,125
B14: (Autumn_Temperature___C_=34)					
	-1,38629	1,80278	-0,769	0,44191	0,25
B15: (Autumn_Temperature___C_=35)					
	0,22314	1,28452	0,174	0,86209	1,25
B1: (Time_out_to_pasture=6)					
	1,24865	1,12486	1,11	0,26698	3,48562
B2: (Time_out_to_pasture=7)					
	0,42547	0,23156	1,837	0,06614	1,53031
B3: (Time_out_to_pasture=8)					
	-0,02179	0,22025	-0,099	0,92119	0,97845
B4: (Time_out_to_pasture=9)					
	0,42877	0,29998	1,429	0,1529	1,53537
B1: (Time_into_stable=2)					
	0,2301	0,33069	0,696	0,48654	1,25873
B2: (Time_into_stable=3)					
	0,32191	0,24629	1,307	0,19121	1,37976
B3: (Time_into_stable=4)					
	-0,20358	0,24361	-0,836	0,40333	0,81581
B4: (Time_into_stable=5)					
	0,78914	0,298	2,648	0,00809	2,2015
B5: (Time_into_stable=6)					
	9,06511	99,63059	0,091	0,9275	8648,26494
B6: (Time_into_stable=7)					
	9,06511	99,63059	0,091	0,9275	8648,26494
B1: (Pasture_percentage=10)					
	-1,02715	1,18526	-0,867	0,38616	0,35803

B2: (Pasture_percentage=20)					
	-0,00265	0,4689	-0,006	0,99549	0,99735
B3: (Pasture_percentage=40)					
	0,07146	0,31574	0,226	0,82095	1,07407
B4: (Pasture_percentage=50)					
	0,91876	0,43654	2,105	0,03532	2,50617
B5: (Pasture_percentage=60)					
	0,47692	0,31369	1,52	0,12842	1,61111
B6: (Pasture_percentage=70)					
	0,73644	0,39452	1,867	0,06195	2,08848
B7: (Pasture_percentage=80)					
	0,26774	0,30573	0,876	0,38117	1,30701
B8: (Pasture_percentage=90)					
	0,48084	0,30182	1,593	0,11113	1,61743
B9: (Pasture_percentage=95)					
	0,21806	0,41183	0,529	0,59646	1,24366
B10: (Pasture_percentage=100)					
	0,68499	0,29538	2,319	0,02039	1,98375
B1: (Managed__Shared_Alone_="S")					
	-0,65925	0,35437	-1,86	0,06284	0,51724
B1: (X__Adult_Horses=4)					
	-0,69315	1,87083	-0,371	0,71101	0,5
B2: (X__Adult_Horses=5)					
	0,81093	1,53659	0,528	0,59768	2,25
B3: (X__Adult_Horses=6)					
	1,09861	1,82574	0,602	0,54735	3
B4: (X__Adult_Horses=7)					
	-0,11778	1,49536	-0,079	0,93722	0,88889
B5: (X__Adult_Horses=8)					
	0,81093	1,47667	0,549	0,58289	2,25
B6: (X__Adult_Horses=9)					
	0,52325	1,44898	0,361	0,71801	1,6875
B7: (X__Adult_Horses=10)					
	2,19722	1,76383	1,246	0,21287	9
B8: (X__Adult_Horses=11)					
	0	2	0	1	1
B9: (X__Adult_Horses=12)					
	0	1,4676	0	1	1
B10: (X__Adult_Horses=13)					
	0,33647	1,53064	0,22	0,82601	1,4
B11: (X__Adult_Horses=14)					
	0,60614	1,45904	0,415	0,67782	1,83333
B12: (X__Adult_Horses=15)					
	0,13353	1,4608	0,091	0,92717	1,14286
B13: (X__Adult_Horses=16)					
	0,95551	1,50895	0,633	0,52658	2,6
B14: (X__Adult_Horses=17)					

	-0,64185	1,46718	-0,437	0,66177	0,52632
B15: (X__Adult_Horses=18)					
	0,22314	1,49164	0,15	0,88108	1,25
B16: (X__Adult_Horses=19)					
	-0,25131	1,50132	-0,167	0,86706	0,77778
B17: (X__Adult_Horses=22)					
	0,63252	1,44571	0,438	0,66174	1,88235
B18: (X__Adult_Horses=23)					
	-10,20284	164,26684	-0,062	0,95047	0,00004
B19: (X__Adult_Horses=24)					
	0,55962	1,54689	0,362	0,71752	1,75
B20: (X__Adult_Horses=25)					
	-0,40547	1,55456	-0,261	0,79423	0,66667
B21: (X__Adult_Horses=26)					
	0,33647	1,53064	0,22	0,82601	1,4
B22: (X__Adult_Horses=28)					
	0,18232	1,5384	0,119	0,90566	1,2
B23: (X__Adult_Horses=30)					
	0,75377	1,47778	0,51	0,61	2,125
B24: (X__Adult_Horses=32)					
	10,20284	164,26684	0,062	0,95047	10000+
B25: (X__Adult_Horses=35)					
	0,27444	1,44659	0,19	0,84953	1,31579
B26: (X__Adult_Horses=36)					
	0,8473	1,57359	0,538	0,59027	2,33333
B27: (X__Adult_Horses=37)					
	-0,55962	1,54689	-0,362	0,71752	0,57143
B28: (X__Adult_Horses=38)					
	0	1,63299	0	1	1
B29: (X__Adult_Horses=45)					
	-0,52325	1,44898	-0,361	0,71801	0,59259
B30: (X__Adult_Horses=50)					
	-10,20284	164,26684	-0,062	0,95047	0,00004
B31: (X__Adult_Horses=70)					
	-0,8473	1,57359	-0,538	0,59027	0,42857
B32: (X__Adult_Horses=120)					
	1,38629	1,55456	0,892	0,37252	4
B1: (Discipline_Usage="Breeding/Show jumping")					
	-0,56265	1,42011	-0,396	0,69195	0,5697
B2: (Discipline_Usage="Breeding/Showing")					
	10,64022	270,8189	0,039	0,96866	10000+
B3: (Discipline_Usage="Breeding/retired")					
	10,64022	270,8189	0,039	0,96866	10000+
B4: (Discipline_Usage="Dressage")					
	-1,37358	0,61466	-2,235	0,02544	0,2532
B5: (Discipline_Usage="Dressage, Show Jumping, General Pleasure Riding")					
	0,1305	0,71882	0,182	0,85594	1,13939

B6: (Discipline_Usage="Dressage, Showing, Breeding")					
	-11,76552	270,8189	-0,043	0,96535	0,00001
B7: (Discipline_Usage="Dressage, Showing, Show Jumping")					
	10,64022	270,8189	0,039	0,96866	10000+
B8: (Discipline_Usage="Dressage, show jumping")					
	0,53596	1,16191	0,461	0,6446	1,70909
B9: (Discipline_Usage="Dressage, show jumping, eventing, pleasure riding")					
	-0,56265	1,42011	-0,396	0,69195	0,5697
B10: (Discipline_Usage="Dressage, showing")					
	10,64022	270,8189	0,039	0,96866	10000+
B11: (Discipline_Usage="Dressage, showing, show jumping")					
	10,64022	191,49791	0,056	0,95569	10000+
B12: (Discipline_Usage="Dressage/Breeding")					
	-11,76552	270,8189	-0,043	0,96535	0,00001
B13: (Discipline_Usage="Dressage/Show jumping/Eventing")					
	0,53596	1,16191	0,461	0,6446	1,70909
B14: (Discipline_Usage="Endurance")					
	0,16559	0,35016	0,473	0,63629	1,18009
B15: (Discipline_Usage="Endurance Riding")					
	10,64022	270,8189	0,039	0,96866	10000+
B16: (Discipline_Usage="Equestrian event")					
	-0,43809	0,1895	-2,312	0,02079	0,64527
B17: (Discipline_Usage="General pleasure")					
	-0,42381	0,23768	-1,783	0,07457	0,65455
B18: (Discipline_Usage="General pleasure riding")					
	-0,23886	0,18329	-1,303	0,19251	0,78752
B19: (Discipline_Usage="General pleasure riding/Breeding")					
	-0,56265	1,42011	-0,396	0,69195	0,5697
B20: (Discipline_Usage="General pleasure riding/Endurance Riding")					
	10,64022	270,8189	0,039	0,96866	10000+
B21: (Discipline_Usage="Not in Work")					
	10,64022	270,8189	0,039	0,96866	10000+
B22: (Discipline_Usage="Not in work")					
	-0,99343	0,37897	-2,621	0,00876	0,3703
B23: (Discipline_Usage="Other")					
	0,87243	0,37484	2,327	0,01994	2,39273
B24: (Discipline_Usage="Polo")					
	-0,15719	0,92197	-0,17	0,86463	0,85455
B25: (Discipline_Usage="Racing")					
	-1,19126	0,45647	-2,61	0,00906	0,30384
B26: (Discipline_Usage="Retired")					
	0,33814	0,35337	0,957	0,33863	1,40233
B27: (Discipline_Usage="Show Jumping")					
	-1,04816	0,46757	-2,242	0,02498	0,35058
B28: (Discipline_Usage="Show Jumping, General Pleasure Riding")					
	10,64022	270,8189	0,039	0,96866	10000+
B29: (Discipline_Usage="Showing")					

	-0,46734	0,45564	-1,026	0,30504	0,62667
B30: (Discipline_Usage="Showing, Dressage")					
	-0,56265	1,00831	-0,558	0,57684	0,5697
B31: (Discipline_Usage="Showing, Dressage, Show Jumping, General Pleasure Riding")					
	0,1305	1,23154	0,106	0,91561	1,13939
B32: (Discipline_Usage="Showing, Dressage, Show jumping, Breeding")					
	0,1305	1,23154	0,106	0,91561	1,13939
B33: (Discipline_Usage="Showing, Show Jumping")					
	-11,76552	270,8189	-0,043	0,96535	0,00001
B34: (Discipline_Usage="Showing, Show jumping")					
	10,64022	270,8189	0,039	0,96866	10000+
B35: (Discipline_Usage="Showing, dressage, show jumping")					
	-11,76552	270,8189	-0,043	0,96535	0,00001
B36: (Discipline_Usage="Showing, not in work")					
	-11,76552	156,3574	-0,075	0,94002	0,00001
B37: (Discipline_Usage="Wild")					
	-11,76552	270,8189	-0,043	0,96535	0,00001
B38: (Discipline_Usage="Work horse")					
	0,20648	0,30614	0,674	0,50001	1,22935
B1: (Age=1)	0	331,68403	0	1	1
B2: (Age=1,4)	0	331,68403	0	1	1
B3: (Age=1,5)	22,40575	331,68403	0,068	0,94614	10000+
B4: (Age=2)	10,50973	191,49917	0,055	0,95623	10000+
B5: (Age=2,5)	10,50973	191,50178	0,055	0,95623	10000+
B6: (Age=3)	10,59674	191,49853	0,055	0,95587	10000+
B7: (Age=3,5)	11,89602	191,50178	0,062	0,95047	10000+
B8: (Age=4)	10,97973	191,49816	0,057	0,95428	10000+
B9: (Age=4,5)	10,79741	191,50004	0,056	0,95504	10000+
B10: (Age=5)	11,37277	191,49804	0,059	0,95264	10000+
B11: (Age=5,5)	10,50973	191,50178	0,055	0,95623	10000+
B12: (Age=6)	11,20287	191,49801	0,059	0,95335	10000+
B13: (Age=7)	11,08225	191,49799	0,058	0,95385	10000+
B14: (Age=8)	11,1212	191,49797	0,058	0,95369	10000+
B15: (Age=9)	11,64916	191,498	0,061	0,95149	10000+
B16: (Age=10)	11,81467	191,49799	0,062	0,9508	10000+
B17: (Age=11)	11,82191	191,498	0,062	0,95077	10000+
B18: (Age=12)	11,82456	191,498	0,062	0,95076	10000+
B19: (Age=13)	11,63574	191,49804	0,061	0,95155	10000+
B20: (Age=14)	12,14114	191,49806	0,063	0,94945	10000+
B21: (Age=15)	11,5706	191,49802	0,06	0,95182	10000+
B22: (Age=16)	12,03912	191,49815	0,063	0,94987	10000+
B23: (Age=17)	11,60834	191,49813	0,061	0,95166	10000+
B24: (Age=18)	11,8568	191,49817	0,062	0,95063	10000+
B25: (Age=19)	11,53935	191,49831	0,06	0,95195	10000+
B26: (Age=20)	12,19612	191,49822	0,064	0,94922	10000+
B27: (Age=21)	12,99463	191,49938	0,068	0,9459	10000+
B28: (Age=22)	13,21778	191,49934	0,069	0,94497	10000+

B29: (Age=23)	11,38519	191,49882	0,059	0,95259	10000+
B30: (Age=24)	11,49056	191,49938	0,06	0,95215	10000+
B31: (Age=25)	11,60834	191,50004	0,061	0,95166	10000+
B32: (Age=26)	10,79741	191,50004	0,056	0,95504	10000+
B33: (Age=27)	0	331,68403	0	1	1
B34: (Age=28)	10,50973	191,50178	0,055	0,95623	10000+
B35: (Age=30)	22,40575	270,81887	0,083	0,93406	10000+
B36: (Age=31)	22,40575	331,68403	0,068	0,94614	10000+
B37: (Age=32)	22,40575	331,68403	0,068	0,94614	10000+
B38: (Age=38)	22,40575	331,68403	0,068	0,94614	10000+
B39: (Age="27 months")					
	0	331,68403	0	1	1
B1: (Breed="Appaloosa")					
	-7,90345	99,63264	-0,079	0,93677	0,00037
B2: (Breed="Arabian")					
	-8,35544	99,63081	-0,084	0,93316	0,00024
B3: (Breed="Boerperd")					
	-8,17312	99,63187	-0,082	0,93462	0,00028
B4: (Breed="Connemara")					
	-7,25683	99,63625	-0,073	0,94194	0,00071
B5: (Breed="Crossbreed")					
	-8,86518	99,63058	-0,089	0,9291	0,00014
B6: (Breed="Fresian")					
	-8,10412	99,6372	-0,081	0,93517	0,0003
B7: (Breed="Irish Sport Horse")					
	-9,20274	99,64055	-0,092	0,92641	0,0001
B8: (Breed="Miniature Pony")					
	-9,89588	99,63804	-0,099	0,92089	0,00005
B9: (Breed="Nooitgedacht")					
	-8,32727	99,63075	-0,084	0,93339	0,00024
B10: (Breed="Noriker")					
	-18,40547	122,02196	-0,151	0,8801	0
B11: (Breed="Pony")					
	-8,66374	99,63089	-0,087	0,9307	0,00017
B12: (Breed="Quarter Horse")					
	0	140,89882	0	1	1
B13: (Breed="Thoroughbred")					
	-8,89879	99,63056	-0,089	0,92883	0,00014
B14: (Breed="Warmblood")					
	-9,49487	99,63067	-0,095	0,92408	0,00008
B15: (Breed="Welsh Pony")					
	-10,30135	99,63386	-0,103	0,91765	0,00003
B1: (Gender="Mare")					
	0,17623	0,1211	1,455	0,14559	1,19271
B2: (Gender="Stallion")					
	-0,32328	0,31092	-1,04	0,29845	0,72377