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Characterization of zoonotic flavi- and alphaviruses in sentinel animals in South Africa

by

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DECLARATION

I, **Stacey Human**, declare that the dissertation, which I hereby submit for the degree Master of Science, is my own original work followed from research carried out in the **Department of Medical Virology**, University of Pretoria, under the supervision of **Prof. Marietjie Venter**. These results have not previously been submitted by me for a degree at this, or any other tertiary institution.

Signed: _____ this _____the day _____2011

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SELECTED ABBREVIATIONS USED IN TEXT

°C	temperature in degrees Celsius
µg	microgram
µl	microliter
µm	micrometre
µM	micromolar
AHS (V)	African horse sickness (virus)
BANV	Banzi virus
BBB	blood brain barrier
BHK	baby hamster kidney cells
BLAST	basic local alignment search tool
BOUV	Bouboui virus
bp	base pair
BSL-3	biohazard safety level 3
C6/36	*Aedes albopictus* (Asian tiger mosquito) cell line
cDNA	complementary DNA
CHIKV	Chikungunya virus
CNS	central nervous system
CPE	cytopathic effect
CS	cyclization sequence
CSF	cerebrospinal fluid
CYS	cysteine residue
D	aspartic acid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
E	glutamic acid
EDTA	ethylene diamine tetra acetic acid
EE V	equine encephalosis virus
EHV	equine herpes virus
EHV	Edge hill virus
ELISA	enzyme linked immunosorbent assay
EMEM	Eagles minimal essential media
ER	endoplasmic reticulum
EtOH	ethanol
FCS	foetal calf serum
FRET	fluorescence resonance energy transfer

g	grams
GATU	genome annotation transfer utility
HI	haemagglutination inhibition
IFA	Immunofluorescence assay
Ig	immunoglobulin
JEV	Japanese encephalitis virus
JUGV	Jugra virus
K	Lysine
KZN	Kwa-Zulu Natal
L1	lineage 1 West Nile virus strains
L2	lineage 2 West Nile virus strains
mg	milligram
$MgCl_2$	magnesium chloride
MIDV	Middelburg virus
min	time in minutes
ml	millilitre
ML	maximum likelihood
Mr	molecular mass
mRNA	messenger RNA
NaCl	sodium chloride
ng	nanogram
NICD	National Institute for Communicable Diseases
NKV	no known vector
nm	nanometre
NS	non-structural *Flavivirus* protein
nsP	non-structural *Alphavirus* protein
nt	nucleotide
NY	New York
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute
PBS	phosphate buffered saline
PM	post-mortem
POTV	Potiskum virus
R	arginine
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction

RVF	Rift Valley fever
RWSL	repeat cyclization sequences in the Wesselsbron 3' non-coding region
s	time in seconds
SABV	Saboya virus
SA	South Africa
SEPV	Sepik virus
SHUV	Shuni virus
SINV	Sindbis virus
SPU	Special Pathogens Unit
TCID	tissue culture infectious dose
TGN	trans-golgi network
Tm	melting temperature
U	unit
UGSV	Uganda S virus
UTR	untranslated region
USA	United States of America
UV	ultraviolet
VERO	green monkey kidney cells
WEE (V)	Western equine encephalitis (virus)
WNF	West Nile fever
WNV	West Nile virus
WSLV	Wesselsbron virus
YFV	yellow fever virus

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ABSTRACT

Characterization of zoonotic flavi- and alphaviruses in sentinel animals in South Africa

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In South Africa (SA), the arboviruses West Nile virus (WNV), Wesselsbron virus (WSLV), Sindbis virus (SINV) and Middelburg virus (MIDV) are considered the most important flavi- and alphaviruses. Clinical presentation and importance of these viruses as animal pathogens in SA remains ambiguous. Although widely endemic in SA, lineage 2 (L2) WNV has rarely been associated with cases of neurological disease and was therefore assumed to be non-pathogenic. However, fatal encephalitis in a foal was diagnosed as L2 WNV in SA, 1996, leading to the thought that L2 cases were possibly being missed. As the above-mentioned arboviruses have the same transmission vectors, *Culex* mosquitoes for WNV and SINV and *Aedes* mosquitoes for WSLV and MIDV, co-screening for these viruses is important.

We hypothesise that horses could be used as sentinels for virus activity in SA and cases of unexplained neurological disease or fever in animals overlooked, rather than being non-existent. To this end, the study aimed to screen horses displaying unexplained neurological disease or fever with *Flavivirus* family-specific RT-PCR. Additionally, samples were screened with an *Alphavirus* family-specific RT-PCR to determine whether co-circulating viruses could be responsible for neurological symptoms in horses. The results would aid in establishing the molecular epidemiology and disease description of each virus, virus distribution and disease seasonality in SA.

In total 261 clinical specimens were collected from horses displaying these symptoms (2008 - 2010). Samples were screened with *Flavi-* and *Alphavirus* differential diagnostic RT-PCR and acute serum was screened for WNV-IgM and neutralizing antibodies.

Serological screening (WNV haemagglutination inhibition, WNV IgG and/or WNV neutralization) identified 62 suspected WNV cases while 34 cases could be confirmed by RT-

PCR (16/34), WNV IgM and neutralization assays (18/34) and virus isolation. Neurological disease made up 91% (31/34) of the cases, mortality was calculated at 44% (15/34). Phylogenetically 12/16 RT-PCR positives grouped with L2 SA strains. The first detection of L1 WNV and horse-associated abortion in SA was reported when a pregnant mare aborted her foetus in Ceres, Western Cape.

The first cases of WSLV-associated disease in horses were identified by sequencing *Flavivirus* RT-PCR positive products from 2 horses displaying severe neurological disease; one being fatal. This suggests missed cases in the past. To elucidate virulence factors of WSLV, a human encephalitic strain AV259, was subjected to Roche FLX454 full-genome sequencing and compared to a previously sequenced febrile strain (H177). Several structural amino acid changes occurred in proteins NS2A, NS4B and NS5 of AV259; necessary for *Flavivirus* replication. Phylogenetically AV259, clinical horse strains and WSLV strains previously isolated from animals, humans and arthropods were similar. Additionally and in concurrence with other studies, WSLV clusters with Sepik virus (SEPV) within the YFV group of the *Flaviviridae* family.

Alphavirus screening identified 17 cases; 6/17 SINV and 11/17 MIDV. SINV-WNV co-infections resulted in fatal neurological disease; remaining SINV cases recovered after displaying fever and/or mild neurological disease. MIDV symptoms varied from “three-day-stiffness” to severe neurological symptoms, with 2 fatalities. Co-infections with equine encephalosis and Shuni virus were identified. MIDV strains identified in this study were phylogenetically distinct from older strains.

Results highlight the use of horses as sentinels for virus activity and suggest that these arboviruses may have been previously missed as horse pathogens in Africa. These viruses should be considered as the aetiological agents in animals displaying unexplained neurological or hepatic disease, fevers or abortions. Awareness of flavi- and alphaviruses and the disease manifestation they may have in horses was illustrated. These findings suggest that a WNV vaccine may be beneficial for horses in SA.

CHAPTER 1
LITERATURE REVIEW

1.1. INTRODUCTION

The *Flavivirus* genus of viruses, *Flaviviridae*, comprise 70 members (Lindenbach *et al.*, 2007), and include well known arboviruses that are frequently associated with disease outbreaks in humans and animals. These viruses include Japanese encephalitis virus (JEV), dengue virus and yellow fever virus (YFV). The *Alphavirus* genus forms part of the *Togaviridae* family of viruses (Powers *et al.*, 2001), has approximately 40 members which are primarily arthropod-borne (Strauss & Strauss, 1994) and include viruses of medical importance, one such being Chikungunya virus (CHIKV) which causes extensive outbreaks in humans in Africa (Attoui *et al.*, 2007; Gould *et al.*, 2009; Powers *et al.*, 2001). Many members of these families have been classified as emerging pathogens. This is defined as the appearance of a disease in a population for the first time or the reappearance of a disease that may have existed previously but is rapidly increasing in geographic range or incidence (Morse, 1995); such as West Nile virus (WNV). Arboviral diseases are among the most important of the emerging infectious diseases (Gubler, 2001).

In sub-Saharan Africa, 22 mosquito-borne/arboviruses have been isolated, 10 of which are considered to be of medical and veterinary importance (Jupp, 2005; McIntosh, 1986). In South Africa (SA), the four most important mosquito-borne viruses from a public health and veterinary perspective include members of the *Flaviviridae*, WNV and Wesselsbron virus (WSLV), and *Togaviridae*, Sindbis virus (SINV) and Middelburg virus (MIDV) (Jupp, 2005; McIntosh, 1986; van der Riet *et al.*, 1985). The epidemiology of these viruses, as well as their role as animal pathogens (other than in livestock with regards to WSLV) in SA is, however, not well defined.

WNV for example, is distributed extensively worldwide (Africa, Asia, Europe, the Caribbean and the Americas) (Campbell *et al.*, 2002; Dauphin *et al.*, 2004) and is divided into two main lineages, lineage 1(L1) and lineage 2 (L2). L1 stains, circulating in the Western and Northern hemispheres, were always thought to be more neuroinvasive than L2 strains, consisting mainly of enzootic viruses from southern Africa and Madagascar (Campbell *et al.*, 2002). Recent studies have demonstrated that pathogenicity is genotype dependent and is not related to lineage or geographic distribution and that highly and less neuroinvasive strains exist in

both lineages (Beasley *et al.*, 2002; Venter *et al.*, 2005). Additionally, L2 strains have been associated with severe disease in humans and encephalitis in a foal in SA. However, very few reports of L2 WNV disease are documented annually in humans in SA and none in horses, apart from the foal in 1996 (Burt *et al.*, 2002), suggesting that WNV disease may be underreported in SA.

1.1.1. Flaviviruses in South Africa

As WNV has the highest incidence and is serologically widespread throughout SA (McIntosh *et al.*, 1962), the question of whether WNV affected horses in SA, and if so, were they causing disease and how did the disease present in horses. WNV causes fatal disease in birds in the USA, leading to the use of birds as sentinels for virus activity in America. Birds in SA are genetically resistant to WNV infection and therefore would not serve well as sentinels here. Horses being highly sensitive to WNV could therefore be used as alternative sentinel animals for virus surveillance in SA.

In addition to WNV, another frequently detected *Flavivirus* in SA, is WSLV (McIntosh, 1986). WSLV is transmitted by *Aedes* mosquito vectors and is chiefly a disease of livestock, causing a mild febrile illness in adult animals except in the case of pregnant sheep where it has been associated with abortions, hepatitis and death in new-born lambs (Coetzer *et al.*, 1979; Weiss *et al.*, 1956). In humans, disease is rare and is associated with a mild febrile illness, though encephalitis has been reported once (Jupp & Kemp, 1998). Viral isolations have been made throughout sub-Saharan Africa, particularly in SA during outbreaks. Another question raised was whether other flaviviruses that are serologically well-known, such as WSLV, could afford partial protection against WNV disease, perhaps being the reason that so few cases of WNV were reported in horses. Although implicated in neurological disease, WSLV is excluded from routine diagnostic tests. Additionally, its disease interaction with, and presentation in, humans and animals other than livestock, is poorly described. For this reason, many WSLV cases may be missed or incorrectly diagnosed. WSLV has not been characterised genomically and thus it is unknown whether differences in the genetic makeup determines differences in disease severity.

Other commonly circulating and readily detected arboviruses in SA are the alphaviruses, Sindbis virus (SINV) and Middelburg virus (MIDV) (McIntosh, 1986).

1.1.2. Alphaviruses in South Africa

The alphaviruses are divided into Old World and New World viruses (Weaver *et al.*, 1993). The Old World alphaviruses are primarily restricted to Africa, Europe, and Australia, causing disease characterized by fever, headaches, rash and arthritis. Encephalitis has rarely been reported. New World viruses in comparison are primarily restricted to the Americas, causing large outbreaks of encephalitis in both humans and horses (Weaver & Barrett, 2004; Zacks & Paessler, 2010). These include Eastern, Western and Venezuelan equine encephalitis (Weaver *et al.*, 1997). In SA two commonly identified Old World alphaviruses of medical importance include Chikungunya virus (CHIKV) and SINV (McIntosh, 1986). Another Old World virus, MIDV is also frequently detected serologically, but has never been implicated in human or animal disease (Kokernot *et al.*, 1957). CHIKV has caused infrequent outbreaks in humans in SA (1956, 1975/76, 1977), primarily after heavy rainfall (McIntosh, 1986), in the Limpopo, Mpumalanga and Northern Provinces as well as in northern Kwa-Zulu Natal (Jupp, 2005). CHIKV is maintained in a feral cycle between *A. furcifer* and *A. cordellieri mosquitoes* and vervet monkeys and baboons. Disease is characterized by fever and severe joint pain which may persist for years after acute illness (Lloyd, 2004). These symptoms are shared by SINV infections.

As the flavi- and alphaviruses are transmitted by the same mosquito vectors, WNV and SINV by *Culex* mosquitoes (McIntosh, 1986) and WSLV and MIDV by *Aedes* mosquitoes (Kokernot *et al.*, 1960), it is important to screen for all of these viruses in cases of unexplained fever and neurological disease as they often cause clinical disease that is indistinguishable (McIntosh, 1986). At present no treatment or vaccinations exist for these viruses. The role of alphaviruses as animal pathogens in Africa is unknown.

1.2. TRANSMISSION VECTORS AND MAINTENANCE IN NATURE

1.2.1. Mosquito Vectors

Due to the mosquito vectors being a common denominator for two different virus families, outbreaks of both diseases may occur (Jupp *et al.*, 1986a), as factors that favour one virus would likely favour the other.

1.2.1.1. *Mosquito survival mechanisms*

Each genus has distinct survival mechanisms during unfavourable environmental conditions. *Aedes* species have drought resistant eggs which require minimal moisture; while *Culex*

mosquitoes survive by means of quiescent larvae and pupae in permanent water collections or as dormant adult females (Jupp, 2005; McIntosh, 1986).

1.2.2. Maintenance in Nature

1.2.2.1. *WNV and SINV maintenance*

In SA, WNV and SINV are maintained in nature in an enzootic fashion being passed from infected *C. univitattus* mosquitoes to susceptible bird hosts (Figure 1.1). Both migratory and non-migratory birds, terrestrial and wetland, may act as natural reservoir or amplification hosts for these viruses (Dusek *et al.*, 2009; Jourdain *et al.*, 2011). Although highly viraemic when infected, birds may display little to no signs of clinical illness. Infected birds usually survive, particularly in WNV and SINV endemic areas, and develop a lifelong immunity to the virus. Seasonal migration of birds may play a role in introducing these viruses into new geographic locations leading to major disease outbreaks (Malkinson *et al.*, 2002).

WNV and SINV may "spill over" into dead end hosts such as humans and equines, usually following climatic events such as flooding. These dead-end hosts do not develop a high enough viraemia to transmit the virus back to mosquito vectors and therefore play no significant role in the life cycle of the virus (Beasley, 2005). Dead-end-hosts often develop clinical disease which can range from a non-specific mild febrile illness to fatal encephalitis in terms of WNV (Hayes *et al.*, 2005) (Figure 1.1).

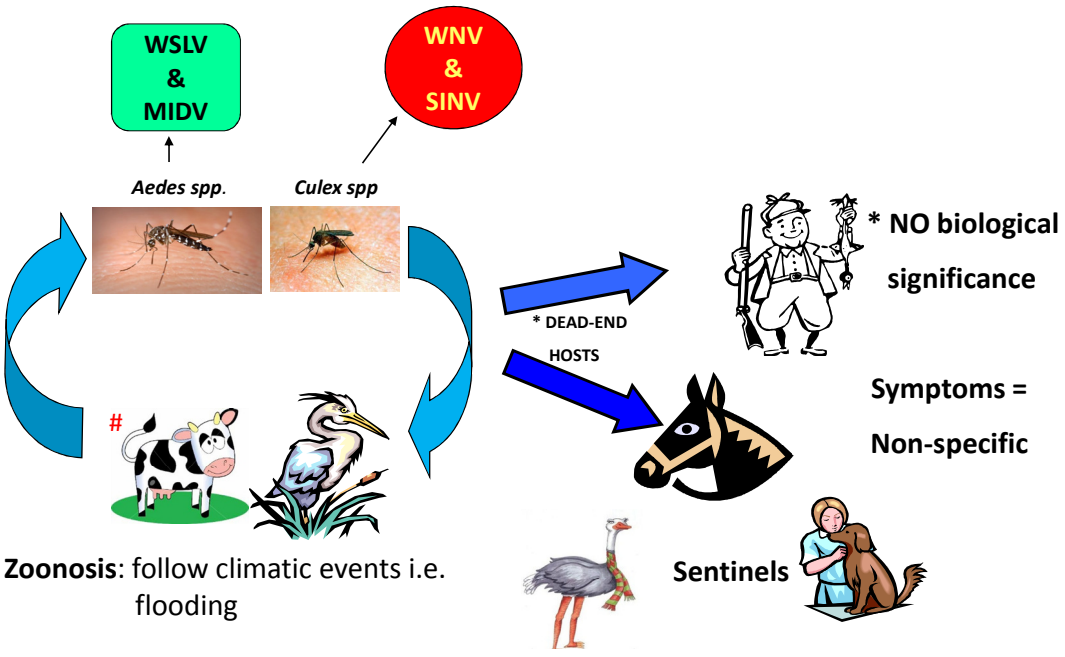

Figure 1.1 Maintenance cycles of the most important arboviruses in South Africa

1.2.2.2. *WSLV and MIDV maintenance*

WSLV and MIDV are maintained in nature in the same manner as WNV and SINV except that they are transmitted by *Aedes* mosquito vectors. WSLV is transmitted to livestock, especially sheep which serve as amplification vectors (CFSPH, 2006), while sheep are thought to play a role in MIDV maintenance (Kokernot *et al.*, 1957), however this remains unclear. Arbovirus activity is seasonal, being detected in the rainfall season (summer to mid-Autumn) (McIntosh *et al.*, 1964).

1.3. THE *FLAVIVIRIDAE* FAMILY OF VIRUSES

1.3.1. Family Classification

The *Flaviviridae* family of viruses, a positive-sense RNA virus group, consists of three genera; Flavivirus, Hepacivirus and Pestivirus and comprises over 94 members (Lindenbach *et al.*, 2007). The *Flavivirus* genus includes mosquito-borne viruses associated with major disease outbreaks in humans and animals which cause morbidity and mortality worldwide.

1.3.2. *Flavivirus* Genus

The *Flavivirus* genus consists of over 70 viruses, the majority of which are arboviruses (Cook & Holmes, 2006). Historically, the flaviviruses were serologically classified into 12 antigenic complexes; however several viruses including the prototype, YFV, did not fit into any of these complexes (Calisher *et al.*, 1989) and were therefore grouped as “unassigned”. The flaviviruses are divided into three groups: tick-borne, mosquito-borne and no known vector (NKV) (Cook & Holmes, 2006; Kuno *et al.*, 1998). Currently, 39 viruses are assigned to the mosquito-borne virus group within the genus (Weissenbock *et al.*, 2010).

A phylogenetic study by Gaunt and others (Gaunt *et al.*, 2001), using partial sequences of the E and NS5 regions of the *Flavivirus* genome revealed that the mosquito-borne virus clade forms two distinct epidemiological groups according to their invertebrate vector and vertebrate hosts: the encephalitic viruses associated with *Culex* mosquitoes and bird reservoirs; and the haemorrhagic viruses associated with *Aedes* mosquitoes and primate hosts (Gaunt *et al.*, 2001). The flaviviruses are some of the major emerging pathogens of this family and include important human pathogens such as JEV, YFV and WNV. In SA, well described flaviviruses include WSLV, WNV, Spondweni virus and Banzi virus (McIntosh, 1986). However the most frequently encountered flaviviruses in SA are WNV and WSLV. These viruses cause a variety of diseases including encephalitis, headache and fever.

Additionally, jaundice and abortions in sheep due to WSLV is regularly recorded. WNV and WSLV are associated with disease outbreaks in humans and animals (Heymann *et al.*, 1958; McIntosh, 1986; Weiss *et al.*, 1956).

1.3.3. Virion Properties and Viral Replication

1.3.3.1. *Virion structure*

Mature *Flavivirus* virions are smooth spheres of ~50 nm in diameter and contain a host-derived lipid bilayer. Two virus encoded proteins (prM and E) are embedded in the lipid bilayer and envelops the nucleocapsid which houses one single-stranded, positive-sense RNA molecule (Beasley, 2005; Castillo-Olivares & Wood, 2004) of approximately 11kb (Rossi *et al.*, 2010).

1.3.3.2. *Virus genome*

The RNA molecule is capped ($m^7GpppAmpN_2$) but lacks a polyadenylated tail on the 3' end (Valle & Falgout, 1998). The viral RNA encodes a single open reading frame (ORF) which is flanked by 5' and 3' non-coding regions (NCR) of ~132 and ~700 nucleotides respectively (Proutski *et al.*, 1997). The ORF is translated directly into a polyprotein which is co-and post-translationally cleaved to form three structural proteins [the core (C), envelope (E) and pre-membrane (prM)] and seven non-structural proteins (NS1 - NS5) (Mukhopadhyay *et al.*, 2005) (Figure. 1.2). The 5' NCR is not well conserved between the flaviviruses, but contains conserved cyclization sequences hypothesised to be involved in genome cyclization and replication (Khromykh *et al.*, 2001a). The 3' NCR is extensively heterogeneous in both size and sequence within the genus (Poidinger *et al.*, 1996); however, conserved secondary structures have been identified among the mosquito-borne flaviviruses.

1.3.3.3. *Endocytosis, replication, virus assembly and budding*

Virions bind, are internalised via clathrin-mediated endocytosis (Chu & Ng, 2004) and trafficked to lysosomes where the acidic pH induces fusion between virus lipid membranes and host cell membranes, releasing viral RNA into the cytoplasm (Stiasny & Heinz, 2006). Replication is semi-conservative and associated with the endoplasmic reticulum (ER). Immature virions assemble at the ER membrane and are transported to the host cell plasma membrane via the trans-Golgi network (TGN) (Mukhopadhyay *et al.*, 2005), where mature infectious particles are exocytosed (Ng *et al.*, 2001).

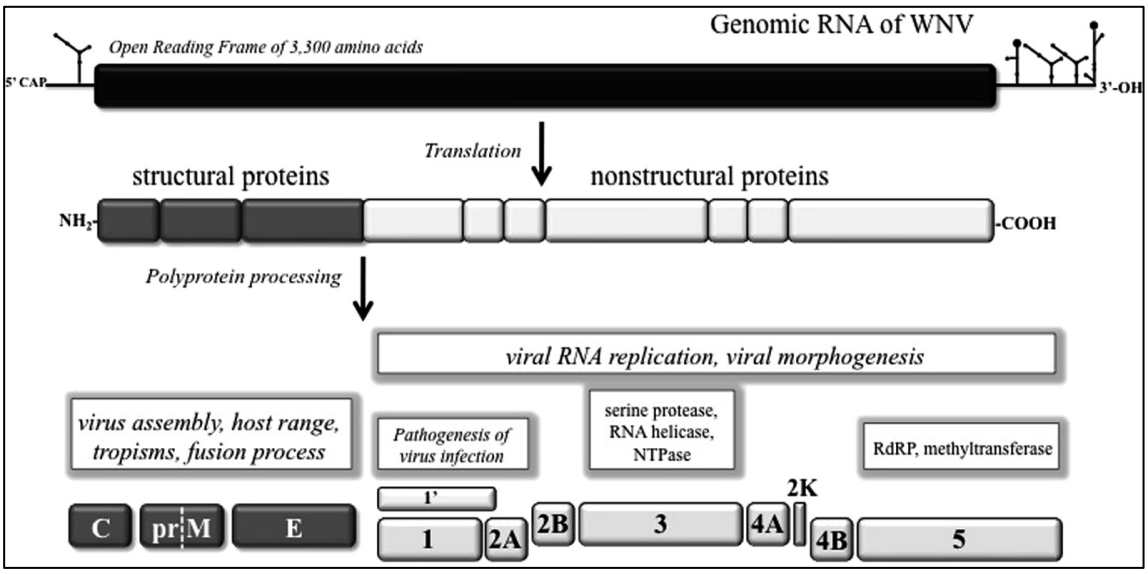

Figure 1.2 Schematic representation of the *Flavivirus* genome using West Nile virus as an example. The 5' structural and 3' non-structural proteins are shown and their functions indicated. Adapted from Murray and others, 2010 (Murray *et al.*, 2010).

1.3.4. Protein Function and Importance

The importance and functions of the *Flavivirus* proteins are shown in Table 1.1. The gene order is as follows: 5'NCR-C-preM-M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'NCR (Figure. 1.2). The structural proteins form infectious virions and interact with viral RNA and host lipid membranes. Importantly the E-protein and is the target for neutralizing antibody production in infected hosts (Chu *et al.*, 2007; Khromykh *et al.*, 2001a; Lindenbach *et al.*, 2007; Volk *et al.*, 2009). The NS proteins are believed to play a role in viral RNA replication (Koonin, 1993; Leung *et al.*, 2008; Valle & Falgout, 1998; Wu *et al.*, 2005) and evasion of the innate host immune response (Munoz-Jordan *et al.*, 2003). Mutations in the NS proteins have been documented to reduce replication efficiency (NS5 and NS4B genes) and virion assembly and budding (NS2A) (Liu *et al.*, 2003). The NS5 forms the core of the replicase complex (Bollati *et al.*, 2009; Koonin, 1993; Lindenbach *et al.*, 2007) and mutation of this protein has led to reduced virulence in mice (Xie *et al.*, 1998) and has been implicated in reduced secretion of infectious Kunjin virions (Khromykh *et al.*, 2001b). Additionally, mutations in the E-protein, NS5 and NS2 proteins may affect tissue tropism (Ding *et al.*, 2005).

Table 1.1 *Flavivirus* proteins and their functions

	Protein	Function
Structural	C	Nucleocapsid, contains conserved cyclization sequences
	prM	Chaperon for E-protein (prevents conformational change during transport of immature virions to host plasma membrane
	E*	Mediates virion insertion into host cell membrane, receptor binding, neutralizing antibody epitopes.
Non-structural	NS1	Involved in protein translation, cellular processing, induces a strong humoral response in host
	NS2A	Interacts with NS5-NS3 replication complex, virion assembly and release, biogenesis of virus-induced membranes in infected cells.
	NS2B	Co-factor for NS3 serine protease
	NS3	Serine protease (complex NS2B/NS3), helicase, nucleoside triphosphatase and RNA triphosphatase activity. Important for polyprotein cleavage.
	NS4A	Interacts with NS5-NS3 replication complex
	NS4B	Co-localized with NS3and dsRNA at replication sites therefore believed to be involved in early replication events.
	NS5*	RNA-dependent-RNA-polymerase (RdRp), methyltransferase

* Proteins targeted for *Flavivirus* diagnostic screening and phylogenetic analysis

1.4. WEST NILE VIRUS

1.4.1. WNV Phylogenetic Classification

WNV falls into the JEV serocomplex within the *Flaviviridae* along with Kunjin (sub-clade of WNV), Cacipacore, JEV, Koutango, Alfuy, Murray Valley encephalitis, St. Louis encephalitis, Usutu and Yaounde viruses (Kuno *et al.*, 1998; Poidinger *et al.*, 1996).

1.4.2. WNV Lineages

WNV strains are classified into at least 5 genetic lineages (Bondre *et al.*, 2007). The two major lineages are those of lineage 1 (L1) and lineage 2 (L2) (Berthet *et al.*, 1997), however a number of putative new lineages have been proposed:

L1 strains are widely distributed, being found in Northern Africa, the Americas, Canada, Asia, Australia, the Caribbean and Europe (Campbell *et al.*, 2002).

Meanwhile L2 typically consists of enzootic viruses from sub-Saharan Africa and Madagascar and are the main flaviviruses implicated in infections in SA (Burt *et al.*, 2002). However, outbreaks of L2 WNV have been associated with neuroinvasive disease in wild

birds, horses and humans in Hungary, Russia and Austria (Bakonyi *et al.*, 2006; Kutasi *et al.*, 2011; Platonov *et al.*, 2008). More recently, outbreaks of L2 WNV neuroinvasive disease have been reported in humans in Greece (ECDC, 2010; Papa *et al.*, 2010).

Lineage 3 WNV (Rabensburg virus) was isolated in 1997 from *C. pipiens* mosquitoes on the Czech republic-Austrian border (Bakonyi *et al.*, 2005; Hubalek *et al.*, 1998), and lineage 4 (LEIVKrnd88-190) from *D. marginatus* ticks in the Caucasus Mountains in 1998 (Lvov *et al.*, 2004). Lineage 5 (WNV804994) was isolated from India (Bondre *et al.*, 2007). A new lineage, lineage 6, has been proposed for the Sarawak Kunjin virus strain (Mackenzie & Williams, 2009) as it is significantly different to Australian Kunjin strains (Scherret *et al.*, 2001). A seventh lineage has been suggested for Koutango virus, an African virus which is closely related to WNV (Mackenzie & Williams, 2009). Additionally, a putative new lineage of WNV has been proposed following the isolation of WNV from a pool of *C. pipiens* mosquitoes in Spain in 2006 (Vazquez *et al.*, 2010).

1.4.2.1. L1 WNV vs. L2 WNV

Following its emergence in the USA, WNV L1 strains were postulated to be more pathogenic than L2 strains due to the high degree of neuroinvasive disease reported in humans, birds and horses during the 1999 and 2002 outbreaks recorded in America (Ostlund *et al.*, 2001; Ward, 2006). In comparison to the sparse WNV outbreaks recorded for L2 disease in SA. Comparisons of the relationships between North American and SA WNV strains and their ability to induce neuroinvasive disease in mice demonstrated that virulence is genotype specific and not related to lineage, geographic location, isolate source, passage level or year of isolation. Importation and exposure of new neuroinvasive WNV strains to a previously immunologically naïve population is the most likely cause for the perceived virulence difference (Beasley *et al.*, 2002; Venter *et al.*, 2005).

1.4.3. WNV Geographic Distribution and Epidemiology

WNV is distributed extensively throughout Africa, the Middle East, Asia, Europe, Australia, North and South America, the Caribbean and Madagascar and may infect a wide range of hosts, making it one of the most widespread human arbovirus infections described (Hayes *et al.*, 2005).

1.4.3.1. Epidemiology

WNV in the Old World (Africa, Asia and Europe)

WNV was first isolated from the blood of a woman presenting with a febrile illness in 1937 in the West Nile Province of Uganda in East Africa (Campbell *et al.*, 2002). Between its original isolation until the mid-1990's WNV was not considered a major pathogen of humans or animals due to infrequent outbreaks and low level neuroinvasive disease (Campbell *et al.*, 2002). Human WNV outbreaks first occurred in Israel in the 1950's and subsequently in France (1962- 1964) (Murgue *et al.*, 2001a) and SA in 1974 (Jupp *et al.*, 1986a), followed by very little reported virus activity over a 20-year period (Campbell *et al.*, 2002). A significant increase in frequency and severity of WNV outbreaks have been documented since 1994 in both humans and equines (Table 1.2) (Dauphin *et al.*, 2004; Kramer *et al.*, 2008; Marfin *et al.*, 2001). Sporadic cases of WNV in birds have also occurred, most notably, WNV was isolated from birds with encephalitis in Israel, Hungary, Austria and Russia (Bakonyi *et al.*, 2006; Platonov *et al.*, 2008; Wodak *et al.*, 2011) (Table 1.1). Recently, outbreaks of L2 WNV neuroinvasive disease have been reported in humans in Greece (Papa *et al.*, 2010) and neurological disease documented in horses (ECDC, 2010). L2 WNV strains have been isolated from birds (Valiakos *et al.*, 2011) and pools of *C. pipiens* mosquitoes (Papa *et al.*, 2011) in the same region as human and horse disease occurrence.

Table 1.2 WNV outbreaks in the Mediterranean basin and Europe (1994 – 2011)

Country	Year			Reference
	Human	Equine	Bird	
Algeria	1994			(Murgue *et al.*, 2001a)
Morocco	1996	1996		(Schuffenecker *et al.*, 2005)
Romania	1996, 2010	1996		(Savage *et al.*, 1999; Sirbu *et al.*, 2010)
Tunisia	1997, 2003			(Abroug *et al.*, 2006)
Italy	2008-2009	1998, 2008 - 2009		(Monaco *et al.*, 2011; Savini *et al.*, 2008)
Russia	1999, 2000-2001		1999, 2000-2004, 2007	(Platonov *et al.*, 2008)
Israel	1999, 2000	1998 - 2000	1997-1998	(Weinberger *et al.*, 2001)
France	1962, 1964, 2000, 2003	1962, 1964-1965, 2000, 2003, 2004, 2006	2001-2002	(Charrel *et al.*, 2001; Del Giudice *et al.*, 2004; Murgue *et al.*, 2001b; Murgue *et al.*, 2002)
Hungary	2003-2004	2007, 2008, 2011	2003-2005, 2009	(Bakonyi *et al.*, 2006; Erdelyi *et al.*, 2007; Kutasi *et al.*, 2011)
Greece	2010	2010		(Papa *et al.*, 2011; Papa *et al.*, 2010; Valiakos *et al.*, 2011)

WNV in the New World (The Americas, Canada, Australia)

WNV was first detected in America in 1999 during an epidemic of meningoencephalitis in New York City (Lanciotti *et al.*, 1999), where 59 people were hospitalized and 7 died. Concurrently WNV caused extensive fatalities birds (especially crows [*Corvidae*], blue jays and several exotic species) and equine cases were also reported (Ostlund *et al.*, 2001). The strain isolated (NY99) was phylogenetically similar to a strain identified in geese in Israel in 1998 (Lanciotti *et al.*, 1999). During the 2002 WNV outbreak over 4156 human, 16500 bird and 15257 equine cases were reported, making WNV responsible for the largest outbreak of arboviral encephalitis ever recorded in the Western hemisphere; as well as the largest outbreak of WNV encephalitis recorded in the world (O'Leary *et al.*, 2004). According to the Center for Disease Control (CDC, 2010), recent statistics show that from 1999 to December 2010, over 30000 cases of WNV had been reported in the United States of America (USA) alone, of which 12711 were neuroinvasive cases and 1206 people died (www.cdc.gov/ncidod/dvbid/westnile/Mapsativity/sur&conrol10MapsAnybyState.htm).

WNV was first detected in Canada in 2001 and had spread to every Canadian province by 2004 except for British Columbia, where the first WNV disease activity was detected in 2009 (Morshed *et al.*, 2010). In comparison, Kunjin virus which is endemic to Australia, is considered to be a WNV subtype (Berthet *et al.*, 1997; Scherret *et al.*, 2001) and belongs to clade b of the L1 strains (Mackenzie & Williams, 2009). Kunjin is generally considered a mild febrile disease in humans but has been associated with neurological disease in horses (Hall *et al.*, 2001) and humans (Charles *et al.*, 2001).

WNV in Latin America and the Caribbean

From 2001 – 2004 there has been widespread circulation of WNV in avian and equine populations in Mexico and many Caribbean countries (Blitvich, 2008; Kramer *et al.*, 2008). Since 2004 seroconversion to WNV in horses and birds has been reported in many South American countries (Bosch *et al.*, 2007; Mattar *et al.*, 2005) and isolated reports of human, equine and avian WNV illness have been reported in Latin America and the Caribbean (Komar & Clark, 2006). WNV activity in Central America has been detected from 2001 – 2006 (Cruz *et al.*, 2005; Hobson-Peters *et al.*, 2011; Morales-Betoulle *et al.*, 2006).

1.4.4. WNV Pathogenesis in Humans and Horses

Initial viral replication occurs within skin dendritic cells at the site of inoculation (Samuel & Diamond, 2006). Virus then spreads to surrounding lymph nodes, resulting in primary

viraemia and subsequent infection of peripheral organs such as the spleen and kidneys (Blitvich, 2008). Viraemia is extremely short, ranging ~2 days pre and 4 days post illness onset. Virus titer in horses is extremely low (Bunning *et al.*, 2002). Secondary viraemia is thought to seed the central nervous system (CNS) (Wang *et al.*, 2004). In humans, WNV predominantly infects neurons in the brainstem and ventral horns but can disseminate throughout the CNS of immune compromised individuals (Guarner *et al.*, 2004). Most non-fatal infections are cleared by the host immune response (Samuel & Diamond, 2006); however, viral persistence has been documented in immune compromised hosts (Brenner *et al.*, 2005). It is unknown whether persistent WNV infections occur in naturally infected horses (Castillo-Olivares & Wood, 2004). The incubation period for WNV is between 2 – 6 days but may extend to 14 days, after which symptoms appear (Nash *et al.*, 2001).

1.4.5. Clinical Manifestations in Humans in the USA

Although most WNV infections in humans are sub-clinical, approximately 20% of people will develop a non-specific flu-like illness known as West Nile fever (WNF) (Debiasi, 2011), whereas neuroinvasive disease will develop in <1% of these individuals (Hayes *et al.*, 2005; Mostashari *et al.*, 2001). WNF, characterized by an acute onset of fever, fatigue, headache, myalgia, arthralgia, anorexia, diahorrea, lymphadenopathy, a sore throat, severe chest pain and dizziness lasts approximately 5 days (McIntosh *et al.*, 1964). Severe non-neurologic manifestations of the disease may occur, including fatal hepatitis (Burt *et al.*, 2002), myocarditis and pancreatitis (Sampathkumar, 2003). WNV neuroinvasive disease is characterized by meningoencephalitis, meningitis and acute flaccid paralysis (Debiasi, 2011; Sejvar, 2007) and is most severe in the elderly or immune suppressed (Brenner *et al.*, 2005). Clinical severity of WNV encephalitis ranges from mild disorientation to coma and death (Hayes *et al.*, 2005). Muscle tremours, ataxia and Parkinsonism are also common (Sejvar *et al.*, 2003). Overall fatality rate for neuroinvasive disease is approximately 10% with long term neurological sequelae occurring in ~50% of patients (O'Leary *et al.*, 2004).

1.4.6. WNV in Horses in the USA

Horses are readily susceptible to infections with WNV. After the initial introduction of WNV into America during 1999, 25 WNV cases were confirmed in horses in New York City (Trock *et al.*, 2001). A steady rise in cases was detected each year after that until 2002 when over fifteen thousand horses in 40 American states were reported (O'Leary *et al.*, 2004).

Outbreaks in Morocco and Italy (Cantile *et al.*, 2000) recorded fatality rates of approximately 45 and 43 percent respectively. The introduction of an equine WNV vaccination and increased vector control helped to decrease WNV infections in equines in the USA.

1.4.6.1. *Clinical features of WNV infections in horses*

WNV infections in horses are thought to be largely asymptomatic (Farfan-Ale *et al.*, 2006; Nielsen *et al.*, 2008), however statistics of equine WNV disease in the USA show that between 10 - 40% of exposed horses develop neurological disease (Castillo-Olivares & Wood, 2004), and of these, the fatality rate may be as high as 40% (Ostlund *et al.*, 2001). Other studies have shown that approximately 20% of horse infections are symptomatic of which 90% are neurological and 42% fatal. Recovery takes between 3 – 4 weeks post infection (Cantile *et al.*, 2000; Salazar *et al.*, 2004); however some horses may take up to 6 months to return to normal function. At least 10% of horse owners report that long term neurological deficits may persist (Ward *et al.*, 2006), limiting the athletic potential of the horses. Severe disease is characterized by an acute onset of neurological abnormalities (Castillo-Olivares & Wood, 2004) which may result in coma and death. A number of studies were carried out in the US to determine which of these symptoms were associated with a poor prognosis of the infected animal. In three studies, it was calculated that recumbency, caudal paresis, inability to rise and horses older than 5 years of age have a poor prognosis for survival (Salazar *et al.*, 2004). Similarly, a study carried out in Texas during 2002 (Ward, 2006) recorded fever, hind-limb ataxia, recumbency, depression, muscle fasciculation/tremor and abnormal gait as the most common symptoms. A poor prognosis was associated with recumbency, lip-droop, lameness and limb in-coordination (Ward *et al.*, 2006). Comparable symptoms were identified in WNV outbreaks in Italy (Cantile *et al.*, 2000), and the original outbreak in New York City (Trock *et al.*, 2001). Behavioral changes such as feed refusal which may lead to anorexia, colic, teeth grinding and hyperaesthesia have been noted (Ostlund *et al.*, 2001).

1.4.6.2. *Diagnosis of WNV in horses*

WNV detection in field cases is hampered by the short duration and low titer of viraemia in horses (Bunning *et al.*, 2002); therefore a negative result should not be regarded as an absence of WNV (Castillo-Olivares & Wood, 2004). WNV diagnosis is dependent on clinical suspicion as well as a positive result by either virus isolation, nested reverse-transcriptase (RT) polymerase chain reaction (PCR) or real-time PCR from blood, tissues or cerebrospinal

fluid (CSF) (Kleiboeker *et al.*, 2004). Nested PCR assays are required for diagnosis in horses due to the low level of viraemia in these animals. Detection of WNV immunoglobulin-M (IgM) antibodies (confirmed by serum neutralization) or a 4-fold rise in neutralizing antibody titers in paired sera (Kleiboeker *et al.*, 2004) is also considered a recent infection. IgM is the first antibody produced and can be detected in the vertebrate host roughly 6 - 10 days after infection (Bunning *et al.*, 2002). Since IgM does not cross the blood-brain-barrier (BBB), presence of IgM in the CSF indicates a breach of the BBB and neuroinvasion, providing evidence of the virus in encephalitic cases (Debiasi, 2011). A study undertaken in 2010 demonstrated that antibody profiles in horses are similar whether infected with L1 or L2 WNV strains and that WNV neutralizing antibodies last for a significantly longer amount of time than WNV-specific IgM antibodies. This may be useful for identifying and differentiating recent infection from previous exposure (Castillo-Olivares *et al.*, 2011). Postmortem diagnosis includes immunohistochemistry, immunofluorescence with WNV-specific antibodies on fixed tissue (Castillo-Olivares *et al.*, 2011) as well as nested RT-PCR and real-time RT-PCR. When testing the CNS, it is important to extract RNA from several areas of the brain and spinal cord (Cantile *et al.*, 2000). Virus isolation can be attempted from the blood, CSF and tissues of infected animals on VERO cells; however the cytopathic effect is not always evident

1.4.7. WNV in South Africa

1.4.7.1. *WNV in humans in SA*

In SA, WNV is extensively endemic in areas where the principal vector, *C univittatus,* and avian hosts of the virus are present. Antibody surveys (1950s - 1970s), carried out on the inland plateau (Figure 1.3), on human and animal sera revealed that WNV had the widest distribution and highest incidence in SA (McIntosh *et al.*, 1962). WNV neutralizing antibodies in human sera were found more frequently on the inland plateau (17.1% in the Karoo and 8% on the Highveld) than the coastal plain (2%) (Jupp, 2001). Human cases/epidemics of WNV in SA are sporadic, possibly as the main transmission vector *C. univitattus* is weakly anthropophilic, and occur annually during summer rainfall months on the Highveld (McIntosh *et al.*, 1964). Most human WNV infections have been characterized by fever, arthralgia, myalgia and a maculopapular rash (Kokernot & McIntosh, 1959), however, severe disease has been documented: renal failure, necrotic hepatitis and non-fatal encephalitis (Burt *et al.*, 2002) (Table 1.3).

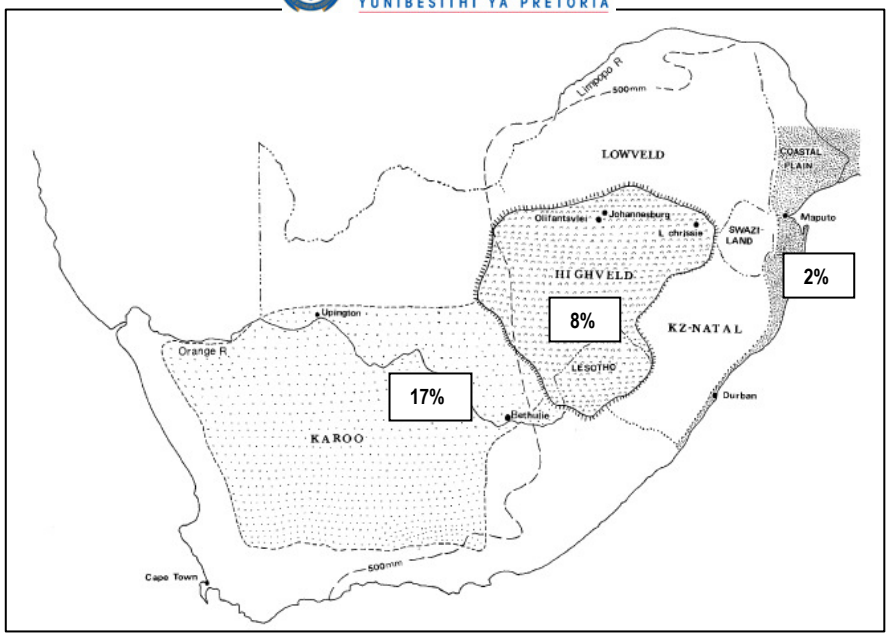

Figure 1.3 Map of South Africa depicting the inland plateau (Highveld and Karoo) and the coastal Kwa-Zulu Natal plain. The 500mm isohyets bisect the country into the moist eastern and arid western regions. Figure adapted from Jupp, 2001 (Jupp, 2001)**.**

Table 1.3 Published WNV strains isolated from human patients in South Africa (1958 - 2001)

Strain	Year	City, Province	Disease	Outcome
H442*	1958	Ndumo, KZN	Fever, myalgia, arthralgia, rash	Survived
H912	1964	Middelburg, Mpumulanga	Fever, myalgia, arthralgia, rash	Survived
H1127	1968	Johannesburg, Gauteng	Fever, myalgia, arthralgia, rash	Survived
SPU101/89	1989	Bloemfontein, Free State	Fever, coagulopathy, haemoglobinuria, renal failure	Survived
SPU116/89*	1989	Pretoria, Gauteng	Necrotic hepatitis	Died
SA381/00#	2000	Naboomspruit, Limpopo	Fever, myalgia, arthralgia, rash	Survived
SA93/01#	2001	Johannesburg, Gauteng	Fever, myalgia, rash, encephalitis	Survived

* WNV strains demonstrated to be of high neuroinvasiveness in mice, # WNV strains demonstrated to be of low neuroinvasiveness in mice

1.4.7.1.1. Outbreaks

In 1974 the largest concurrent WNV-SINV outbreak ever recorded in SA occurred over extensive areas of the Karoo (especially Upington), just after a flood period, where tens of thousands of human cases were estimated (Jupp *et al.*, 1986a; McIntosh, 1986). Most infections were detected between the months of February – April (McIntosh, 1986) and seroconversion ranged between 50% – 80% in certain towns in the Karoo. No deaths were recorded during the outbreak and only one meningoencephalitis case was observed. In the summer of 1983-84 an epizootic of WNV and SINV occurred in the Witwatersrand-Pretoria region of the Gauteng province. The number of human cases was much higher for SINV (Jupp *et al.*, 1986a). Since these outbreaks human WNV infections in SA have been constant at approximately 5 – 15 cases per year (Burt *et al.*, 2002). However, only a small percentage of cases are thought to be submitted for laboratory investigation. When considering the historical seroprevalence of WNV in humans and horses, WNV infection rate is high and therefore it is more likely that infections are underreported rather than non-existent. No recent WNV serological data are available for SA.

Although some avian species, such as ostrich chicks, show susceptibility to the disease experimental infection of 13 species of wild birds from SA were able to support virus replication without becoming sick or dying (Burt *et al.*, 2002). It is then unsurprising that the large WNV epidemics in SA were associated with epizootics in birds; for example, a 53% seroprevalence was observed in wild birds after the 1974 outbreak and no excessive bird deaths were recorded (Jupp, 2001).

1.4.7.1.2. Molecular epidemiology of SA WNV strains

Phylogenetically, the homology between South African isolates ranged between 86.3% – 100%. Identical isolates originated from different sources, isolation years or regions in some instances (Burt *et al.*, 2002). Minimal genetic diversity strongly supports that WNV is endemic in SA and does not depend on annual reintroduction by migrating birds.

1.4.7.2. *WNV in horses in SA*

A study carried out in thoroughbred horses in SA in 2003 showed that 11% of yearlings and 75% of their dams were positive for WNV by serology (Guthrie *et al.*, 2003). As these horses had no previous record of disease, it was postulated that L2 WNV strains, which are endemic to SA, do not cause neuroinvasive disease. Additionally, three seronegative horses were

inoculated with a L2 strain (SA381/00), which was isolated from a human with benign disease (Burt *et al.*, 2002). Following inoculation the horses failed to develop clinical disease (Guthrie *et al.*, 2003). In a subsequent study it was shown that the strain used was mild and less neuroinvasive in mice when compared to other SA WNV strains (Venter *et al.*, 2005). In the United States (US), subclinical infections in horses are frequently reported (Nielsen *et al.*, 2008). L2 WNV was diagnosed in a thoroughbred foal with fatal encephalitis in the Somerset West district in the Western Cape (Burt *et al.*, 2002). This raised the question as to whether neurological disease may be missed in horses in SA and indicated the need to investigate unexplained neurological disease or fever in equines.

1.4.8 Differential Diagnosis of Neurological Horse Disease and Unexplained Fatalities in SA

Clinically, all horses appear to be equally susceptible to WNV encephalomyelitis, regardless of age, breed or regional location (Bunning *et al.*, 2002); suggesting that horses in SA would also be susceptible to WNV infection. Differential diagnosis for WNV should include other frequently encountered horse pathogens in SA, such as: rabies virus, alphaviruses (SINV and MIDV), EHV-1, EEV and AHSV (MacLachlan & Guthrie, 2010). Additionally**,** other potential *Flavivirus* pathogens should be considered. In SA, many flaviviruses have been identified as disease causing agents, including Spondweni virus, Banzi virus and WSLV (McIntosh, 1986). WSLV is frequently detected in livestock (Weiss *et al.*, 1956) and is serologically widespread in SA (McIntosh *et al.*, 1962), making it the most important of these viruses to screen for in addition to WNV.

1.5. WESSELSBRON VIRUS

1.5.1. Phylogenetic Classification

WSLV is grouped with the “unassigned” viruses, which includes the prototype flavivirus, YFV (Gaunt *et al.*, 2001). Most recently phylogenetic analysis has determined that WLSV is most closely related to another “unassigned” virus, Sepik virus (SEPV) that was isolated in Papua New Guinea (Grard *et al.*, 2010). Currently, the “unassigned” group of viruses include: YFV, WSLV, SEPV, Banzi, Edge Hill, Jugra, Saboya, Potiskum, Uganda S and Bouboui.

1.5.2. Geographic Distribution

First isolated in the Wesselsbron district of the Free State province of SA in 1955 (Weiss *et al.*, 1956), WSLV has now been isolated from several African countries including Botswana

(Mushi *et al.*, 1998), the Cameroon, Nigeria, Uganda, Senegal, the Central African Republic, Cote d' Voire, Mozambique and Zimbabwe. Serological surveys suggest that this virus is widely distributed in other African countries (Baba *et al.*, 1999), and Madagascar (Morvan *et al.*, 1990). Additionally, WSLV has also been isolated from mosquitoes in Thailand (CFSPH, 2006).

1.5.3. WSLV in South Africa

WSLV is widespread in southern Africa, but clinical disease is sporadic and usually associated with outbreaks characterized by abortions in sheep and goats (Mushi *et al.*, 1998; Weiss *et al.*, 1956). Human disease had been documented to cause encephalitis (Jupp & Kemp, 1998) and febrile disease (Smithburn *et al.*, 1957). Serological studies have shown WSLV to predominate in the more tropical parts of SA, such as Kwa-Zulu Natal (McIntosh *et al.*, 1962) particularly in cattle, sheep, goats, and donkeys (Kokernot *et al.*, 1958; Kokernot *et al.*, 1960). The more temperate plateau regions such as Gauteng, the Karoo and the Free State province have lower antibody titers in comparison. WSLV antibodies are nevertheless widespread in both humans and animals in the temperate regions (Dickinson *et al.*, 1965). WSLV neutralizing antibodies have been found in white and black rhinoceros, giraffes, zebras, ostriches, African elephants and most ruminants except impala (Barnard & Voges, 1986). Although the reservoir host in unknown, it is thought to include domestic ruminants, wild birds and wild rodents such as gerbils. WSLV is readily isolated from mosquitoes in SA (Kokernot *et al.*, 1960).

1.5.4. WSLV Animal Disease in South Africa

1.5.4.1. *Sheep*

WSLV was first isolated from an eight-day-old lamb (Coetzer *et al.*, 1978; Weiss *et al.*, 1956), from a flock of sheep that at the time consisted of ewes in advanced stages of pregnancy, several of which aborted at full term and/or died. It was also noted that many new born lambs were dying during the first week of life; however, no clinical signs of the disease occurred in the yearlings or wethers (castrated male sheep). The outbreak was thought to be due to a vaccine strain of Rift valley fever (RVF) virus. WSLV and RVF have similar ecologies as they are transmitted by the same mosquito vectors and characteristically cause disease after periods of heavy rainfall; the virus isolated from the lamb, however, was shown to be distinct from RVF (Weiss *et al.*, 1956).

The incubation period in animals ranges between 1 and 4 days. WSLV disease causes an acute, biphasic, febrile illness in livestock and has a broad host range occasionally causing illness in ostriches, pigs, cattle, goats and man (Allwright *et al.*, 1995; Jupp & Kemp, 1998; Verwoerd, 2000). The virus has also been isolated from a camel and detected serologically in lemurs in Madagascar. In adult sheep and cattle it is sub-clinical, except in the case of pregnant ewes where it may cause hepatitis, haemorrhages and abortions associated with neurological defects and *hydrops amnii* which is an excessive build-up of fluid in the amniotic cavity (Coetzer & Barnard, 1977). Complications during the abortion or due to a prolonged pregnancy may cause death in the ewe. Foetuses aborted due to *hydrops amnii* are malformed and show signs of arthrogryposis, brachygnathy inferior, hydraencephaly, hypoplasia and neurogenic aplasia of the spinal cord. In an atypical outbreak in SA in 1957, symptoms in adult animals included nasal discharge, diarrhoea, jaundice and subcutaneous oedema of the head (CFSPH, 2006).

In new-born lambs WSLV is hepatotropic and primarily causes a fatal disease. In the unborn foetus of both sheep and cattle, this disease is neurotropic resulting in brain teratology (Coetzer *et al.*, 1979). Outbreaks of abortions amongst sheep and goats as well as substantial mortality among new-born lambs and kids (roughly 30%) (Coetzer & Theodoridis, 1982; Coetzer *et al.*, 1978) attract considerable attention, especially as these losses lead to a heavy economic burden amongst farmers. In new-born lambs clinical signs may include anorexia, weakness, listlessness, an increased respiratory rate, lethargy, sunken flanks and respiration being mostly abdominal.

1.5.4.2. *Other species*

WSLV infections in adult goats, horses, cattle and pigs are usually unapparent and limited to fever and/or low viraemia (Weiss *et al.*, 1956), although WSLV has been implicated in abortions in goats from Botswana (Mushi *et al.*, 1998). Experimental infection in Nigeria of Red Sokoto goats with a WSLV strain isolated from a camel resulted in biphasic febrile reaction which coincided with viraemia that lasted 3 – 4 days. Weakness, swaying of the hindquarters, mucoid diarrhoea, oedema of the head and death was observed in 50% of the infected animals (Baba, 1993). Experimental infection of 15 pregnant cattle with WSLV (Coetzer *et al.*, 1979), lead to only 2 abortions. The main disease feature being a slight fever in adult animals with low or absent viraemia. Of the calves that did abort, marked porencephaly and cerebellar hydroplasia was present (Coetzer *et al.*, 1979).

1.5.5. Laboratory Diagnosis

WSLV infections are positive if virus is isolated from viraemic specimens (in suckling mice, chick embryo's, baby hamster kidney (BHK) or lamb kidney cells), or if the HI assay is positive and confirmed by serum neutralization. An HI assay adapted to detect WSLV IgM antibodies has been used to identify infections in Nigeria (Baba *et al.*, 1999). Meanwhile immunohistochemical staining on formalin-fixed liver specimens is used in post-mortem diagnosis to detect antigen (van der Lugt *et al.*, 1995). As yet, no WSLV-specific PCR has been published.

1.6. THE ALPHAVRUSES, *TOGAVIRIDAE* FAMILY

The *Alphavirus* and *Rubivirus* genera make up the *Togaviridae* family of viruses (Powers *et al.*, 2001). The alphaviruses are primarily arthropod-borne and are serologically distinct from the rubiviruses (Strauss & Strauss, 1994).

1.6.1. Genus Classification

All medically important arthropod-borne togaviruses come from the *Alphavirus* genus (Lloyd, 2004), and over 40 members have been identified (Luers *et al.*, 2005). Antigenically, the alphaviruses are divided into 8 serocomplexes: Eastern, Western and Venezuelan equine encephalitis, Trocara, Middelburg, Ndumu, Semliki Forest and Barmah Forest complexes (Gould *et al.*, 2009). These groupings reflect differences in the envelope-1 (E1) proteins (Powers *et al.*, 2001). The alphaviruses are divided into Old World and New World viruses (Weaver *et al.*, 1993). Antigenic complexes accurately reflect clades of viruses that share medically important characteristics (Powers *et al.*, 2001).

1.6.1.1. *Old World viruses*

Old World alphaviruses are primarily restricted to Africa, Europe, Australia and cause disease characterized by fever, headaches, myositis, myalgia, arthralgia, rash, arthritis. Although viraemia is usually short-lived, arthralgia and myalgia may persist for months following infection (Laine *et al.*, 2002). Old World viruses include the prototype *Alphavirus* Sindbis virus (SINV), Semlliki Forest, Barmah Forest, Middelburg and Ndumu viruses.

1.6.1.2. *New World viruses*

New World viruses primarily cause fatal encephalitis and degeneration and necrosis of neurons in their dead-end hosts; and include Eastern and Venezuelan equine encephalitis viruses. These viruses have been associated with encephalitis in humans and horses in the Americas (Zacks & Paessler, 2010). Western equine encephalitis virus (WEEV) is a recombinant that derived its non-structural genes from an Eastern equine encephalitis virus ancestor and its structural proteins from a SINV ancestor (Weaver *et al.*, 1997).

1.6.2. Virion properties and Viral Replication

1.6.2.1. *Virion structure*

Alphaviruses are small spherical particles of ~70 nm in diameter consisting of a host-derived bi-lipid membrane, a nucleocapsid and an outer glycoprotein shell (Strauss & Strauss, 1994). The E1 and E2 glycoproteins form the outer surface of the virion and interact with host-cell receptors and host derived antibodies (Sanchez-San Martin *et al.*, 2009). The envelope is tightly organized around the nucleocapsid which houses a single-stranded, positive-sense RNA molecule of roughly 11.5kb in size (Norder *et al.*, 1996).

1.6.2.2 *Alphavirus genome*

Genomic RNA is capped (7-methylguanosine) at the 5'terminus, polyadenylated at the 3' terminus (Figure 1.4) and is flanked by a 5' NCR (Pfeffer *et al.*, 1998). The genome is divided into two parts: the 5' two-thirds encode four non-structural proteins (nsP1-nsP4) while the 3' one-third encodes the structural proteins; the capsid (C), envelope (E2 and E1), and 6K (Lloyd, 2004). Some alphaviruses have a third envelope protein, E3 (Strauss & Strauss, 1994), however this is absent in SINV.

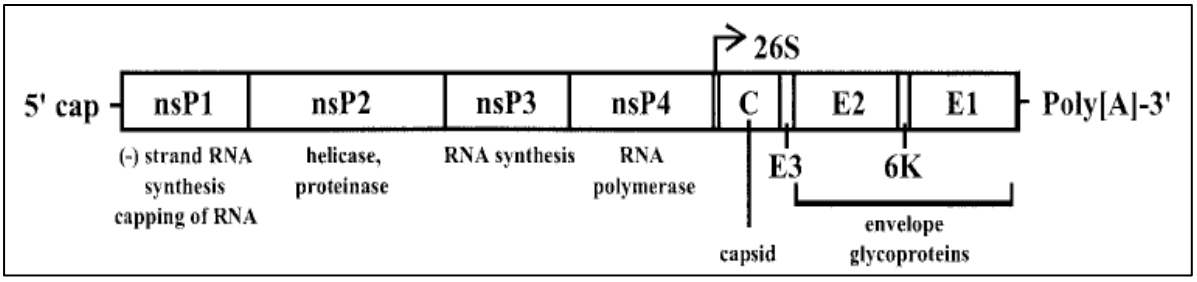

Figure 1.4 Schematic representation of the *Alphavirus* genome organization. Non-structural (5' end) and structural (3' end) proteins as well as their functions are indicated. Unlike the flaviviruses, alphaviruses are poly-adenylated on the 3' end. Figure adapter from Powers and others, 2001 (Powers *et al.*, 2001)**.**

1.6.2.3 *Entry, replication, packaging and budding*

Following clathrin-mediated endocytosis (DeTulleo & Kirchhausen, 1998), acidification induces membrane insertion (Marquardt *et al.*, 1993) and release of viral RNA into the host cytoplasm. Replication occurs in association with endosome and lysosome surfaces and is asymmetric. A "packaging sequence" has been identified in SINV RNA that promotes encapsidation of the viral RNA. Virions are transported via the golgi network to the plasma membrane where they bud out of the cell (Strauss & Strauss, 1994).

1.6.3 Protein Function and Importance

Alphavirus gene order is as follows: 5'NCR-nsP1-nsP2-nsP3-nsP4-C-E3-E2-6K-E1-polyA-3' (Figure 1.4). The nsPs are multifunctional and are required for viral RNA replication (Lloyd, 2004), while structural proteins are responsible for virion formation (Boege *et al.*, 1983; Zhu *et al.*, 2010) (Table 1.4).

Table 1.4: *Alphavirus* proteins and their functions

	Protein	Function
Non-structural (ns)	**nsP1**	Methyltransferase and guanylyltransferase (needed for capping mRNA and viral genome), involved in negative strand synthesis, interacts with nsP4 during replication
	nsP2	Nucleoside triphosphatase, helicase, RNA triphosphatase, serine protease (involved in processing the nonstructural polyprotein precursor)
	nsP3	Negative strand RNA synthesis and neurovirulence
	nsP4	RNA-dependent RNA polymerase, core of the replicase complex
Structural	**C**	Encapsidates the genomic RNA (nucleocapsid), autoprotease (processes the subgenomic 26S RNA molecule into functioning structural proteins)
	E2	Receptor mediated attachment, major neutralizing epitopes, associated with virulence
	6K	Involved in virus budding and virion assembly
	E1	Membrane fusion, conserved cross-reactive epitopes

1.7. SINDBIS VIRUS

1.7.1. SINV Phylogeny: The WEEV Sero-Complex

SINV falls within the WEE sero-complex (Luers *et al.*, 2005) along with Aura, Highlands J, Buggy creek, Fort Morgan and WEE viruses. Phylogeny based on the E1 protein has demonstrated that the WEE clade is monophyletic and that viruses most closely related to

WEEV are other New World members, Fort Morgan, Buggy Creek and Highlands J viruses (WEEV complex recombinants); while Old World viruses SINV, Babanki, Ockelbo and New World Aura virus are more distantly related. (Weaver *et al.*, 1993) Re-evaluation of neutralization classifications (Calisher *et al.*, 1988) has distinguished WEE "complex recombinants" apart from other complex members (Powers *et al.*, 2001). Phylogenetic studies carried out on the nsP4 gene confirm this (Weaver *et al.*, 1993). Old world viruses within this complex do not appear to have recombinant genomes.

1.7.1.1. *SINV genetic diversity*

Several subtypes have been included in the SINV complex: Karelian fever, Ockelbo, Babanki, Kyzylagach and Wataroah viruses. Aura virus is the only New World virus to be included in this complex, but as a distinct virus species (Rumenapf *et al.*, 1995). A Recent SINV phylogenetic study by Lundstrom and Pfeffer (2010) (Lundstrom & Pfeffer, 2010) using a limited region of the E2 protein identified 5 tentative SINV genotypes. The SIN-I genotype includes strains from Africa and Europe responsible for disease outbreaks in sub-Saharan Africa and northern Europe (Jost *et al.*, 2010; Norder *et al.*, 1996; Shirako *et al.*, 1991; Simpson *et al.*, 1996), including the SA Girdwood strain that caused disease in a human (Simpson *et al.*, 1996). Strains from Madagascar and central Europe that have not caused human disease form a sub-cluster within this genotype. This indicates a strong association between human pathogenicity and the genetic signature of SINV strains and suggests that the SIN-I genotype may be more pathogenic to humans than other genotypes (Lundstrom & Pfeffer, 2010). SIN-II included strains from Australia (Saleh *et al.*, 2003; Sammels *et al.*, 1999) and SIN-III strains from East Asia, while SIN-V contained a single isolate from New Zealand. Isolates from China and Azerbaijan make up SIN-IV. These SIN genotypes as well as the sub-clusters correlate with major bird migratory patterns (Lundstrom & Pfeffer, 2010).

1.7.2. SINV Geographic Distribution

SINV, originally isolated from a pool of *C. univitattus and C. pipiens* mosquitoes in the Egyptian village of Sindbis near Cairo in 1952 (Taylor *et al.*, 1955), has now been identified worldwide (Jost *et al.*, 2010; Liang *et al.*, 2000). SINV-subtypes are found in Northern Europe; Ockelbo virus in Sweden, Pogosta virus in Finland (Kurkela *et al.*, 2004) and Karelian fever virus in the Karelian regions of Russia and Finland. Babanki and Kyzylagach subtypes are found in central and West Africa and Azerbaijan respectively. Taylor and others

(1955) (Taylor *et al.*, 1955) found evidence of SINV antibodies in a number of domestic animals in Egypt including sheep, goats, cows, donkeys and horses and in humans.

1.7.3. SINV Disease in Humans

SINV is among the least virulent of the alphaviruses and infections are typically mild, ranging from a mild flu-like illness to one characterized by rash and arthralgia or polyarthritis in more serious cases (Laine *et al.*, 2004). One SINV case has been associated with haemorrhagic disease in Australia (Guard *et al.*, 1982) the first isolation from humans being in 1961 in Uganda, and again in 1962 from 5 people displaying generalized SINV symptoms as reported by staff members of the East Africa Virus Research Institute (Malherbe *et al.*, 1963). The SA Girdwood strain was isolated from vesicle fluid of skin lesions taken from a patient with an acute rash and arthritis (Malherbe *et al.*, 1963) and strain YN87448 isolated from a serum sample of a febrile patient in China (Zhou *et al.*, 1999). SINV-subtypes have been implicated in SINV-like disease in Europe; Ockelbo disease in Sweden (Horling *et al.*, 1993) and Karelian fever in the Karelian regions of Russia and Finland. Strains from Finland have been isolated from whole blood and skin lesions of patients during acute Pogosta disease (Kurkela *et al.*, 2004).

1.7.4. SINV in South Africa

SINV is endemic and widespread across SA (Dickinson *et al.*, 1965; McIntosh *et al.*, 1962). Neutralizing antibodies to this virus in human sera are found extensively in the Cape Province (Dickinson *et al.*, 1965) and Highveld (McIntosh *et al.*, 1962) temperate zones. However, neutralizing antibody titers in livestock in the same regions were much lower, suggesting that these animals do not play a significant role in the maintenance of this virus. Most SINV cases are characterized by fever, joint pain and a rash and are detected annually in SA in the summer months, particularly in the moister regions of the Highveld and Orange Free State (Malherbe *et al.*, 1963). It is postulated that the absence of large-scale outbreaks is because the vector for this virus, *C. univitattus* is weakly anthropophilic (Jupp, 2001). However, extensive disease due to SINV was recorded in the Karoo and Cape Province during the summer of 1973-74, after periods of heavy rainfall when the largest epidemic of mosquito-borne virus (WNV and SINV) disease was described in SA (McIntosh *et al.*, 1964). Similarly an epizootic of SINV and WNV occurred in the summer of 1983-84 in the Witwatersrand-Pretoria region of SA. In this particular epizootic thousands of human cases were recorded, most of which were SINV (Jupp *et al.*, 1986a).

1.7.5. SINV Infection in Animals

Experimental infection in suckling mice produces a fatal encephalitic-type disease. Death occurs 2-3 days post-inoculation, preceded by a brief period of paralysis. Encephalitic disease is age dependent in these models (Malherbe *et al.*, 1963). Studies have shown that SINV induces apoptosis in infected neurons (Lewis *et al.*, 1996); however maturation of neurons increases resistance to apoptosis induction, correlating to the age-dependent mortality observed in mice. However, an amino acid substitution of histidine for glutamine at E2-55 increases neurovirulence in older animals by inducing widespread apoptosis in the brain and spinal cords of these mice (Griffin, 2005; Johnston *et al.*, 2001; Tucker *et al.*, 1993); indicating that the E2 protein is the most important virulence determinant. As it is responsible for virus fusion and is the major neutralizing epitope, mutation of this protein could reduce the ability of the virus to infect host cells (Pierro *et al.*, 2008) or induce a protective response in the host (Johnston *et al.*, 2001). Various SINV strains have differing host virulence. Strain SAAR86 is virulent in both adult and neonatal mice (Russell *et al.*, 1989), unlike other SINV strains.

1.7.5.1. *Diagnosis*

Virus has been isolated from skin biopsies/lesions (Malherbe *et al.*, 1963), whole blood (Kurkela *et al.*, 2004) and serum samples (Liang *et al.*, 2000; Lloyd, 2004). Antibodies appear ~10 days post illness onset and may be detected by HI, ELISA (Sane *et al.*, 2011), serum neutralization (McIntosh *et al.*, 1962) and immunofluorescence. SINV IgM can be detected for prolonged periods following acute infection, suggesting a continual expression of viral antigen (Laine *et al.*, 2004). This may aggravate and enhance the development of chronic musculoskeletal symptoms. Positive IgM results therefore must be taken into account along with clinical symptoms and travel history. Molecular methods including genus-specific RT-PCR can detect all alphaviruses using degenerate primers (Sanchez-Seco *et al.*, 2001). Alphaviruses can be isolated or propagates in suckling or older mice, monkey kidney (VERO LLC-MK2), BHK-21 cells) and mosquito (C6/36; *Aedes albopictus*) cell lines (Calisher *et al.*, 1999; Malherbe *et al.*, 1963)

1.8. MIDDELBURG VIRUS

The first isolation of Middelburg virus (MIDV) from *A caballus* mosquitoes occurred in 1957 in the Middelburg region of the Eastern Cape province in SA during an epizootic in sheep where many animals succumbed to the disease (Kokernot *et al.*, 1957). MIDV forms its own

serocomplex and is the least divergent of all the *Alphavirus* antigenic serocomplexes (Powers *et al.*, 2001).

1.8.1. Geographic Distribution

Serological data suggest that MIDV is extensively endemic to SA (McIntosh *et al.*, 1962). MIDV has been isolated from mosquitoes in Senegal (Robin *et al.*, 1969) and haemagglutinating antibodies have been identified in migratory birds in Romania (Antipa *et al.*, 1984).

1.8.2. Human Infection

Human infection has not been recorded (Lloyd, 2004), however, neutralizing antibodies have been found in laboratory field workers (Kokernot *et al.*, 1957).

1.8.3. Experimental Infections in Animals

Suckling mice are susceptible to the virus by intracerebral or intraperitoneal inoculation and show signs of illness 40 hours after incubation. Prior to death, generalized peripheral vasoconstriction and extensor rigidity is observed. Infections in one day old chicks produce viraemia but no clinical disease. Lambs are susceptible, maintaining a “hunched” stance, with their heads held low and may exhibit mild rigor. Viraemia coincided with fever, lasting 3 days in some cases (Kokernot *et al.*, 1957). Virus isolation from mammalian or avian hosts has been unsuccessful.

1.8.4. Natural Infection in Animals

In 1993, MIDV was isolated from the spleen of a horse in Zimbabwe displaying symptoms clinically similar to severe AHSV infection. Symptoms included increased body temperature, tachycardia, pulmonary infection and generalized oedema of the head and neck (Attoui *et al.*, 2007). This finding is important as it indicates that MIDV can cause severe disease in equids. Little clinical information was available in this study.

1.9. STUDY OBJECTIVES

The long term objectives of this project were to determine whether WNV and/or other flavi- and alphaviruses contribute to neurological disease or fever in horses; and if horses can be used as sentinels for virus activity in South Africa, as alternatives to birds.

To address these questions, short term objectives were:

- To establish a network between the Zoonosis Unit at the Medical Virology laboratory and veterinarians in South Africa in order to receive samples from horses displaying unexplained neurological disease and fever from all regions of the country, and attempt identification of acute cases that may be linked to clinical disease;
- To screen serum/plasma and central nervous system tissue specimens from horses that present with unexplained febrile and/or neurological symptoms for flavi- and alphaviruses using family-specific RT-PCR, and specifically for WNV using WNV-specific RT-PCR, WNV IgM ELISA and WNV serum neutralization;
- To carry out molecular epidemiological analysis on *Flavi*- and *Alphavirus* positive cases;
- To describe the disease characteristics of *Flavi*- and *Alphavirus* infections in horses in South Africa;
- To conduct molecular epidemiology and phylogenetic characterization of WSLV by sequencing the E and NS5 genes of a selection of pathogenic WSLV isolates and compare them to other flaviviruses;
- To sequence the complete genome of an encephalitic strain of WLSV (AV259) and compare it to a strain that caused mild febrile disease in a human (H177) to identify pathogenicity markers.

CHAPTER 2

WEST NILE VIRUS IN HORSES IN SOUTH AFRICA

2.1. INTRODUCTION

West Nile virus (WNV) strains cluster into at least 5 genetic lineages (Bondre *et al.*, 2007); the major two being lineage 1 (L1) and lineage 2 (L2) (Berthet *et al.*, 1997). Historically, L2 strains were restricted to sub-Saharan Africa and Madagascar; however outbreaks among wild birds in Hungary in 2004 marked the first detection of L2 WNV outside of Africa. Recently outbreaks of L2 WNV in humans (Papa *et al.*, 2011; Papa *et al.*, 2010) and neuroinvasive disease in horses have been reported in Greece (ECDC, 2010). Additionally, L2 WNV strains have been isolated in pools of mosquitoes and birds (Valiakos *et al.*, 2011) in the same geographic locations as human and horse disease, suggesting that L2 WNV may have established in the region. Humans and horses are incidental hosts of this virus (Campbell *et al.*, 2002) and disease presentation ranges from sub-clinical to severely neuroinvasive disease in both of these hosts. Although only 5-15 WNV cases are reported per annum in humans in South Africa (SA), serious human disease including fatal hepatitis and non-fatal encephalitis, as well as fatal neurological disease in a foal in 1996 were all shown to be WNV L2 infections (Burt *et al.*, 2002). It is thought that only a proportion of WNV cases are submitted for laboratory investigation, suggesting that L2 WNV infections may be underestimated and under-detected in SA.

A study in horses in SA (Guthrie *et al.*, 2003), postulated that L2 WNV strains were non-pathogenic after 3 horses, experimentally inoculated with a L2 WNV strain (SA381/00), did not develop any signs of clinical disease. In the same study thoroughbred horses were surveyed for WNV antibodies revealing that 75% of the dams and 10% of their yearlings had prior exposure to WNV, which suggests that sub-clinical or mild disease may have occurred in these horses. In comparison, North America saw the largest outbreak of L1 WNV encephalitis in horses during 2002 with over 15000 cases of neurological disease being reported in 40 states (O'Leary *et al.*, 2004). This outbreak resulted in the hypothesis that L1 WNV strains are more virulent than L2 strains and that L2 strains are not pathogenic in horses. Experimental infection of 12 horses with a highly virulent L1 strain also resulted in clinical disease in 1% of the animals (Bunning *et al.*, 2002) which questions the conclusions drawn from Guthrie's (Guthrie *et al.*, 2003) study. Subsequent studies have demonstrated that only 20% of horses exposed to L1 WNV will develop symptoms with the majority of

infections being asymptomatic (Nielsen *et al.*, 2008). Of the symptomatic cases, 90% will display neurological signs of which approximately 30 - 40% will succumb to the disease (Ward *et al.*, 2006). Comparisons of the relationships between North American and SA WNV strains and their ability to induce neuroinvasiveness in mice demonstrated that virulence is genotype specific and not related to lineage, geographic location, source of isolate, passage level or year of isolation (Beasley *et al.*, 2002; Burt *et al.*, 2002; Venter *et al.*, 2005). It was shown that strain SA381/00, used to infect the thoroughbred horses in the study undertaken by Guthrie and others (Guthrie *et al.*, 2003), was of low neuroinvasiveness (Beasley *et al.*, 2002; Venter *et al.*, 2005) and could therefore explain why these horses did not develop disease.

In SA, African horse sickness (AHS) is the major cause of morbidity and mortality in horses (Bremer & Viljoen, 1998) and fatal cases that cannot be diagnosed are often assumed to be AHS. As WNV was not considered to cause severe disease in SA, this virus was not routinely included in the differential diagnosis of horses displaying unexplained neurological disease or fever. Additionally, no surveillance program existed for WNV in SA, therefore limiting the knowledge of how the disease manifests in horses.

The aim of this study was therefore to test the hypothesis that L2 WNV may produce a similar disease presentation in horses in SA as observed for L1 WNV in North America and to determine whether WNV cases are being missed. To address this, horses displaying unexplained fever and neurological disease were tested for the presence of WNV by real-time RT-PCR and/or serology (neutralization and IgM assays). Positive cases were further characterized by epidemiological and phylogenetic analysis to elucidate the genotypes and strains involved as well as disease seasonality and horse breeds affected.

2.2. MATERIALS AND METHODS

2.2.1. Clinical Specimen Collection

Serum, plasma, cerebrospinal fluid (CSF) and/or post mortem central nervous system (CNS) tissue (fresh and fixed) was collected from horses displaying unexplained neurological symptoms and fever (January 2008 - December 2010). Specimens were received from the Onderstepoort Veterinary Institute (OVI), the University of Pretoria's Department of Paraclinical Sciences (Faculty of Veterinary Sciences) and private equine veterinarians

throughout SA. Horses displaying symptoms typical of an AHSV infection were excluded from the study. Specimen collection was ethically approved by the University of Pretoria's Animal Use and Care Committee (protocol number H017-09).

2.2.2. Sample Preparation and Viral RNA Extraction

2.2.2.1. *Serum, plasma, CSF and cell culture supernatant*

EDTA (ethylene diamine-tetra acetic acid) and clotted blood was spun for 10 min at 640g and 1440g respectively, for efficient separation of plasma and serum from whole blood. The buffy coat from plasma specimens was used for extraction. Cell culture supernatant was centrifuged at 640g for 10 min to remove cellular debris and then passed through a 0.2µm filter (Corning, Lowell, MA) to remove bacterial contaminants. Viral RNA was extracted using the QIAmp viral RNA mini kit (Qiagen, Valencia, CA) according to the manufacturers' recommendations.

2.2.2.2. *Tissue*

Frozen and fresh tissue samples were processed and extracted using the RNeasy Plus mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Fixed tissue samples were processed and viral RNA extracted as described in 3.2.1.

2.2.3. Primer Design

Sequences for nested equine encephalosis virus (EEV) primers were based on alignments of reference strains described in (van Niekerk *et al.*, 2003) and designed with Primer 3 (v0.4.0).

2.2.4. *Flavivirus* and WNV Detection

2.2.4.1. *Conventional Flavivirus-family RT-PCR*

A first round diagnostic *Flavivirus* family-specific reverse transcription (RT) PCR directed at the NS5 gene, using primers FU1 and CFD2 (Scaramozzino *et al.*, 2001), was conducted using the Titan One Tube RT-PCR kit (Roche, Mannheim, Germany) according to manufacturers' instructions. Each PCR reaction contained 10 µl template RNA and 0.8µM of each primer in a final volume of 50µl. Reverse transcription commenced at 50°C for 30 min followed by a single step of 94°C for 2 min; and 35 cycles of 94°C for 10s, 50°C for 30s and 68°C for 1 min with a final extension of 68°C for 7 min, yielding a 265 base pair (bp) product

(genome position 9043–9308 relative to strain SA93/01, accession number EF429198). (Table 2.1 - *Flavivirus* primers).

2.2.4.2. *WNV-specific real-time RT-PCR*

A semi-nested real-time RT-PCR using fluorescence resonance energy transfer (FRET) probe analysis that distinguishes between L1 and L2 WNV was carried out using primers FS778 and CFD2 and probe set WN9177S and WN9201A as described in (Zaayman *et al.*, 2009) on *Flavivirus* first round products generated in 2.2.4.1. The LightCycler DNA FastStart Master^{PLUS} HybProbe kit (Roche, Mannheim Germany) was used to detect WNV positive specimens. Each PCR reaction contained 2µl first round product, 0.2µM of each probe, 0.5µM of each primer and 4µl probe master mix in a final reaction volume of 20µl. Reactions were performed on the Lightcycler 2 instrument (Roche, Mannheim, Germany). Cycling commenced at 95°C for 10 min followed by 45 cycles of 92°C for 10s, 53°C for 8s and 72°C for 8s, in single acquisition mode yielding a product of approximately 214 bp (genome position 9094 – 9308 relative to SA93/01) and ended off with melting curve analysis (30°C - 80°C) at a temperature ramp rate of 0.1°C/s. (Table 2.1 - WNV primer and probe details).

2.2.5. Additional WNV Amplification for Phylogenetic Analysis

2.2.5.1. *WNV E-protein amplification*

For confirmation and phylogenetic analysis, an E-protein fragment of 1105 bp was amplified in a first round reaction using the Titan One Tube RT-PCR kit (Roche, Mannheim, Germany) according to manufacturers' instructions. Each reaction contained 10µl template RNA and 0.8µM of each primer (WNV132F and WNV2468R) (Table 2.1) in a final volume of 50µl. Cycling commenced at 50°C for 30 min and 94°C for 2min; and 35 cycles of 94°C for 10s, 52°C for 30 s and 68°C for 1 min with a final extension of 68°C for 7 min. The Expand High Fidelity^{PLUS} PCR system was used in a semi-nested PCR to amplify a 339 bp fragment (genome positions 1363 – 1702 relative to SA93/01), according to manufacturers' instructions (Roche, Mannheim, Germany). Each PCR reaction contained 2µl of first round template and 0.8µM of each primer (WNV132F and WNV240R) (Berthet *et al.*, 1997; Burt *et al.*, 2002) in a final reaction volume of 50µl. Cycling commenced at 94°C for 2 min and 35 cycles of 94°C for 10s, 52°C for 30s and 72°C for 1 min with a final elongation of 72°C for 7 min.

2.2.5.2. *WNV NS5/3' UTR amplification*

For phylogenetic investigation, the Titan One-Tube RT-PCR system (Roche, Mannheim, Germany) was used to amplify a 1250 bp fragment of the NS5/3'UTR region according to manufacturers' instructions with the following PCR conditions: 10µl of RNA template and 0.8µM of each primer FU1 and WNV10962R (Botha, 2008) in a total volume of 50µl. Reactions were incubated at 50°C for 30 min, followed by 94°C for 2 min and 35 cycles of 94°C for 10s, 51°C for 30s and 68°C for 1 min, with a final extension of 68°C for 7 min. A nested PCR amplified a 740 bp region (genome position 10105 – 10845 relative to SA93/01) using the Expand High Fidelity^{PLUS} PCR system according to manufacturers' instructions (Roche, Mannheim, Germany). Each PCR reaction contained 2µl of first round PCR product and 0.8µM of each primer EMF1 and VD8 (Pierre *et al.*, 1994) (Table 2.1) in a final volume of 50µl. PCR commenced at 94°C for 2 min and 35 cycles of 94°C for 10s, 55°C for 30s and 72°C for 1 min with a final extension of 72°C for 7 min.

2.2.6. Differential Diagnosis

Alphaviruses (Chapter 4), AHS, EEV, equine herpes virus (EHV) and Shuni virus (SHUV) screening was carried out as differential diagnoses on clinical specimens:

2.2.6.1. *Control viruses used for differential diagnosis*

Sindbis virus (SINV) strain SA-AR86 RNA was obtained from the Special Pathogens Unit (SPU), National Institute for Communicable Diseases (NICD), Sandringham, SA. Kunjin virus MRM16, WNV L1b, was provided by Dr D Beasley and Dr R Shope of the University of Texas Medical Branch, Galveston, Texas. AHS and EHV were donated by Dr T Gerdes of the Virology Department at the OVI, Pretoria, SA. EEV and SHUV were isolated from clinical specimens in our laboratory and the OVI, respectively.

2.2.6.2. *AHS and EEV RNA preparation*

For AHS and EEV screening, samples were extracted using either the QIAgen viral RNA mini kit or QIAgen RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the supplier's recommendations'. Following this, double stranded viral RNA was denatured at 95°C for 5 min and snap frozen on ice prior to being added to the respective reaction tube. Table 2.2 summarizes primer details (AHS, EEV and EHV and SHUV).

2.2.6.3 *AHS NS-1 PCR*

The Titan One Tube PCR system kit (Roche, Mannheim, Germany) was used according to manufacturers' recommendations. In a total volume of 50µl, 0.8µM of each primer AHS-F and AHS-R (Rodriguez-Sanchez *et al.*, 2008) and 10µl of template RNA was added. RT-PCR was initiated at 94°C for 2 min followed by 50°C for 30 min; and 40 cycles of 94°C for 10s, 51°C for 30s and 68°C for 45s, with a final extension of 68°C for 7 min.

2.2.6.4. *EEV NS-3 PCR*

PCR reactions contained 0.8µM of each primer EEV-F and EEV-R (van Niekerk *et al.*, 2003) and 10µl of template RNA in a total volume of 50µl using the Titan One Tube PCR system (Roche, Mannheim, Germany) according to suppliers recommendations'. PCR cycling conditions were similar to 2.2.6.3 with the exception that the RT step began with 50°C for 30 min followed by 94°C tor 2 min, with an annealing temperature of 53°C. A semi-nested PCR was carried out using primers EEV-Nf and EEV-Nr, in an Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) reaction following manufacturer's instructions. Each PCR reaction contained 2µl of first round template, 2µl of each primer in a final reaction volume of 50µl. Reactions cycled at 94°C for 2 min and 35 cycles of 94°C for 10s, 54°C for 30s and 72°C for 1 min with a final elongation step of 72°C for 7 min.

2.2.6.5. *EHV glycoprotein-B PCR*

The Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) was used according to manufacturers' recommendations. Each reaction contained 10µl of RNA template and 0.8µM of each primer EHV-F and EHV-R (Kirisawa *et al.*, 1993) in a final volume of 50µl. Cycling commenced at 94°C for 2 min and 35 sets of 94°C for 10, 55°C for 30s and 72°C for 1 min with a final extension of 72°C for 7 min. This PCR was designed specific to EHV-1 and EHV-4.

2.2.6.6. *SHUV S-segment PCR*

The Titan One Tube PCR system (Roche, Mannheim, Germany) was used in a first round PCR using primers Shu111+ and Shu668- (Van Eeden C. *et al.*, In press) according to the supplier's instructions. Cycling began at 50°C for 30 min, 94°C for 2 min; and 35 cycles of 94°C for 30s, 55°C for 1 min and 68°C for 1 min with a final extension of 68°C for 7 min.

The Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) was used in a nested PCR using primers Shu178+ and Shu611- (Van Eeden C. *et al.*, In press); cycling beginning at 95°C for 2 min; and 35 cycles of 95°C for 30min, 50°C for 1 and 72°C for 1 with a final extension of 72°C for 7 min.

2.2.7. PCR Product Analysis

PCR products were resolved on a 1.5% SeaKem®LE agarose gel (Lonza, Basal, Switzerland) and visualized on a UV transilluminator. DNA molecular weight markers XIV (100 bp) and XVII (500 bp) were used to determine amplicon size (Roche, Mannheim, Germany).

2.2.8. PCR Product Purification

PCR products were excised from a 1% agarose gel (Lonza, Basal, Switzerland) and purified using the Wizard SV and PCR cleanup kit (Promega, Madison, WI) according to manufacturers' recommendations. The concentration and quality of the recovered product was estimated by comparison of 1µg of a 100 bp molecular weight marker (DNA molecular marker XIV (Roche, Mannheim, Germany)) on a 2% agarose gel (Lonza, Basal, Switzerland).

2.2.9. Sequencing and Phylogenetic Analysis

Positive amplicons were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Carlsbad, CA) according to manufacturers' instructions and the following cycling conditions: 94°C for 3 min and 25 cycles of 94°C for 30s, 50°C for 5s and 60°C for 4 min. Fragments were purified with Centri-Sep^{TM} spin columns (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions prior to being resolved on an ABI3130 sequencer (Applied Biosystems, Carlsbad, CA). Sequences were compiled using Sequencher (v4.6) (Gene Codes Corporation, Ann Arbor, MI), aligned with MAFFT (V6) (Katoh *et al.*, 2009) and edited using BioEdit (v7.0.9.0) (Hall, 1999). Maximum-likelihood (ML) trees were drawn using PhyML (Guindon *et al.*, 2009) with a bootstrap replicate value of 100. Nucleotide and amino acid divergence was calculated using MEGA (v5) (Tamura *et al.*, 2007) with the P-distance analysis option.

2.2.10. WNV IgM-competitive ELISA

A commercial WNV IgM-competitive ELISA kit (ID Vet, Montpellier, France) was used to screen horse serum samples for anti-prM-E antibodies according to manufacturer's instructions.

2.2.11. Virus Titration

2.2.11.1. *Preparation of virus and determining VERO cell concentration*

Kunjin MR161C, WNV L1b, was serially diluted by the addition of 900µl Eagle Minimum Essential Medium (EMEM) (Lonza, Basal, Switzerland) supplemented with 2% foetal calf serum (FCS) (Invitrogen, Carlsbad, CA) 100IU/ml penicillin, 100µg/ml streptomycin (Lonza, Basal, Switzerland) and 1mg/ml glutamine (Lonza, Basal, Switzerland) and 100µL virus (10^0 – 10^{-8}), homogenizing by vortex. The Sigma-Aldrich (St. Louis, MO, USA) trypan blue method was followed to count VERO cells and the total number of cells per milliliter of supernatant was calculated using the formula: **no. cells X 10 000 (standard) X dilution factor (2) = cells/ml**. This total cell concentration was then used in the equation $\mathbf{C_1V_1 = C_2V_2}$ to determine the volume of cells required for a final concentration of 1 x 10^5 cells/ml.

2.2.11.2. *Virus titration*

In a flat-bottomed 96 well microtiter plate (Corning, Lowell, MA) row A was used as the cell control for each dilution. Rows B – J were used for dilutions 10^0 – 10^{-8}, 4 replicates of each. First, serum-free EMEM (50µl/well) was added to each well, followed by 50µl/well of the appropriate dilution. Finally, 100µl/well of [1x10^5 cells/ml] VERO cells (prepared in 2% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin and 100µg/ml Streptomycin (Lonza, Basal, Switzerland)) was added to each well and incubated at 37°C, 5% CO_2 and 80% humidity for 72 hours. The cell control row therefore contained 50µl 2% EMEM (in place of virus), 50µl serum-free EMEM and 100µl VERO cells. Cells were inspected daily for the presence of cytopathic effect (CPE). The tissue culture infectious dose where 50% of the cells had died ($TCID_{50}$) was calculated using the formula: $\mathbf{TCID_{50}/vol = X_0 - d/2 + \Sigma xi/n}$, where: **$X_0$ is the highest dilution at which all replicates react, d/2 is the difference between dilutions, i.e. 0.5** and **$\Sigma xi/n$ is the number of wells at X_0 and above.** The $TCID_{50}$ for Kunjin was calculated as an antilog value of 3162 which was used to determine the volume of Kunjin virus stock required for neutralization assays.

2.2.12. Serum Neutralization

To detect the presence of WNV specific neutralizing antibodies in serum or CSF of WNV IgM positive samples, a modified version of a previously published method was used (Grandien *et al.*, 1989). Before experimentation, horse serum samples were heat inactivated at 56°C for 30 min in a water bath. Decomplemented FCS (Invitrogen, Carlsbad, CA) was used as the negative control, and serum from a known-positive horse sample was used as the positive control. Sera were diluted to a 1:10 concentration in serum-free EMEM. A 96-well culture plate was labeled as follows: column A was the serum control while columns B – H were used for dilutions. Row 1 and 2 were used for the positive and negative controls. Briefly, all wells except for the second column received 50µl serum-free EMEM. Diluted sera including positive and negative controls (50µl/well) were added to wells 1, 2 and 3 of each row and titrated from column 3 to 8 by transferring 50µl each time to make 2-fold serial dilutions (1:10 - 1:640). The last 50µl, from column 8, was discarded. $100TCID_{50}$ U/ml Kunjin virus (as described in 2.2.11) prepared in 2% FCS (Invitrogen, Carlsbad, CA), was incubated together with 2-fold dilutions of horse sera (1:10-1:640).

A back titration is required as a quality control of virus strength. Briefly, a dilution series was made, using the $100TCID_{50}$ virus preparation as the starting stock, to produce three 10-fold virus dilutions ($10TCID_{50}$, $1TCID_{50}$, $0TCID_{50}$). Using the last two rows of the plate, 50µl of serum-free media and 50µl of each dilution was added to the appropriate wells.

The plate was incubated at 37°C, 5% CO_2 and 80% humidity for 1 hour. Following this, 1 volume of VERO cells (prepared in 2% FCS (Invitrogen), 100 U/ml Penicillin and 100µg/ml Streptomycin (Lonza, Basal, Switzerland)) was added to the virus/serum combination at a concentration of $1x10^5$ cells/ml and incubated at 37°C, 5% CO_2 and 80% humidity for 72 hours. Each well was then examined for the presence of CPE and deemed to be positive for neutralization if less than 25% of the cells in each well displayed CPE.

Table 2.1: *Flavivirus* and West Nile virus primers and probes used in this study

Primer/Probe* Name	Gene Targeted/PCR	Primer Orientation	Sequence (5' – 3')	Tm (°C)	Reference
FU1	NS5 Diagnostic PCR	Sense	TAC AAC ATG ATG GGA AAG AGA GAG AA	50	Scaramozzino *et al.*, 2001
CFD2		Antisense	GTG TCC CAG CCG GCG GTG TCA TCA GC	50	
FS778		Sense	AA(AG) GG(CTA) AG(CT) (CA)C(TAG) GC(CTA) AT(CTA) TGGT	53	
* WN9177S		Sense	AAG ACC ACT GGC TTG GAA GAA AG-Fluo	53	Zaayman *et al.*, 2009
* WN9201A		Antisense	LCRed-640-ACT CAG GAG GAG GAG TCG AGG GCTT-Pho	53	
Seq9103F		Sense	AGA GCC ATC TGG TTC ATG T	50	This study
WN10962R	NS5	Antisense	AGA TCC TGT GTT CTA CCA	51	Botha, 2008
EMF-1		Sense	TGG ATG AC(CG) AC(TG) GA(AG) GA(CT) ATG	55	Pierre *et al.*, 1994
VD-8		Antisense	GGG TCT CCT CTA ACC TCT AG	55	
WN132F	E	Sense	GAA AAC ATC AAG TAT GAG G	52	Berthet *et al.*, 1997
WN2468R		Antisense	ATG GCA CAC CCA GTG TCA G	52	
WN240R		Antisense	GAG GTT CTT CAA ACT CCA T	52	

Table 2.2: Primers used for differential diagnosis of equine diseases in SA

Primer name	Virus	Gene targeted	Orientation	Sequence (5' – 3')	Tm (°C)	Reference
AHS-F	African horse sickness	NS-1	Sense	GTT GAC CTC GCT CTG CTT GAC	56	Rodriguez-Sanchez *et al.*, 2008
AHS-R			Antisense	AAT CGC TTC CTT CGT TGT ATG AC	57	
EEV-F	Equine encephalosis	NS-3	Sense	GTT AAG TTT CTG CGC CAT GTA T	58	Van Niekerk *et al.*, 2003
EEV-R			Antisense	CCG AAC TGG TAC GGT A	58	
EEV-nF			Sense	GGD GCR GAT GAR TGT GAT AA	59	This study
EEV-nR			Antisense	TTK CTA ATY CTA TCC GCG TTC	59	
EHV-F	Equine herpes	Glycoprotein-B	Sense	CTT GTG AGA TCT AAC CGC ACC TA	60	Kirisawa *et al.*, 1993
EHV-R			Antisense	GTG GCA ATT CCC TGC ATA AT	60	
Shu111+	Shuni	S-segment	Sense	CGA TAC CGT TAG AGT CTT CTT CC	55	Van Eeden *et al.*, in press
Shu668-			Antisense	CGA ATT GGG CAA GGA AAG T	53	
Shu178+			Sense	CCG AGT GTT GAT CTT ACA TTT GGT	56	
Shu611-			Antisense	GCT GCA CGG ACA GCA TCT A	57	

2.2.13. Virus Culture

WNV isolations should be carried out in a BSL-3 laboratory and as such was not attempted on routine specimens. The OVI obtained an isolate from horse brain tissue (HS101/08) submitted to their Virology laboratory, which was identified by RT-PCR as WNV in this study. WNV isolate HS101/08 was cultured at the OVI using the following protocol: filtered supernatant from a 10% brain tissue suspension was used to infect VERO cell monolayers supplemented with EMEM (Lonza, Basal, Switzerland) containing 2% FCS (Invitrogen, Carlsbad, CA), 100IU/ml penicillin, 100µg/ml streptomycin (Lonza, Basal, Switzerland) and 1mg/ml glutamine (Invitrogen, Carlsbad, CA). Infection was allowed to proceed until a CPE of 80% became apparent. Virus was harvested using cell scrapers (Greiner Bio-One, Frickenhausen, Germany) and supernatant centrifuged at 640g for 10 min, passed through a 0.2µm filter (Corning, Lowell, MA) and stored at -70°C until further use. Additional PCR positive specimens were sent to DeltaMune and the SPU at the NICD; however no isolates were returned.

2.3. RESULTS

2.3.1. Specimen Collection

Over three consecutive seasons (January 2008 - December 2010), 261 horse samples were collected (Table 2.3). Blood samples made up the bulk of the submissions (68%), while tissue samples (CNS, spleen and liver) contributed 32%.

2.3.2. WNV Positive Clinical Specimens

WNV cases were defined as isolates, a positive WNV RT-PCR result or a positive WNV IgM confirmed by WNV neutralization. Thirty-four WNV positive samples were identified, 32 of which displayed neurological disease (94%). Neurological disease percentage recorded per year is indicated in Table 2.3. The overall mortality rate for WNV infected horses was 44% (15/34).

Table 2.3: Summary of clinical samples received and total number of WNV positive cases identified in horses in South Africa (2008 – 2010)

	2008		2009		2010		Total
Total # specimens received	79		41		141		**261**
Neurological (%)	50	63%	35	74%	110	78%	**195**
Fever (%)	29	37%	6	13%	26	18%	**61**
Hepatic (%)	-	-	-	-	4	3%	**4**
Other (%)	-	-	6	13%	1	0.7%	**7**
WNV positives/year	13		6		15		**34**
Neurological disease (WNV cases)	13	100%	6	100%	13	87%	**32**
WNV real-time RT-PCR	6		3		7		**16**
WNV IgM & Neutralization	7		3		8		**18**
Total deaths/year	6		3		6		**15**

2.3.2.1. *WNV positive cases*

Sixteen WNV RT-PCR cases were identified over three consecutive seasons (Table 2.3). L2 WNV was amplified by real-time RT-PCR from the brain tissue of 11 horses displaying severe neurological disease, all of which were either euthanized or died (Table 2.4, Table 2.5, Table 2.6A, Table 2.6B; Appendix 1). Of the 3 strains amplified from plasma, SAE134/08 and SAE28/10 displayed neurological disease, while SAE81/10 displayed only fever. In 2010, two L1 strains were amplified from the brain tissue of a mare (and her 7.5 month gestational foetus) displaying severe neurological disease. All 18 WNV IgM cases were confirmed by WNV neutralization; 17/18 displayed neurological disease and death was recorded in 2/18 (Table 2.4, Table 2.5, Table 2.6B; Appendix 1). Additionally, 3 IgM cases could not be confirmed by WNV serum neutralization and were therefore regarded as suspected cases.

2.3.2.2. *Demographics*

Geographically, WNV cases were distributed throughout SA (Figure 2.1, Table 2.7). WNV cases predominated in Gauteng during 2008 and 2009. In 2010, although more evenly distributed between the provinces, WNV cases remained highest in Gauteng (Table 2.7). The percentage of WNV cases per province is indicated.

Table 2.7 Distribution of WNV cases in South African Provinces (2008-2010)

Province	2008	2009	2010
Free State	-	-	20%
Gauteng	71.4%	67%	27%
Kwa-Zulu Natal	-	33%	20%
Northern Cape	14.6%	-	20%
North West	7%	-	-
Western Cape	-	-	13%
***Tanzania**	7%	-	-

* Tanzania is a country on the East coast of Africa; it does not fall under South Africa.

Horse ages ranged from 4 months to 24 years and breeds included thoroughbreds, warmbloods, Lippizaners, Welsh ponies, Arabians, Friesians, American saddlers and cross breeds.

Figure 2.1 Geographic distribution of WNV positive cases detected over 3 consecutive seasons in South Africa. Black dots indicate WNV lineage 2 cases and white dots indicate WNV lineage 1 cases. The number of cases detected in each area is indicated.

2.3.2.3. *Co-infections*

Table 2.8 Co-infections detected in WNV cases

Infectious agent	2008	2009	2010
ASHV	2	-	-
EHV	-	-	1
T. brucei	1	-	-
SINV	-	2	-
SHUV	-	-	1

Co-infections were identified in 7 WNV-confirmed cases during differential diagnosis screening, including, AHS in spleen tissue, *Trypanosoma brucei* in CSF, SINV in brain tissue, EHV and SHUV in plasma (Table 2.8).

2.3.3. WNV Clinical Disease Description

Confirmed WNV infections were identified in horses displaying both fever and severe neurological disease (n=32). Horses displaying only fever survived the illness (n=2), while fifteen (44%) horses displaying neurological disease either died or were euthanized. The majority of ill horses were reportedly ataxic (25/34), with other prevalent signs including recumbency and muscle weakness, front and hind limb paralysis and muscle fasciculation. Additional symptoms are summarized in Table 2.9.

A WNV isolate (HS101/08) was obtained from a frozen tissue sample by the OVI Virology department. This horse became recumbent due to quadriplegia and displayed teeth grinding, muscle twitching and limb paddling which progressed for 3 days into a severe form, mimicking rabies, i.e. seizures, chewing, coma and death. The rabies fluorescent antigen test (FAT) on brain tissue was negative. *Flavivirus* immunohistochemistry (IHC) staining detected antigen in the lumbar spinal cord and in several grey matter axons. All PCR positive specimens identified in 2008 and 2009 were shown to be L2.

In 2010, L1 WNV was identified in an 8-year-old thoroughbred mare and her 7.5 month gestational foetus stabled on a farm in Ceres in the Western Cape. The mare was recumbent, showed severe hindquarter in-coordination, mild tongue fasciculation and had slightly toxic mucous membranes. She was unable to rise on day 4 and 5, aborted her foetus on day 6 and died on day 7. This was the first and only L1 WNV case detected in SA.

Table 2.9: Summary of the symptoms most commonly reported for WNV positive horses

Clinical Sign	2008	2009	2010	TOTAL	Percentage (n/34) %
Ataxia/stumbling	8	6	11	**25**	**73**
Fever	6	6	4	**16**	**47**
Recumbent	1	4	3	**8**	**23**
Muscle weakness	1	3	3	**7**	**21**
Hepatitis/icterus	2	2	1	**5**	**15**
Paralysis	3	-	1	**4**	**12**
Muscle fasciculation's	2	2	-	**4**	**12**
Fever only	-	-	3	**3**	**9**
Weak tail tone/tail pull		2	1	**3**	**9**
Partial blindness	2	1	-	**3**	**9**
Seizures	2	-	-	**2**	**6**
Head hanging/pulling left	2	-	-	**2**	**6**
Laminitis stance	-	1	1	**2**	**6**
Depressed	-		2	**2**	**6**
No tongue tone		-	1	**1**	**3**

2.3.4. Suspected WNV Cases

Suspected WNV cases were defined as samples positive by WNV neutralization, WNV HI and/or WNV IgG assays that fit the clinical description identified for WNV cases. WNV IgM positives that could not be confirmed by serum neutralization were also regarded as suspected cases.

Table 2.10 Suspected WNV cases identified in horses (2008-2010)

	2008	2009	2010	Total positive
WNV neutralization only	7	8	28	43
WNV HI only	3	4	-	7
WNV IgM +ve, neutralization -ve	1	-	2	3
WNV neutralization & WNV HI +ve	3	1	-	4
WNV IgG & WNV neutralization +ve	-	-	4	4
WNV IgG, WNV neutralization & WNV HI +ve	-	1		1
TOTAL # specimens	**14**	**14**	**34**	**62**

Sixty-two suspected WNV cases were identified (Table 2.10). Most cases were based on WNV neutralizing results; however these cases were negative by IgM ELISA resulting in an unconfirmed infection. Although WNV disease could not be confirmed, recent reports suggest that WNV neutralizing antibodies may persist for longer than IgM responses in serum following WNV infection (Castillo-Olivares *et al.*, 2011); therefore defining these cases as suspicious WNV infections. Three IgM positive cases could not be confirmed by serum neutralization and were therefore also regarded as suspected WNV cases.

Fever and neurological disease (predominantly ataxia) were recorded for these horses. Other symptoms included stiff/swaying gait, hind limb and forequarter paresis, recumbency, falling and feet dragging. In severe cases, collapse, horizontal nystagmus, non-responsive pupils, lateral recumbency, violent spasms in the hind limbs and in-coordinated forelimbs were reported. Due to the high seroprevalence of WNV in SA,

these cases will require further investigation to determine the aetiological agents involved.

2.3.5. WNV Seasonality

Most WNV cases were detected from late summer to autumn (March – May) during the rainfall months; which is considered the arbovirus season in SA (Figure 2.2).

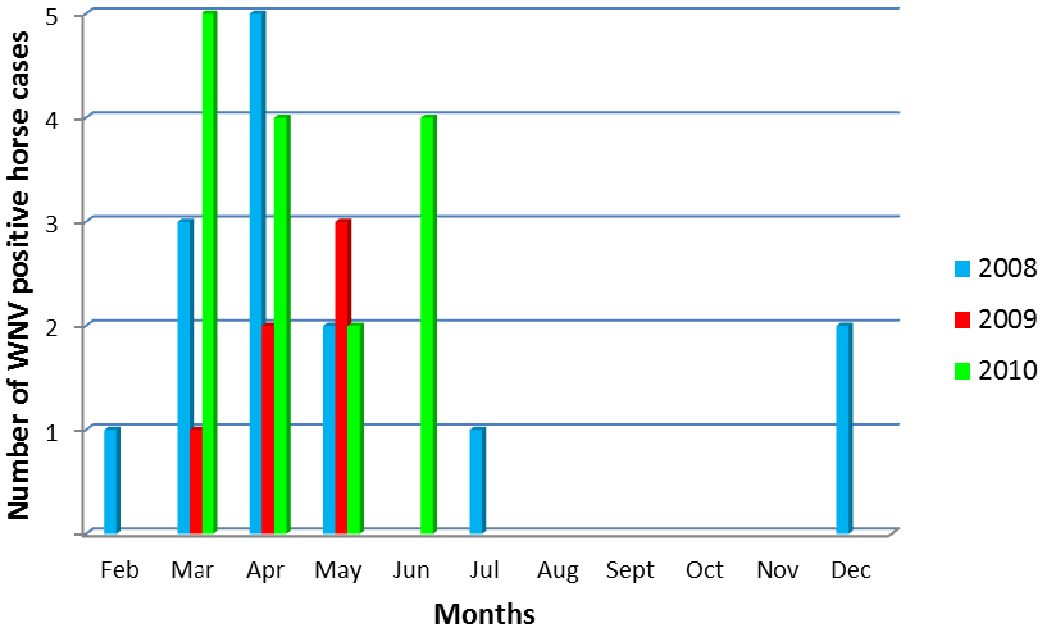

Figure 2.2: **WNV cases identified over three consecutive seasons in horses from South Africa.** The year is indicated in the legend on the right hand side of the graph.

2.3.6. Sequence Confirmation and Phylogenetic Analysis

2.3.6.1. *Phylogenetic analysis of the diagnostic NS5 region*

Phylogenetic comparison of a 189 bp fragment of the NS5 gene (diagnostic PCR) with representative sequences of currently recognized WNV lineages confirmed that 12 horse strains clustered with L2 strains previously isolated from humans in SA, while SAE75/10 clustered with L1 strains (Figure 2.3). The remaining L2 (SAE22/10 and SAE28/10) and L1 (SAE76/10) horse strains which were positive by WNV real-time RT-PCR could not be sequenced due to low viral RNA concentration. L2 horse strains SAE126/08 and SAE139/08 were the most divergent; SAE126/08 grouping with the previously isolated SA93/01 strain (Figure 2.3). Nucleotide p-distance analysis revealed a 1.8% divergence between strains SAE126/08 and SAE139/08. SAE126/08 and

SAE139/08 differed by 1.2% and 0.6% to other L2 horse strains respectively, which were otherwise highly similar. Nucleotide differences ranged between 1.2% - 3.6% when comparing L2 horse strains to previously isolated SA strains, a L2 Hungarian strain and the original Kenyan strain; while diverging18.7% - 19.3% to the Madagascar strain. The L1 horse strain, SAE75/10 grouped most closely with, but separately to, a Tunisian strain (PAH001) within the L1a clade. PAH001 was isolated in 1997 from a human that succumbed to neurological disease (Murgue *et al.*, 2001a). SAE75/10 diverged by 4.2% to strains PAH001 and NY385-99 on a nucleotide level, while differing by 18% to L2 strains.

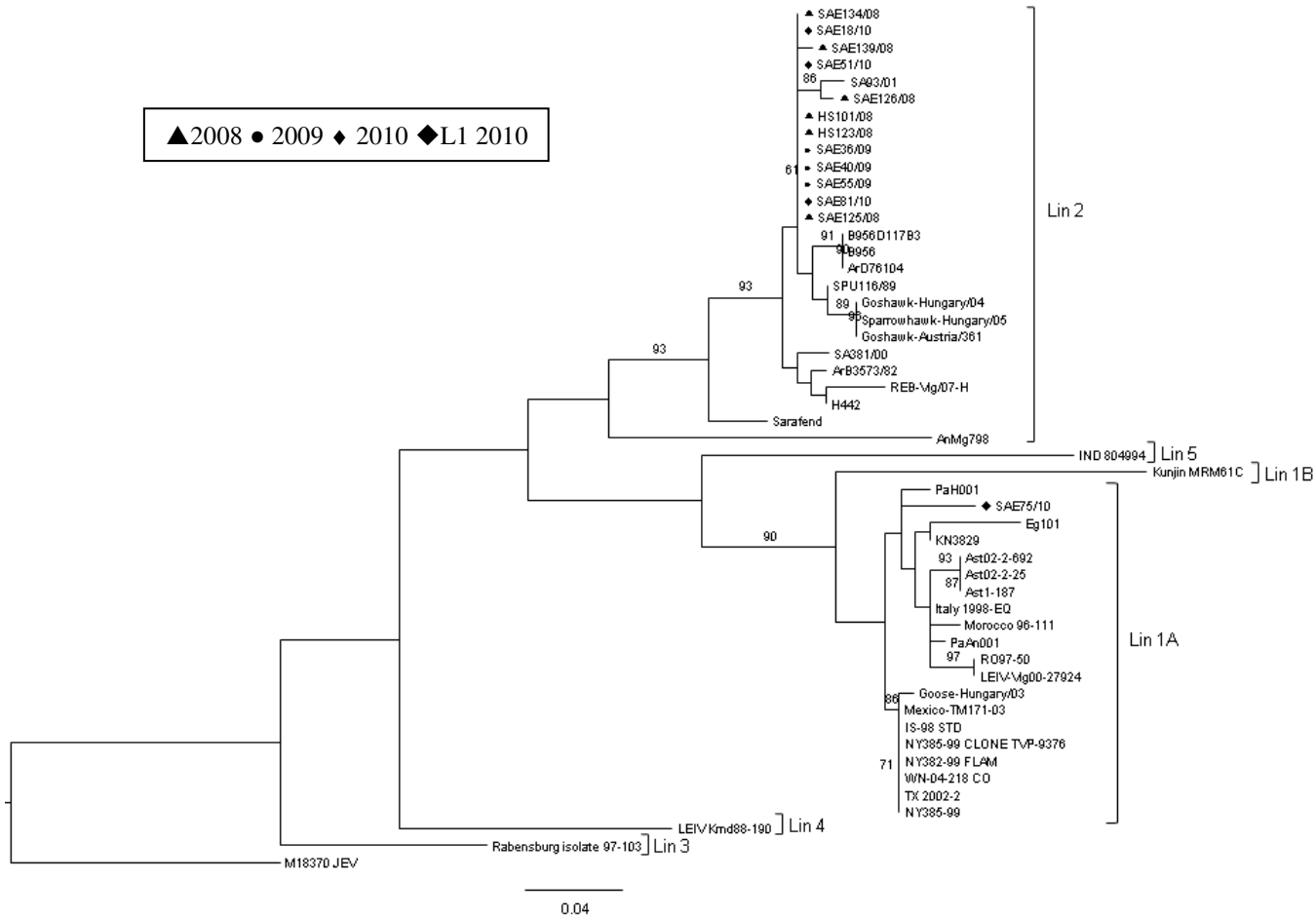

Figure 2.3: **Maximum-likelihood comparison of a partial region of the NS5 protein amplified for WNV positive horses over three consecutive seasons in South Africa (January 2008 – December 2010).** The tree was constructed using the HKY codon position substitution model using PhyML (Guindon *et al.*, 2009) and drawn to scale with the bar indicating 0.04 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. Only values of >60 are shown on the phylogram. Strains identified in this study are indicated: ▲2008, ● 2009 and ♦ 2010. The South African lineage 1 strain identified in 2010 is indicated by a large diamond◆. See Table 2.11a and Table 2.11b in Appendix 1 for GenBank accession numbers.

Amino acid divergence between L2 horse strains and previously isolated SA WNV strains ranged between 1.9% - 5.6%, while showing the least divergence from the L2 Hungarian strain (0% - 1.9%) and the most divergence from the Madagascar strain (5.6% - 7.4%). SAE75/10 differed between 3.7% - 5.6% to L2 horse strains on an amino acid level.

2.3.6.2. *E-protein phylogenetic analysis*

A more variable region within the E-protein could be amplified for strains HS101/08, SAE36/09, SAE40/09, SAE55/09, SAE18/10, SAE22/10, SAE51/10 and SAE75/10 (Figure 2.4).

Table 2.12 Nucleotide p-distance divergence between WNV positive cases and previously isolated strains from around the world

	Percentage similarity between strains (%)						
	HS101/08	SAE36/09	SAE40/09	SAE55/09	SAE18/10	SAE22/10	SAE51/10
SA93/01	1.7	1.3	1.7	1.7	2.0	1.3	1.3
H442	1.7	1.3	1.7	1.7	2.0	1.3	1.3
SPU116/89	**0.7**	**0.3**	**0.7**	**0.7**	**1.0**	**0.3**	**0.3**
SA381/00	2.0	1.7	2.0	2.0	2.3	1.7	1.7
Hungary 04	1.7	1.3	1.7	1.7	2.0	1.3	1.3
AnMg798	17.9	18.2	17.9	17.9	18.9	18.2	18.2
SAE75/10	23.8	24.0	23.8	23.8	23.8	24.2	24.2

L2 WNV strains identified in this study grouped closest to SPU116/89 (nucleotide differences of 0.3% - 1%) and SA93/01 strains isolated from humans with fatal hepatic and non-fatal encephalitic disease (Burt *et al.*, 2002). Horse strains SAE40/09 and SAE55/09 formed a separate cluster within the grouping, while strains HS101/08 and SAE18/10 were the most divergent of these strains (Figure 2.4). Nucleotide p-distance analysis revealed an average divergence of 0.4% between L2 horse strains.

Previously isolated SA strains differed to the Hungarian strain by 1.7% – 2.6%, similar to the findings in this study (Table 2.12) which suggests that the Hungarian strains may have originated from southern Africa. On the amino acid level, L2 horse strains were identical to one another and the Hungarian and Ugandan strains, except for SAE18/10 which differed to all strains by 1%.

L1 strain, SAE75/10, clustered closest to L1 strains from Tunisia (2.6% nucleotide difference, identical on amino acid level) and Russia (2.3% - 3% nucleotide differences) (Figure 2.4) Meanwhile P-distance nucleotide comparison of SAE75/10 to L2 horse strains revealed differences of 23.8% - 24.2%, while being 6% different on an amino acid level. All L2 WNV strains amplified were from horses stabled in and around the Gauteng Province, while SAE75/10, the L1 strain, was amplified from a horse stabled in Ceres in the Western Cape.

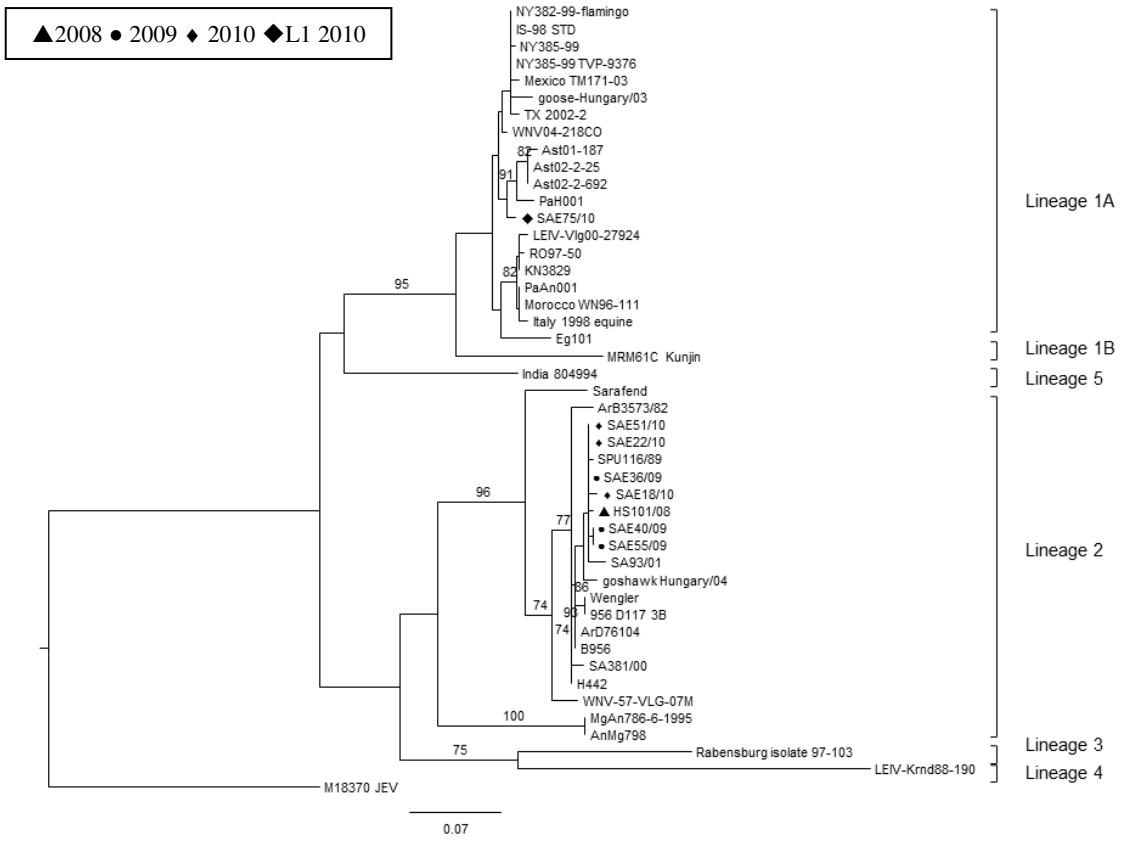

Figure 2.4: Phylogenetic analysis of a partial section of the E-gene of West Nile virus strains identified in horses in South Africa over three consecutive seasons (January 2008 to December 2010). The maximum-likelihood tree was constructed as described in Figure 2.3. The scale the bar indicates 0.07 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. Only values of >70 are shown. Japanese encephalitis virus JEV-XJ169 was used to root the tree. Strains identified in this study are indicated: ▲2008, ● 2009 and ♦2010. The lineage 1 strain identified in horses in South Africa is indicated by◆. See Table 2.11a and Table 2.11b in Appendix 1 for GenBank accession numbers.

2.3.6.3. *NS5/ 3' UTR phylogenetic analysis*

A larger area covering the junction region between the NS5 protein and the 3' UTR of the *Flavivirus* genome (740 bp) was amplified for L2 strains HS101/08, SAE36/09, SAE40/09, SAE55/09, SAE18/01, SAE51/10 and SAE81/10; and L1 strain SAE75/10,

as it is representative of the genetic diversity of the *Flavivirus* genome (Poidinger *et al.*, 1996) (Figure 2.5).

L2 horse strains formed two sub-clusters within the L2 clade and were closely related except for strain SAE55/09 which formed a completely separate branch, grouping closely with SPU116/8 (Figure 2.5). Nucleotide p-distance analysis carried out on the entire fragment (NS5 and 3' UTR) indicated differences of between 0% and 1.4% within the L2 horse strains, having an average divergence of 0.8%.

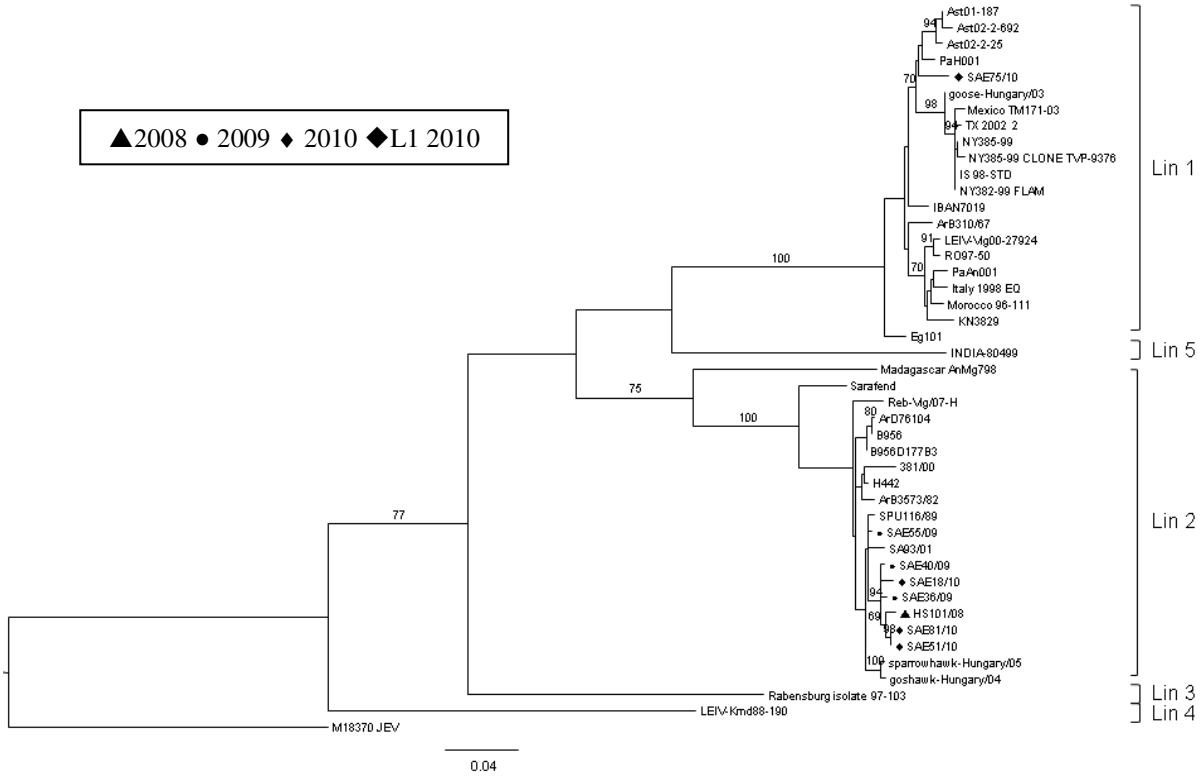

Figure 2.5: Phylogenetic analysis of the junction region between the NS5 and 3' UTR regions of the WNV genomes of strains amplified from horses in South Africa over three consecutive seasons. The tree was constructed as described in Figure 2.3. The scale bar indicates 0.04 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. Only values of >69 are shown. Japanese encephalitis virus strain M18370 was used to root the tree. Strains identified in this study are indicated: ▲ 2008, • 2009 and ♦2010. The lineage 1 strain identified in horses in South Africa is indicated by◆. See Table 2.11a in Appendix 1 for GenBank accession numbers of the *Flavivirus* strains used and Table 2.11b for accession numbers of the WNV strains identified in this study.

In comparison to L2 WNV strains previously isolated in SA, horse strains were most closely related to SPU116/89, with particular reference to strain SAE55/09 which showed only a 0.4% difference at the nucleotide level. Horse strains were most divergent from the SA strain SA381/00 with differences ranging from 2% - 3.6%. On an amino acid level all L2 horse strains were identical in the NS5 coding region.

SAE75/10, grouped closely to the Tunisian strain PaH001 in this tree, having a nucleotide difference of 1.9% and an amino acid difference of 4.4% (Figure 2.5). Differences of between 17% and 22% were seen in comparison to L2 horse strains, with an 11.6% amino acid difference.

2.4. DISCUSSION

The primary aim of our investigation was to establish whether lineage 2 (L2) WNV strains cause neuroinvasive disease in horses in South Africa (SA), and to define the molecular epidemiology and clinical presentation of these strains. Historically WNV L2 strains have been restricted to southern Africa and Madagascar while L1 strains circulate in the Americas, Europe, North Africa and Australia (Berthet *et al.*, 1997). Although endemic to SA, few cases of L2 WNV disease have been reported and therefore the role of this virus as a human and equine pathogen was undecided (Guthrie *et al.*, 2003; Lanciotti *et al.*, 1999). During the first season of this investigation (2008) a pilot study to determine the role of WNV L2 strains in equines in SA, established that this agent caused severe neurological disease in these animals (Venter *et al.*, 2009a). Case investigations were expanded over three years to determine the impact of WNV and the prevalence of neurological cases in horses in SA.

In North America, horses are extremely susceptible to WNV and develop severe neurological disease (Castillo-Olivares & Wood, 2004). A 2008 study (Ward & Scheurmann, 2008), looked at the temporal relationship between human and equine WNV cases. It was established that equine disease occurred significantly earlier than human infection in the same geographic location leading to the use of horses as sentinels for human infection. Despite a high seroprevalence of WNV in resident birds of SA, most do not develop clinical disease which suggests that genetic resistance to WNV exists (Jupp, 2001) thus reducing their usefulness as sentinels. This study demonstrates the value of using horses as sentinels for disease surveillance in South Africa.

Passive surveillance of horses displaying unexplained neurological disease and/or fever was carried out over three consecutive seasons from January 2008 to December 2010. During this time, 261 specimens from horses were submitted from private veterinarians

throughout SA as well as the Onderstepoort Veterinary Institute (OVI) and the Pathology division of the Department of Paraclinical Sciences in the Faculty of Veterinary Sciences at the University of Pretoria. A progressive increase in the number of samples received from the 2008 season (79 samples) to the 2010 season (141 samples) indicates the success and usefulness of a surveillance system such as this.

In total, 34 WNV positive cases were identified by WNV RT-PCR or IgM serology in horses across SA (Figure 2.1, Table 2.7). WNV has a defined seasonality curve beginning in February and tapering off in July with most horse cases peaking in the months of March and April (late summer/autumn) for the three consecutive years (Figure 2.2). This was similar to what was observed in the 1974 outbreak in humans in SA (Jupp *et al.*, 1986a). A serological survey done in Coahuila state in Mexico, found that temperatures ranging from 18°C – 22°C was associated with seroconversion of horses to WNV (Blitvich *et al.*, 2003). Heavy rainfall over an extended period was recorded over the three years, and this together with warmer winters, could explain why cases were seen in June and July; the winter months of the year (Figure 2.2).

The WNV seasonality (Figure 2.2) explained in this study coincides with AHSV and EEV outbreaks in SA, as heavy rainfall favours the breeding of *Culicoides* transmission vectors for these arboviruses (van Niekerk *et al.*, 2003). As both of these agents are widespread in the country (Lord *et al.*, 2002), concurrent circulation of these viruses may therefore contribute to the under-recognition of WNV cases in horses in SA. This is due to the severity of the disease caused by AHSV, and to a lesser extent EEV (Bremer & Viljoen, 1998; Howell *et al.*, 2008). AHS is a highly fatal disease (Quan *et al.*, 2008) characterized by depression, respiratory distress, coughing spasms, fever and severe sweating. Frothy discharge from the nares and periods of recumbency occur in terminal disease (Mellor & Hamblin, 2004). EEV, mostly associated with fever, can also present as neurological disease and cause abortion (MacLachlan & Guthrie, 2010; Viljoen & Huismans, 1989). Although clinical AHS is unlike WNV and is not characteristically neurological, horses that die of unexplained disease may be disregarded as AHS infections (Venter *et al.*, 2009a). Two AHS-WNV co-infections were identified in horses in 2008 (Table 2.8). In most of the WNV cases identified (27/34), no other pathogens were detected. Neurological disease, recorded in 32 of the WNV cases identified in this study (Table 2.3), can be used as the distinguishing feature

of an AHS-WNV co-infection. EEV was not detected in neurological cases; however the Kaalplaas strain was isolated from a number of horses with fevers.

WNV IgM ELISA cases were confirmed by WNV serum neutralization, suggesting a recent WNV infection. Sub-clinical WNV infection in horses have been demonstrated in America (Bunning *et al.*, 2002) where, many of these horses had strong IgM and neutralization responses to WNV. Recently, IgM in horses has been shown to have a limited circulation time (Castillo-Olivares *et al.*, 2011) and therefore serves as a good basis to differentiate between recent and previous exposure to the virus in these animals. Of the 18 IgM and neutralization positive samples, 17 displayed neurological disease. Three IgM positive cases were unable to be verified with serum neutralization (Table 2.10). Although IgM antibodies have a shorter life-span than neutralizing antibodies (Castillo-Olivares *et al.*, 2011), and therefore indicate recent infections, true cases have to be confirmed by serum neutralization, especially in an endemic country such as SA. Further investigations are required to determine the aetiological agents involved in disease progression in these horses.

The mortality rate was calculated at 44% (15/34) in this study which supports results from the recent outbreaks of WNV in Morocco and Italy (Cantile *et al.*, 2000) where fatality rates reached approximately 45% and 43% respectively. Recent outbreaks of L2 WNV in Greece (Papa *et al.*, 2010) and before that in Hungary (Bakonyi *et al.*, 2006), has confirmed that L2 WNV can cause severe neurological disease in equids (Kutasi *et al.*, 2011), supporting the findings in this study.

Twelve of the RT-PCR positive cases were confirmed by DNA sequencing as L2 WNV. These strains were closely related to one another and to recent human isolates from SA (Burt *et al.*, 2002) in this diagnostic NS5 region. The two most divergent strains were SAE126/08 which clustered more closely with SA93/01 (bootstrap confidence of 86%); and SAE139/08 (the Tanzanian strain) which formed its own branch within this L2 cluster (Figure 2.3). SA93/01 was isolated from a patient with encephalitis in 2001 (Burt *et al.*, 2002). Two L2 strains identified in 2010 were only identifiable by FRET probe analysis and could not be sequenced, due to a low virus titer in these samples.

Additional analysis of the junction region between the NS5/3'UTR (Figure 2.5) as well as a region of the E-gene (Figure 2.4, Table 2.12) for some of the L2 horse strains revealed a close relationship to strain SPU116/89 followed by SA93/01, both of which

were previously found to be highly neuroinvasive in mice (Venter *et al.*, 2005). In particular, strain SAE55/09, identified in Pretoria during 2009 is most divergent from the remaining horse strains in both the E and NS5/3'UTR region. It is most closely related to SPU116/89, which was isolated from a patient with fatal hepatitis in 1989 (Burt *et al.*, 2002). Interestingly the sequence characteristics of the NS5/3'UTR junction region of SAE55/09 resembles that of older strains isolated from Kenya (Sarafend and B956), due to a large deletion in the C-terminus of the 3'UTR just after the NS5 stop codon (Figure 2.6, Appendix 1). This is characteristic of the heterogeneity of *Flavivirus* architecture in this region as a result of deletions, insertions and sequence repeats which are mostly concentrated towards the proximal part of the 3'UTR (Proutski *et al.*, 1997). In comparison, the distal region of the 3'UTR is highly organised and forms conserved secondary structures that are essential in *Flavivirus* replication (Proutski *et al.*, 1999). Despite this deletion being observed, SAE55/09 was identified in the brain tissue of a horse displaying signs of severe neurological disease, suggesting that the replication and therefore pathogenicity of this strain was unaffected by the deletion. SINV was also amplified from the brain tissue of this horse. The NS5/3'UTR junction region was chosen as it is representative of the genetic diversity across the entire *Flavivirus* genome (Poidinger *et al.*, 1996) and most accurately describes the relationship between strains.

These additional regions could be amplified in horses displaying only fever as well as those displaying neurological disease. Approximately only 10% of horses that are infected with WNV will develop neurological disease, with the remaining horses having sub-clinical infections (Castillo-Olivares & Wood, 2004).

In 2010, a thoroughbred pregnant mare from Ceres in the Western Cape displaying neurological disease aborted her 7.5 month gestational foetus the day before she died. Both the brain of this mare (SAE75/10) and the aborted foetus (SAE76/10) tested positive for lineage 1 (L1) WNV by FRET probe analysis. DNA sequencing confirmed this. This was the first detection of L1 WNV in SA (Venter *et al.*, 2011). Abortion has not previously been linked directly to WNV infection in horses and has only been recorded in ewes in SA following experimental infection. (Barnard & Voges, 1986) The regions amplified for the NS5 diagnostic PCR, NS5/3'UTR junction and E-protein grouped this strain with a Tunisian strain, PAH001, that was originally isolated from a man with fatal encephalitis (Charrel *et al.*, 2003; Murgue *et al.*, 2001a) (Figure 2.4 and

Figure 2.5). It is postulated that this virus was transported from North Africa to South Africa, possibly via migratory birds.

Except for fever (n=2), WNV disease described in this study is almost exclusively neurological in nature (n=32), as demonstrated by the L1 and L2 WNV infections identified. Clinically, both central nervous signs (blindness, fever, stiff neck, lethargy) and peripheral nervous signs (fasciculation's, fine muscle twitching, muscle weakness/cramps, spasms, loss of balance and co-ordination) were reported (Table 2.9). Severe L1 and L2 WNV disease identified here was not distinguishable from previous studies (Cantile *et al.*, 2000; Ostlund *et al.*, 2001). Zoonotic WNV disease is an important consideration when working with CNS of infected animals (Venter *et al.*, 2009b).

2.5. CONCLUSION

This study suggests that the historical view of L2 WNV strains being non-pathogenic avirulent and non-neuroinvasive is not accurate and should be reviewed by state and private veterinarians, medical clinicians and academics. Horse owners should be made aware of this virus and its disease, its zoonotic potential as well as possible treatment plans that could be applied in the case of infected horses. Increased awareness of WNV disease, its seasonality and differential diagnostic symptoms will aid in correct identification of WNV in unexplained neurological disease in horses, hence increasing the number of cases detected annually. Additionally correct identification of WNV cases will establish peak WNV activity periods as well as "hot spots" in the country, which could lead to further studies focusing on WNV disease progression in horses as well as various environmental factors affecting this. Our data show that disease presentation is the same in L1 or L2 infections and that L2 WNV does cause neurological disease in horses in SA. The detection of L1 WNV in the Western Cape validates WNV as an emerging pathogen and confirms the use of horses as sentinels for WNV activity in SA. This case also confirms the transplacental transmission of WNV in horses and suggests a risk for abortion in pregnant mares with severe neurological disease.

CHAPTER 3

MOLECULAR EPIDEMIOLOGY OF WESSELSBRON VIRUS INFECTIONS IN HUMANS AND HORSES IN SOUTH AFRICA

3.1. INTRODUCTION

Wesselsbron virus (WSLV), an arthropod-borne flavivirus, was first isolated from an 8-day-old lamb in 1955 in the town of Wesselsbron in the Free State province of South Africa (SA) (Coetzer *et al.*, 1978; Weiss *et al.*, 1956). In this flock, many new-borne lambs and pregnant ewes died; however, no clinical signs of the disease were reported in the yearlings or wethers.

WSLV has been isolated from vertebrate and arthropod hosts in several African countries including SA (van der Lugt *et al.*, 1995; Weiss *et al.*, 1956) and Cote d' Voire (Baba *et al.*, 1999), mosquitoes in Thailand and is serologically widespread in other African countries (van der Lugt *et al.*, 1995), including Madagascar (Morvan *et al.*, 1990). WSLV is maintained in nature in an enzootic fashion, being transmitted between infected *Aedes* mosquitoes and herbivore hosts. Human infection may result from mosquito bite (Jupp & Kemp, 1998; Smithburn *et al.*, 1957), laboratory infection or incorrect handling of infected animal tissues (CFSPH, 2006; Heymann *et al.*, 1958; Justines & Shope, 1969; Tomori *et al.*, 1981).

In livestock such as sheep, goats and cattle, WSLV disease causes an acute, biphasic, febrile illness(Smithburn *et al.*, 1957; Weiss *et al.*, 1956). Illness due to WSLV has been reported in ostriches (Allwright *et al.*, 1995; Verwoerd, 2000). Experimental infection of horses with WSLV resulted in mild febrile reactions (Weiss *et al.*, 1956). Although predominantly mild or sub-clinical in adult animals (Coetzer & Theodoridis, 1982), it may cause hepatitis, haemorrhages and abortions associated with *hydrops amnii* in pregnant ewes (Coetzer & Barnard, 1977). Pathological changes in the liver of adult animals are variable and therefore difficult to diagnose (Coetzer *et al.*, 1978). In new born lambs, WSLV is fatal and liver pathology is constant and typical, characterized by fatty infiltration of hepatocytes, bile pigmentation, necrobiosis of hepatocytes as well as the infiltration of neutrophils and lymphocytes (Coetzer *et al.*, 1978; leRoux, 1959).

Spread of WSLV to mosquito vectors in other parts of the world, such as the West Nile virus (WNV) outbreak in North America (Campbell *et al.*, 2002), is of concern as no

preventative treatment is available for WSLV. The use of an attenuated WSLV vaccine on pregnant ewes was discontinued due to severe economic losses resulting from abortions and foetal malformations (encephalitis) in these animals (Coetzer *et al.*, 1979; leRoux, 1959; MVM, 2011)

The *Flavivirus* genome is an 11kb, single-stranded, positive-sense RNA molecule, which encodes three structural proteins (Capsid, membrane and envelope) and seven non-structural proteins (NS1 to NS5) (Campbell *et al.*, 2002). The NS3 (protease and helicase) and NS5 (methyltransferase and RNA-dependent-RNA polymerase) proteins are important for replication (Lindenbach *et al.*, 2007). Other NS proteins as well as the 5' and 3' untranslated regions (UTR) may be involved in replication. Although serological classifications grouped YFV, the prototype flavivirus, and WSLV in an unassigned category, subsequent phylogenetic studies have since defined the relationship between these unassigned viruses and the rest of the flaviviruses (Grard *et al.*, 2010; Kuno *et al.*, 1998; Zanotto *et al.*, 1996). Additionally, phylogenetic differences based on mosquito vector (*Culex* or *Aedes* genera), proposed a genetic basis for virus adaptation to their specific vectors; the *Culex* virus clade being associated more with encephalitic disease and the *Aedes* virus clade associated with haemorrhagic-type diseases (Gaunt *et al.*, 2001). Although strain H177 was recently sequenced (Grard *et al.*, 2010), WSLV remains poorly characterized on a molecular level. No molecular epidemiological data is available and it is not clear whether strain differences exist which may affect the pathogenicity of individual strains.

To determine whether flaviviruses may be associated with unexplained neurological or hepatic disease in horses in SA, acute cases were screened over 36 months using *Flavivirus* family-specific RT-PCR. Real-time probe analysis identified West Nile virus (WNV) cases (Venter *et al.*, 2009a). Any positive cases not detected by WNV-probes were subjected to sequence analysis. Here we report the identification of WSLV in two cases of severe neurological disease in horses; one from plasma and the other formalin-fixed brain tissue. A virus isolate could not be obtained from the formalin-fixed brain tissue. For this reason strain AV259, isolated in 1996 from a human with encephalitis, was subjected to full-genome sequencing and compared to a previously isolated strain that was identified in a febrile human. Additionally, AV259 was compared to the two partial horse sequences and several field isolates from mosquitoes and animals in SA.

This provides the first molecular epidemiological comparison between WSLV strains with different pathogenic profiles from southern Africa.

3.2. MATERIALS AND METHODS

Please refer to 2.2.1 for clinical specimen collection.

3.2.1. Viral RNA Extraction (Formalin-Fixed and Paraffin-Embedded Tissues)

Formalin-fixed tissue, dissected into 25 mg sections, was washed in PBS for 1 min (3 repeats) with soft swirling to remove excess formalin. Paraffin-embedded tissue (25 mg sections) was deparaffinized by the addition of 1200µl xylene, vigorous vortex and centrifugation at maximum speed for 5 min. Following supernatant aspiration, 1200µl 100% ethanol (EtOH) was added to the pellet and gently vortexed to remove surplus xylene. Finally, the pellet was centrifuged (5 min, full-speed) and the EtOH aspirated (three repeats). Tubes were incubated open at 37ºC for 15 min to evaporate excess EtOH, and the pellet resuspended in buffer RTL. Viral RNA was then extracted according to the viral RNeasy mini kit (Qiagen, Valencia, CA). Please refer to 2.2.2.1. for sample preparation and viral RNA extraction from plasma.

3.2.2. Wesselsbron Virus Strains

WSLV strains were obtained from the Special Pathogens Unit (SPU) at the National Institute of Communicable Diseases (NICD), Sandringham, SA (Table 3.1). Strains isolated from humans caused disease characterized by either mild fever (H177[#]) or non-fatal encephalitis (AV259*).

3.2.3. Wesselsbron Virus Cell Culture

AV259, a mouse brain isolate, was cultured as described in 2.2.13. VERO isolates and clinical samples were cultured by inoculating 500µl of clarified and filtered supernatant or plasma, onto a monolayer of VERO cells supplemented as described in 2.2.13. Virus stocks were prepared by supernatant centrifugation and filtration through a 0.2µm filter (Millipore Corporate, Billerica, MA). Additionally, AV259, which was subjected to full-genome sequencing, was concentrated using Centricon–Plus70 columns according to manufacturers' recommendations (Millipore Corporate, Billerica, MA).

3.2.4. AV259 DNAse, RNAse Treatment and Viral RNA Extraction

Contaminating genomic DNA and RNA was removed from concentrated AV259 retentate by the addition of 0.5U/µl DNAse-1 (Roche, Mannheim, Germany) supplemented with 42mM $MgCl_2$ and incubated for 10 min at room temperature. The reaction was stopped by adding 5mM EDTA. Finally, 1µl of RNAse (Roche, Mannheim, Germany) was added, incubated at 37°C for 45 min and the reaction stopped by the addition of 1.03M of NaCl and incubating on ice for 60 min. Once completed, viral RNA was extracted using the Qiagen Viral RNA kit (Qiagen, Valencia, CA) as described in 2.2.2.1.

3.2.5. Clinical Specimen PCR Amplification

3.2.5.1. *Flavivirus family-specific PCR*

Clinical specimens were amplified in a first round PCR with primers FU1 (Scaramozzino *et al.*, 2001) and WNV9317R (Zaayman *et al.*, 2009) using the Titan One Tube PCR kit according to manufacturer's recommendations' (Roche, Mannheim, Germany). The PCR was carried out using 10µl RNA template and 0.8µM of each primer in a final volume of 50µl. Cycling began at 50°C for 30 min followed by 94°C for 2 min; and 35 cycles of 94°C for 10s, 50°C for 30s and 68°C for 1 min with a final extension of 68°C for 7 min. A nested PCR reaction was performed using the Expand High Fidelity kit (Roche, Mannheim, Germany) according to manufacturer's instructions using primers MAMD and CFD2 (Scaramozzino *et al.*, 2001). Nested PCR used 2µl of the first round PCR product and 0.8µM of each primer in a final volume of 50µl. Cycling commenced at 94°C for 2 min and 35 cycles of 94°C for 10s, 53°C for 30s and 72°C for 1 min, and a final extension of 72°C for 7 min. To confirm that WSLV positive specimens were negative for WNV, specimens were reamplified from RNA as described above, and a semi-nested WNV real-time RT-PCR was carried out as described in 2.2.4.2.

3.2.5.2. *WSLV-specific NS5/3'UTR PCR*

Complementary DNA (cDNA) was made with Expand Reverse Transcriptase (Roche Mannheim, Germany) using 10µl of viral RNA and 0.4µM of random primers in a total volume of 50µl according to the cycling program 30°C for 10 min and 42°C for 50 min. A larger region of the NS5/3'UTR junction was amplified for phylogenetic analysis as such: following cDNA synthesis, first round PCR was performed using the Expand

High Fidelity kit (Roche, Mannheim, Germany) according to supplier's instructions. To a final volume of 50µl, 5µl of cDNA and 0.8µM of each primer EMF-1 (5' TGG ATG AC(C/G) AC(T/G) GA(A/G) GA(C/T) ATG 3') and VD8R (5' GGG TCT CCT CTA ACC TCT AG 3') (Pierre *et al.*, 1994) was added. Cycling commenced at 94°C for 2 min and 40 cycles of 94°C for 30s, 55°C for 45s and 72°C for 45s, with a final extension of 72°C for 7 min. A 303 bp fragment (genome position 10294 – 10597 relative to strain H177, accession number EU707555) was amplified using the Expand High Fidelity kit (Roche, Mannheim, Germany) in a nested reaction according to manufacturer's instructions. In a final volume of 50µl, using 5µl of first round product, 5µl Q-solution (Qiagen, Valencia, CA) and 0.8µM of each primer W10294F (5' CAG GGA ACT CGA GGA GAG TG 3') and W10578R (5' CTG GTT TGA GCC TAC CAG GA 3') was added. Template and PCR master mix was denatured for 10 min at 95°C before adding the Expand HiFi enzyme. Cycling began at 94°C for 2 min and 10 cycles of 94°C for 30s, 51°C for 30s and 72°C for 30s; 25 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s +10s/cycle with a final extension of 72°C for 7 min.

3.2.5.3. *WSLV-specific E-protein PCR*

Following cDNA synthesis, described in 3.2.5.2; a first round reaction of 50µl using 5 µl cDNA and 0.8µM of each primer WS1568E+ (5' AGG CTT TGG TCC TGG GT AGT 3') and WS2252E- (5' AAY GCA TAT GAA GCC CAT TG3') was prepared using the Expand High Fidelity kit (Roche, Mannheim, Germany) following manufacturers' recommendations'. Cycling commenced at 94°C for 2 min and 40 cycles of 94°C for 30s, 53°C for 30s and 72°C for 45s, with a final extension of 72°C for 7 min. A 256 bp fragment (genome position 1936 – 2192, relative to strain H177) was amplified in a nested reaction using the Expand High Fidelity kit (Roche, Mannheim, Germany) according to manufacturer's instructions, using 10µl of first round product and 0.8µM of each primer WS1936EN+ (5' CCA CCA TTT GGA GAA AGT TA 3') and WS2173EN- (5' TTG GTT ATC CAA CTG AGA CC 3'). Cycling began at 94°C for 2 min and 10 cycles of 94°C for 10s, 50°C for 30s and 72°C for 30s, 25 cycles of 94°C for 10s, 50°C for 30s and 72°C for 30s +10s/cycle with a final extension of 72°C for 7 min.

3.2.6. Clinical Specimen Differential Diagnosis

Refer to 2.2.6 and 4.2.1 for differential diagnosis of clinical specimens.

3.2.7. WSLV Isolate E-protein Amplification

A partial region of the E-protein was amplified for the field isolates (Table 3.1) with the Titan One Tube PCR kit (Roche, Mannheim, Germany) according to manufacturers' recommendations using primers WS1315F (5' GTG ACC TTT GTT CCA AC CA CA 3') and WS2276R (5'GGC CAG CAC T(T/A)G CAG TAA GG3'). Briefly, 10µl RNA template and 0.8µM of each primer was mixed in a final volume of 50µl and cycling commenced at 50°C for 30 min with a subsequent step of 94°C for 2 min. Thereafter 35 cycles of 94°C for 10s, 55°C for 30s and 68°C for 45s with a final extension of 68°C for 7 min amplified a 980 bp fragment (genome position 1315 – 2295, relative to strain H177).

3.2.8. AV259 Genome Amplification

Three sets of primers specific to the WSLV genome were designed as described in section 2.2.3 based on WSLV strain H177 (EU555707), to amplify three overlapping fragments of ~4000 bp each. Primer names reflect genome position (Table 3.2). cDNA was made using concentrated AV259 viral RNA as described in 3.2.5.2 and fragments were amplified using the Roche Long Template kit (Roche, Mannheim, Germany) according to manufacturers' instructions. Briefly, 2µl of cDNA and 0.8µM of each primer (Table 3.2) were mixed in a final volume of 50µl and amplified according to the following cycling conditions: 92°C for 2min; 10 cycles of 92°C for 10s, **Tm** for 30s and 68°C for 4 min; followed by 25 cycles of 92°C for 15s, **Tm** for 30s and 68°C for 4 min + 20s/cycle, with a final extension of 68°C for 7 min. PCR fragments were analyzed as in section 2.2.7 and purified as described in 2.2.8. The concentration of each purified fragment was determined using a NanoDrop ND1000 machine (Thermo Scientific, Wilmington, DE). Pyrosequencing requires between 200ng – 500ng of PCR product.

3.2.9. Pyrosequencing

Each fragment generated in 3.2.8 was purified (final volume of 20µl) and submitted separately to Inqaba Biotec© for pyrosequencing using the 454FLX technology (Roche, Mannheim, Germany). The final concentration of each fragment as determined by NanoDrop analysis is shown in Table 3.3. Additional primers were designed as in 2.2.3 based on AV259 sequence information to fill gaps left following 454FLX pyrosequencing (Table 3.4).

3.2.10. Phylogenetic Analysis of Clinical Specimens and WSLV Isolates

DNA sequencing and phylogenetic analysis, as described in 2.2.9 confirmed positive RT-PCR products from clinical samples and WSLV isolates.

3.2.11. Genome Assembly and Phylogenetic Analysis of AV259

Genome assembly of AV259 FLX454-fragments was performed by using the CLC genomics workbench (CLC Bio, Århus, Denmark); multiple sequence alignments done using MAFFT v6 (http://align.bmr.kyushu-u.ac.jp/mafft/software/) (Katoh *et al.*, 2009), edited with BioEdit (v 7.0.9) (Hall, 1999) and amino acid analysis using GeneDoc for Windows (Nicholas & Nicholas, 1997). Maximum-likelihood trees were generated using PhyML (Guindon *et al.*, 2009) with the neighbor-joining option and a bootstrap confidence of 100 replicates. Nucleotide and amino acid differences were calculated with MEGA (v5) using the P-distance option (Tamura *et al.*, 2007).

3.2.12. Protein Placement Prediction

The 5' and 3' untranslated regions (UTRs) and the viral open reading frame (ORF) of AV259 were determined by comparing AV259 to WSLV H177 and Sepik virus (SEPV) strain MK4871 (GenBank accession number DQ387642) (Kuno & Chang, 2006). Protein placement was predicted with the genome annotation transfer utility (GATU) program using YFV 17D as the reference strain (http://www.viprbrc.org/brc/gatuStart.do?decorator=flavi) (Tcherepanov V. *et al.*, 2006). Cleavage sites and cysteine (CYS) residues were identified by comparing the AV259 ORF to SEPV (Kuno & Chang, 2006), while host cellular signalase sites were determined using SignalP-NN (http://www.cbs.dtu.dk/services/). N-linked glycosylation sites were also determined (www.cbs.dtu.dk).

3.2.13. Genome Cyclization

To investigate genome cyclization structures formed by interactions between the 5' and 3' UTRs, 190 nt from the 5' UTR and the last 115 nt of the 3'UTR were joined by a 50 base pair poly-A spacer (Khromykh *et al.*, 2001a) and analyzed using the internet-based M-fold program v3.2 (fold temperature = 37°C, 5% suboptimality, [http://mfold.bioinfo.rpi.edu]) (Zuker, 2003). Secondary structures within the 5' UTR and 3'UTR were also determined using the M-fold program, as described above.

Table 3.1: Wesselsbron virus strains used in this study

Virus strain	Location	Date isolated	Host isolated from	Received as isolate
TAR100	Tete Pan, Kwa-Zulu Natal	22 July 1955	Mosquito	VERO
AR778	Middelburg, Mpumalanga	26 April 1957	Mosquito	VERO
AR2209	Ndumu, Kwa-Zulu Natal	16 June 1959	Mosquito	VERO
AR11189	Pearston near Salisbury, Kwa-Zulu Natal	16 July 1969	Mosquito	VERO
H177[#]	Mkuze, Kwa-Zulu Natal	7 March 1974	Human	VERO
AN16210	Graaff Reinet, Western Cape	9 August 1989	Sheep	VERO
AV259*	Bultfontein, Free State	4 April 1996	Human	Mouse brain

*** WSLV strain used for pyrosequencing**

Table 3.2: Primers designed to amplify the genome of WSLV strain AV259

Set no.	Orientation	Primer name	Sequence	Primer Tm (°C)	PCR Tm (°C)	Roche Long Template buffer
1	Sense	W1F	AGT ATA TTC TGC GTG CTA ATC G	56	55	2
	Antisense	W4039R	TGG TTG AGA CGC TGTC AGT C	60		
2	Sense	W3845F	GCT TCC AAA GGA GAT GTT GA	58	55	1,2,3
	Antisense	W7605R	TGC TTT GCC TCT CCT TGT CT	60		
3	Sense	W7472F	GCG GT(G/A) GGA CCA CTC ATT G	55	50	1
	Antisense	W10794R	AGA CAC TAG TTG GTT CTC AAC	51		

Table 3.3: Concentration of purified AV259 fragments submitted for pyrosequencing

Fragment	ng/ul	Final volume (µl)	Final concentration (µg)
1	90.6	20	1.8
2	326.09	20	7.2
3	164.56	20	3.2

Table 3.4: Primers designed to amplify "gaps" in the AV259 genome after pyrosequencing

Primer name	Sense/ Antisense	Sequence	Genome position	PCR fragment size (nt)	Genome "gap" size (nt)	Primer Tm (°C)
G1-F	Sense	ATT TGT GGC AAC CAT GTG GAA	3796 - 3959	164	4	59
G1-R	Antisense	AAG CAG AGC AGA GGC AAC AC				60
G2-F	Sense	GCA GAC TGA CAG CGT CTC AA	4036 - 4185	150	48	60
G2-R	Antisense	GTC TCT CCA AGA GGC CAA GA				59
G3-F	Sense	GAA ATG GTT GTG CTC GGT TC	7448 - 7624	177	95	60
G3-R	Antisense	TGC TTT GCC TCT CCT TGT CT				60
G4-F	Sense	GTG GCA AAA CAG GAG GAA AC	9186 - 9337	152	11	59
G4-R	Antisense	GTA TGC CAG CTC CGT CAC AG				61
G5-F	Sense	TAA AGG GGT TCC TGG TAG GC	10569 - 10771	203	6	60
G5-R	Antisense	ATC TGT GCT TTA GGG CGA GA				59

3.3. RESULTS

3.3.1. Identification of Wesselsbron Virus in Clinical Samples

In total, 261 clinical samples were collected over 36 months; 79 in 2008, 47 in 2009 and 141 in 2010 from horses with acute neurological or hepatic illness or unexplained fever. In 2008, WSLV was identified through *Flavivirus* family-specific RT-PCR and DNA sequencing for 2 (SAE118/08, SAE122/08) specimens submitted due to unexplained neurological disease. In 2008, 50/79 samples were submitted due to unexplained neurological/hepatic disease and 29/79 due to unexplained fever. The positive cases made up 4% (2/50) of the neurological cases identified in 2008 (Table 3.5). No WSLV cases were detected by RT-PCR in other years.

3.3.2. Horse Cases

Both WSLV cases (SAE118/08 and SAE122/08) were associated with severe neurological symptoms (Table 3.5). SAE118/08, a 2-year-old Lipizzaner filly stabled near the Hartbeespoort dam in the North West province, displayed symptoms of mild ataxia, hind-limb in-coordination, poor anal tone, fever (40°C), yellow mucous membranes, jaundice, a raise heart rate and swelling of the limbs and ventral abdomen but maintained a good appetite. Treatment consisted of antibiotics, anti-inflammatories and an isotonic drench and she was discharged after 8 days in the clinic. Ataxia persisted for ~20 days following release.

SAE122/08, a 16½ month-old filly stabled in the Bonnievale area of the Breerivierallei in the Western Cape province, displayed symptoms of acute fever (39.9°C), profuse urination and paresis in the hind-limbs which progressed to limb weakness, in-coordination and eventual paralysis. Euthanasia was elected on the 4th day of illness. Central nervous system (CNS) tissue was submitted to the Western Cape Provincial Veterinary laboratory for post-mortem (PM) investigation and formalin-fixed brain tissue was sent to the Zoonosis Unit, Department Medical Virology for PCR investigation. Histologically, PM findings identified signs of meningitis and mononuclear meningo-encephalitis by the presence of widespread perivascular cuffing around the blood vessels in the neuropil. Meningitis, multifocal neuronal necrosis and gliosis were features of the grey matter. No other organs displayed significant lesions. Results of virus isolation and differential diagnostic tests are summarized in Table 3.5.

3.3.3. Phylogenetic Analysis of Horse Cases

3.3.3.1. *Flavivirus family-specific PCR phylogenetic analysis*

WSLV isolates from previous outbreaks in SA were amplified by RT-PCR and compared to clinical strains identified in horses. A 200 bp region of the NS5 gene was analysed by maximum-likelihood (ML) comparison, which clustered all SA WSLV strains together (Figure 3.1). Clinical strains and isolates formed their own branch within the YFV group. Two sub-clusters can be seen within the WSLV group, one which contains the clinical strains identified in this study. AV259, a strain isolated from a human with encephalitis (Jupp & Kemp, 1998), fell into the second group forming its own branch. While WSLV strains grouped with YFV (Figure 3.1), amino acid P-distance analysis revealed that WSLV is most closely related to SEPV (7.6% divergence), rather than YFV (13.6% - 15.2% difference). On a nucleotide level, clinical strains were most closely related to field isolates from SA in this conserved region, which is used as the diagnostic RT-PCR. SAE118/08 was identical to strain H177 which was isolated from a human with fever in 1955 (Smithburn *et al.*, 1957) while SAE122/08 had a 0.5 % divergence to AR2209, a strain isolated from mosquitoes in 1959. Clinical strains differed from one another by 1%; while an average nucleotide divergence of 3.2% was calculated for all WSLV strains.

3.3.3.2. *WSLV-specific NS5/3'UTR PCR phylogenetic analysis*

To confirm these findings, both WSLV horse strains were re-extracted and reamplified from the original clinical specimens, to produce a fragment of 264 bp of the NS5/3'UTR. Clinical specimens were identical to one another and to H177 and AR2209 (Figure 3.2, Appendix 2). Strains H177 and AR2209 were also identical to one another in this region. SAE118/08 and SAE122/08 showed a 3% difference to AV259 on the nucleotide level and a 6.1% divergence on the amino acid level. The average nucleotide divergence for WSLV strains was 2.4%.

3.3.3.3. *WSLV-specific E-protein PCR phylogenetic analysis*

The E-protein of strain SAE122/08 was successfully amplified from formalin-fixed tissue and had a 6.8%, 7.3% and 9.6% nucleotide divergence to strains H177, AR2209 and AV259 respectively. A 4.6% average divergence was calculated for WSLV strains (Figure 3.3, Appendix 2). Amino acid analysis on this 220 bp fragment revealed an 11% divergence between SAE122/08 and remaining WSLV strains, while being 19.4% different to SEPV. E-protein amplification was unsuccessful on the plasma specimen for clinical strain SAE118/08.

Table 3.5: Positive Wesselsbron virus cases identified in horses in South Africa during 2008

Case no.	Date sample received	Specimen	Age	Location	Disease	Final diagnosis	Virus isolation	Results of other viral tests	Outcome
SAE118/08	5/05/2008	Plasma	2y	Hartbeespoort	Neurologic	WSLV PCR+	No	IFA –ve: AHSV PCR –ve: AHSV, EEV, EHV, alpha, WNV Neut –ve: WNV	Survived
SAE122/08	07/05/2008	Formalin fixed brain tissue	16 ½m	Stellenbosch	Neurologic	WSLV PCR+	No	IMP –ve: Flavivirus, AHSV, EEV, EHV PCR –ve: AHSV, EEV, EHV, alpha, WNV FAT –ve: rabies	Died (euthanised)

*** AHSV, African horse sickness virus; EEV, equine encephalosis virus; EHV, equine herpes virus; WNV, West Nile virus; Alpha, alphaviruses including Sindbis and Middelburg virus; IMP, Immunoperoxidase; Neut, neutralisation assay; FAT, fluorescent antibody tests; -, negative; +, positive.**

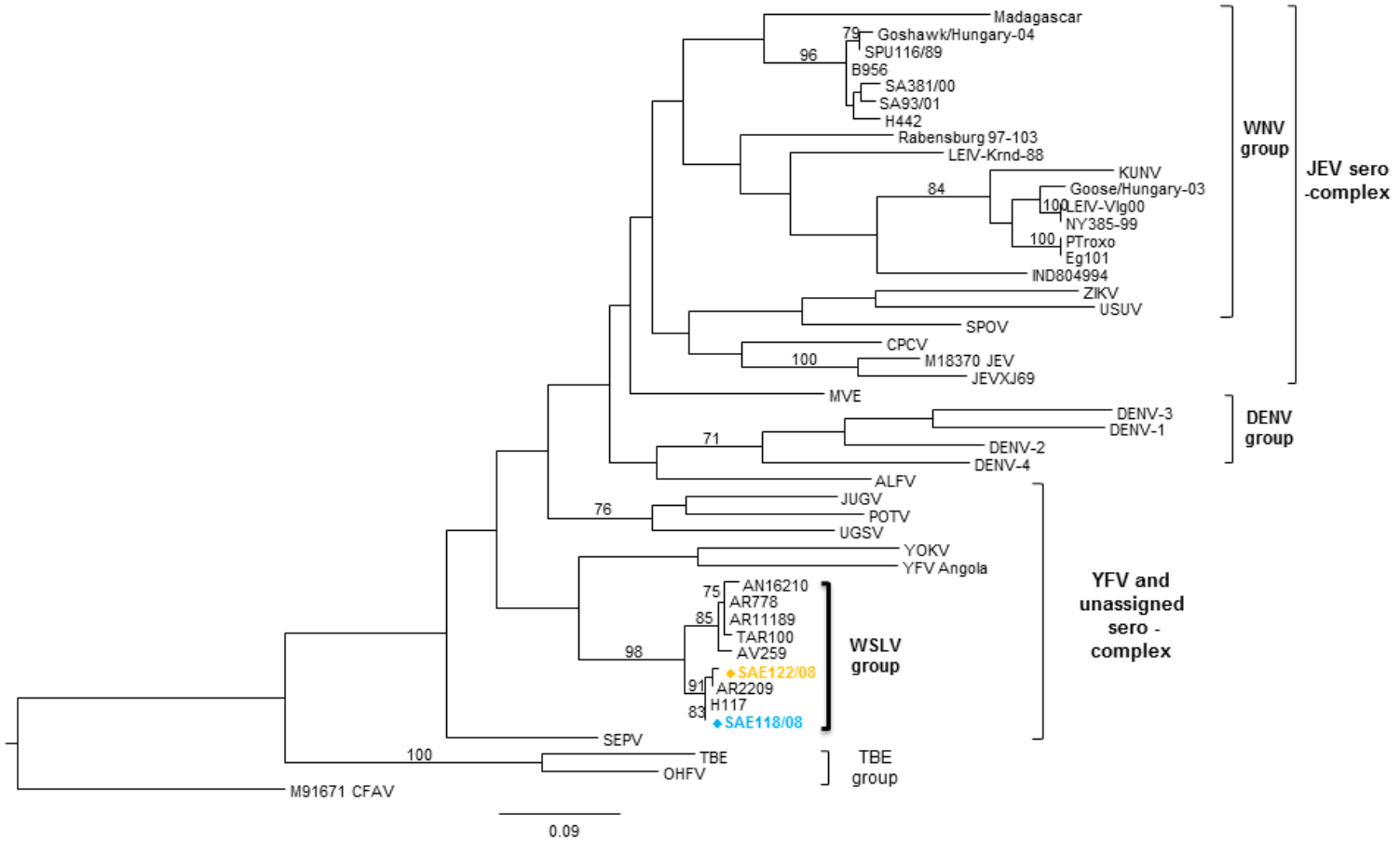

Figure 3.1: Maximum-likelihood comparison of a partial section of the NS5 region amplified for Wesselsbron strains identified in horses in 2008 with representative sequences of other mosquito-borne and tick-borne flaviviruses (Guindon *et al.*, 2009). The tree was constructed using the HKY codon position substitution model using PhyML and drawn to scale with the bar indicating 0.09 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. Only values >70 are shown. The tree was rooted with CFAV. The Wesselsbron isolates identified in this study are indicated by a diamond (♦), strain SAE118/08 is shown in blue and strain SAE122/08 is shown in orange. WSLV isolates used in this study are indicated by ✦ and strain AV259 used for pyrosequencing is highlighted by an asterisk. Accession numbers for strains used in phylogenetic analysis as well as those identified in this study can be found in Table 3.6a and Table 3.6b.

3.3.4 WSLV Isolate E-protein Phylogenetic Analysis

ML analysis of a 940 bp fragment of the E-protein for WSLV isolates confirmed that WSLV is most closely related to SEPV as shown by the clustering of WSLV strains with SEPV (Figure 3.4). Strains AV259 and H177 showed a 5.1% difference on the nucleotide level. P-distance analysis revealed an average divergence of 3.3% for WSLV strains on the nucleotide level; while differing from SEPV between 20.6% – 21.5%, and from YFV between 36.2% - 36.8%. Amino acid divergence of 7.9% - 8.6% was calculated between WSLV and SEPV and ~43% for YFV.

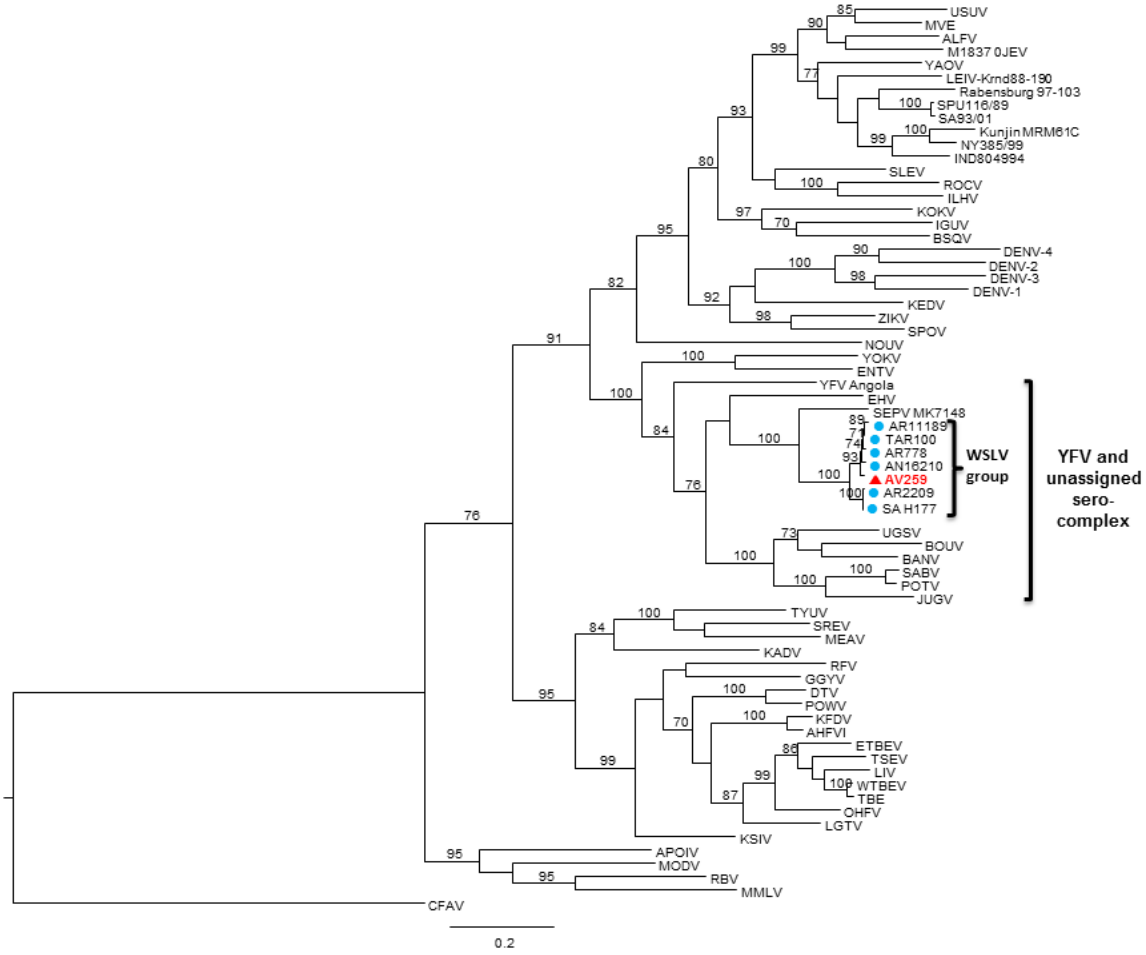

Figure 3.4: Maximum-likelihood comparison of a 940 bp fragment of the E-protein of WSLV isolates from mosquitoes, animals and humans in South Africa. The tree was constructed as described in Figure 3.1 and drawn to scale with the bar indicating 0.2 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. Only values >70 are shown. The tree was rooted with CFAV. WSLV strains isolated in South Africa from previous outbreaks are indicated by a blue circle (●) and the strain sequenced in this study is highlighted in red (AV259) and indicated by a triangle (▲).Accession numbers for strains used in phylogenetic analysis as well as those identified in this study can be found in Table 3.6a and Table 3.6b.

3.3.5. Genome Organization of AV259

In order to investigate the genome composition and differences between encephalitic and febrile WSLV strains, the full genome of strain AV259 was sequenced and compared to a previously sequenced strain, H177, which caused febrile disease in a human (Smithburn *et al.*, 1957).

The complete genome of AV259 was determined to be 10814 nucleotides (nt) in length with an ORF of 10215 nt encoding 3405 amino acids (Table 3.7), and a Mr of 3279476u. The single polyprotein ORF is flanked by 5' and 3' UTRs of 118 nt and 481 nt respectively. The complete sequence of WSLV strain AV259 has been submitted to GenBank under the following accession number: JN226796.

Genome organization of AV259 is similar to other flaviviruses and follows the sequence: 5'UTR-C-PrM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-3'UTR (Table 3.5). The G + C content of the complete genome was calculated as 47.73%, which is similar to WSLV H177 (47.65%) and SEPV (47.29%). YFV has a slightly higher G + C content at 49%. The calculated percentage of each amino acid is 28.6% A, 21.09% C, 26.6% G and 23.6% T.

3.3.6. Cleavage and Glycosylation Sites and Cysteine Residues

3.3.6.1. *Cleavage sites*

Predicted SignalP cleavage sites for proteins C/prM, prM/E, E/NS1 and 2K/NS4B correlated to predicted protein placement on the AV259 genome (Table 3.7). All cleavage sites for AV259 were identical to those of WSLV strain H177. Four cleavage sites were identical in sequence to SEPV MK7148: prM/E, E/NS1, NS1/NS2A and NS4A/2K. The NS1/NS2A site is thought to be cleaved by an unknown cellular enzyme and generally follows the sequence V-X-A (where X is variable) (Rice & Strauss 1990), as can be seen by the sequence VVA in AV259 at the NS1/NS2A junction site.

Table 3.7: Structural (C, prM, M and E) and non-structural (NS 1 – NS5) gene, protein lengths and genome positions are shown along with their predicted cleavage sites for AV259 (see next page). Asterisks (*) indicate cleavage sites which are identical in sequence to those found in SEPV MK4178. Comparisons of the amino acid similarity of each protein to WSLV H177, SEPV MK4178 and YFV-Angola71 are indicated.

Table 3.7: Genome organisation and protein cleavage sites of AV259

Protein	Gene/region	Genome position(s)	Protein cleavage sequence	Size (nt)	Size (aa)	Amino acid & (nucleotide) % similarity		
						H177	SEPV	YFV
Structural	5' UTR	1 – 118	-	118	-	(98)	(89)	(54)
	C	119 – 466	LLAYS↓ATVTR	348	116	98 (96)	76 (74)	40 (52)
	M (prM)	467 – 733	RRSRR↓SAVITP	267	89	100 (96)	90 (76)	57 (60)
	(M)	734 – 958	GPAYS↓THCLG*	225	75			
	E	959 – 2428	TGVGA↓EVGCS*	1470	490	99 (94)	92 (79)	55 (61)
Non-structural	NS1	2429 – 3487	SWVVA↓SKGDV*	1059	353	99 (93)	84 (77)	66 (64)
	NS2A	3488 – 4165	RLPQR↓SWPLG	678	226	98 (95)	82 (76)	39 (51)
	NS2B	4166 – 4555	KTTTR↓SGVLW	390	130	99 (93)	89 (75)	49 (56)
	NS3	4556 – 6424	AEGRR↓SASGI	1869	623	99 (93)	90 (77)	73 (66)
	NS4A	6425 – 6802	PGTQR↓STYDN*	378	126	100 (93)	85 (75)	57 (60)
	2K	6803 – 6871	LLVAA↓NEMEL	69	23	100 (93)	78 (68)	48 (52)
	NS4B	6872 – 7615	KQTRR↓GKAAG	744	248	98 (91)	90 (75)	65 (62)
	NS5	7616 – 10333	GEVLW↓IQQNS	2718	906	99 (94)	86 (75)	69 (66)
	3' UTR	10334 – 10814	-	481	-	(98)	(86)	(65)
Full length genome				10814	3405	-	-	-

3.3.6.2. *Glycosylation sites*

In AV259, two N-linked glycosylation sites (N-LglyS) were predicted for the prM, one for the E-protein and three in the NS1 protein in comparison to SEPV which has only two sites in the NS1 (Table 3.8) (Kuno & Chang, 2006). Identical N-LglyS were identified in strain H177.

Table 3.8: N-linked glycosylation sites of AV259

AV259 gene	No. sites	Residue position	Sequence
prM	2	14, 30	NVTF, NCSV
E	1	154	NHTK
NS1	3	125, 130, 208	NPSR, NGTF, NGTW

3.3.6.3. *Cysteine residues*

Cysteine (CYS) residues are required for stabilizing disulphide bridges in all of the mosquito-borne flaviviruses. Typical *Flavivirus* CYS-residues which form disulphide bonds (Nowak & Wengler, 1987) were found in AV259: six in the prM, 12 highly conserved CYS-residues in the ectodomain of the E-protein and 12 in the NS1. This is consistent with findings in other flaviviruses, including WSLV H177, and corresponds to work done on SEPV (Kuno & Chang, 2006).

3.3.7. Conserved Protein Motifs and Amino Acid Composition

3.3.7.1. *Conserved protein motifs*

Conserved protein motifs were identified in the E, NS3 and NS5 proteins.

E-protein: the conserved *Flavivirus* motif GDD (Kuno *et al.*, 1998; Volk *et al.*, 2009) was found in domain III of the E-protein (residues 377 – 379), and corresponds to the tripeptide which is involved in receptor binding and contains the major neutralizing epitopes (Beasley *et al.*, 2002).

NS3 protein: the *Flavivirus* NS3 protein contains the conserved binding motif DEAH which places it in superfamily-2 of the helicases (Table 3.9) (Wu *et al.*, 2005). In the mosquito-borne flaviviruses, the pentapeptide between residues 298 – 304 is SIAARG; however the Y is replaced by a W in the YFV group as is seen in AV259, H177 and

SEPV. Between residues 225 - 231, the conserved motif LAPTRVV, which was present in H177, was identified (Medeiros *et al.*, 2007). The catalytic triad $H_{48}D_{76}S_{135}$ and substrate binding pocket $G_{127}S_{135}G_{136}$ of the trypsin-like serine protease was observed (Crabtree *et al.*, 2003; Valle & Falgout, 1998).

Table 3.9: Conserved motifs present in the NS3 helicase protein of AV259

Motif	Sequence	Residue
Motif I (Walker A)	GAGKT	201
Motif 2 (Walker B)	LAPTRVVLSEM	226
Motif II	DEAH	289
Motif III	SATPPG	320
Motif IV	FLPSIR	366
Motif V	TDIAEMGAN	413
Motif VI	QRRGRIGR	460

These sequence motifs were also found in WSLV strain H177 as well as SEPV-MK7148. (Kuno & Chang, 2006)

NS-5 protein: The conserved *Flavivirus* peptides YADDTAGWDT (residues 532 – 541), QRGSGQV (residues 600-606), DDCVV (residues 667 – 671), TACL (residues 755 – 758) and YFHRRDLR (residues 770 – 777) were identified (Kuno *et al.*, 1998). A highly conserved mosquito-borne *Flavivirus* peptide was also present in the NS5 protein AMTDTT**P**FGQQRVFKEKVDT (residues 341 – 360) (Medeiros *et al.*, 2007). These motifs were identical in WSLV H177.

3.3.7.2. *Amino acid composition*

Hydrophobic residues contributed to the majority of the genome. A total of 351 negatively charged residues (D & E) and 394 positively charged residues (K & R) made up 21.87% of the total amino acid composition. Comparison of AV259 and H177 revealed a total of 37 amino acid changes across the coding region of AV259. No changes occurred in the prM and NS4A proteins (Table 3. 10). Changes in structural residues (G and P) occurred in the NS2A, NS4B and NS5 proteins which form an integral part of, or are believed to play a role in, viral replication (Lindenbach *et al.*, 2007).

Table 3.10: Amino acid differences between AV259 and H177

Region	Capsid		Envelope					NS1			NS2a					NS2b	NS3				
Aa position	110	113	43	71	153	236	447	140	254	277	103	120	163	164	182	109	58	136	340	360	550
AV259	V	L	V	S	V	Q	V	R	R	R	R	L	M	V	I	I	I	S	I	K	L
H177	I	I	I	T	E	E	I	K	K	K	G	I	V	A	V	T	F	T	V	R	F

Region	NS4b				NS5											
Aa position	47	48	49	209	30	144	245	252	272	280	291	656	834	835	847	897
AV259	L	V	T	N	S	F	S	V	P	P	R	V	V	I	V	E
H177	P	G	N	D	P	L	T	I	T	L	K	A	I	T	I	D

Amino acid differences between AV259, an encephalitic strain of WSLV, and H177 isolated from a patient with fever. Light grey, hydrophobic amino acids; dark grey, hydrophilic amino acids and black, structural determining amino acids. Numbering is according to AV259 and is relative to each protein.

3.3.8. The Non-Coding Regions

3.3.8.1. *The 5' untranslated region (5' UTR)*

The 5' UTR of AV259 is identical in length to H177, being 118 nt long. In comparison, the closely related SEPV 5' UTR, is 116 nt. Secondary structures of the 5' UTR have been predicted for other flaviviruses (Brinton & Dispoto, 1988), each of which display a stem-loop (SL) structure with a side loop. A possible structure for the 5' UTR is depicted in Figure 3.5a. The conserved 5' cyclization (5'-CYC) sequence (TCAATATG) is found within the capsid gene of AV259 (nucleotides 165 – 172).

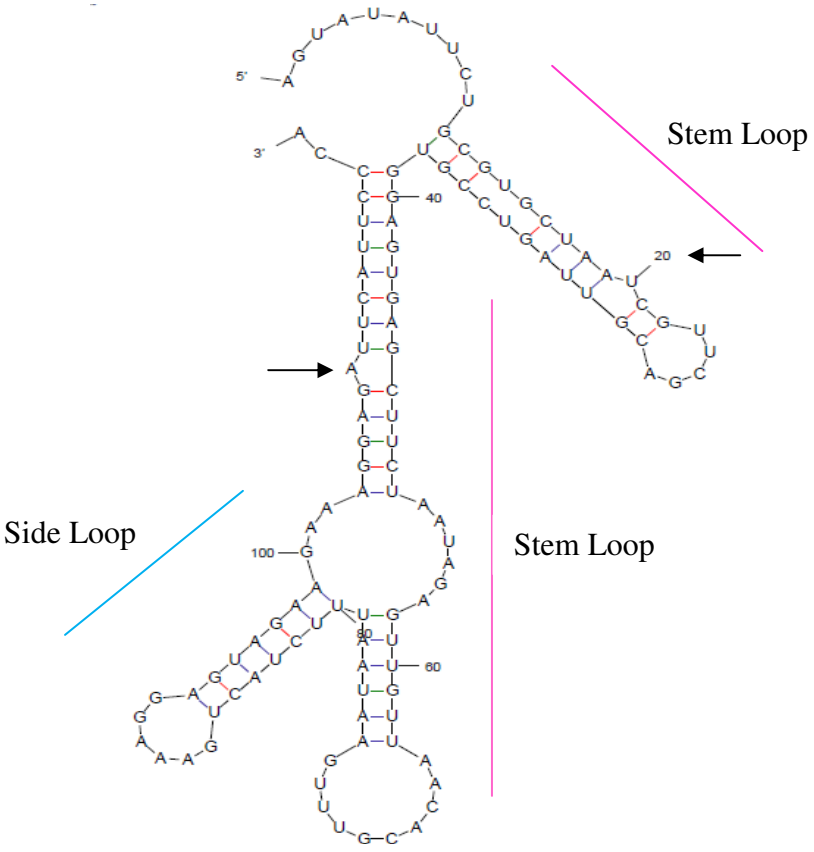

Figure 3.5a: **The predicted 5' UTR structure of Wesselsbron strain AV259 (nucleotides 1 – 118).** The arrows indicate structures called bulges that occur during secondary RNA folding. The stem loop (SL) and side loop is indicated. The structure was determined using the internet based M-fold program (Zuker, 2003).

3.3.8.2. *The 3' untranslated region (3' UTR)*

The 3' UTR of AV259 is identical in length to H177, being 481 nt long, while the 3' UTR of SEPV is 462nt in length. The M-fold program produced 24 putative structures for the AV259 3'UTR (Figure 3.5b). The terminal 84 nt form a long stable hairpin (LSH), a structure conserved among flaviviruses, which contains the conserved

Flavivirus pentapeptide sequence (Wengler & Castle, 1986). Tandem repeat sequences (RYF) are found in the 3' UTR of YFV which share a 15 nt "core sequence" (5' AACCGGGATA[T/A/C]AAAC3') (Mutebi *et al.*, 2004). When compared to the work done on SEPV (Kuno & Chang, 2006), the same tandem repeats were identified in AV259 (RWSL1 – RWSL3). While 2/3 tandem repeats, maintain the core RYF sequence, the last (RWSL-3) is degenerate. Conserved cyclization sequence's (CS1 and CS2) and the complementary 3' cyclization (3'-CYC) sequence (which falls within the CS1 sequence) were also present. The pattern of CS organization (5' → 3') is RWSL1-RWSL2-RWSL3-CS2-CS1 (3-CYC). See Figure 3.5b and Figure 3.5c.

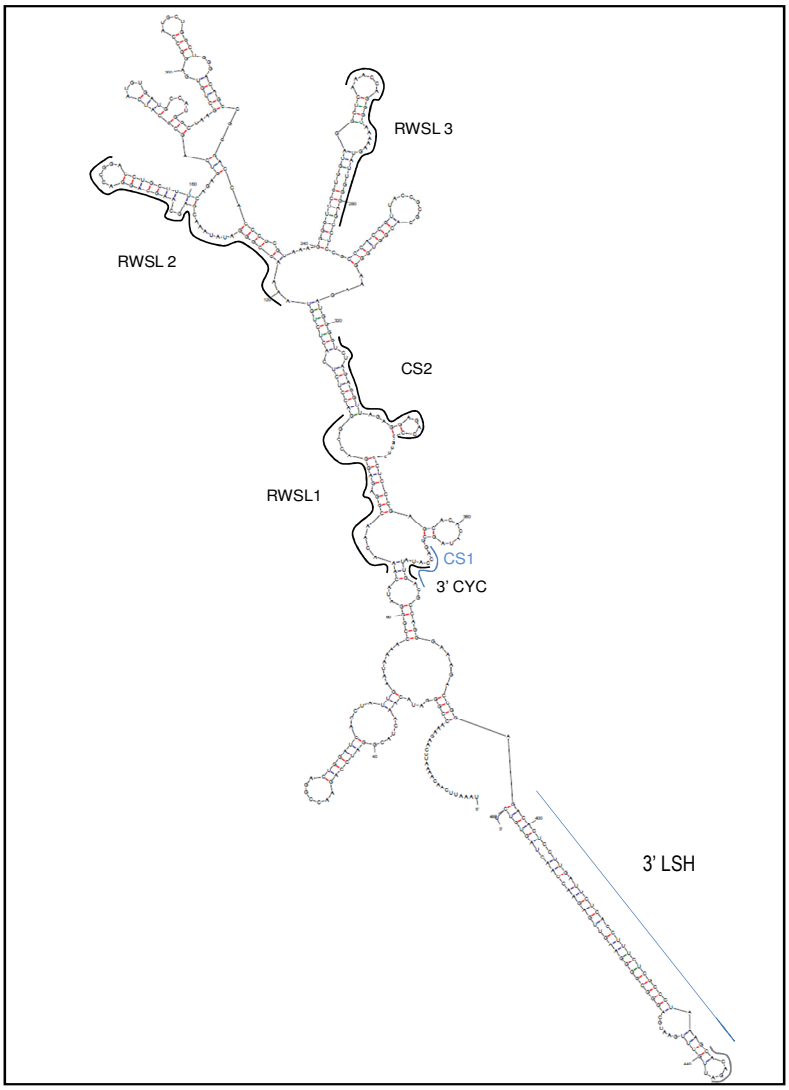

Figure 3.5b: **Secondary folding of the 3' UTR of AV259.** The structure was generated using the internet based M-fold program (Zuker, 2003). RWSL – tandem repeats, CS; conserved sequence, CYC- cyclization sequence, LSH –long stable hairpin. The nucleotide alignment of these sequences can be seen in Figure 3.5c on the next page.

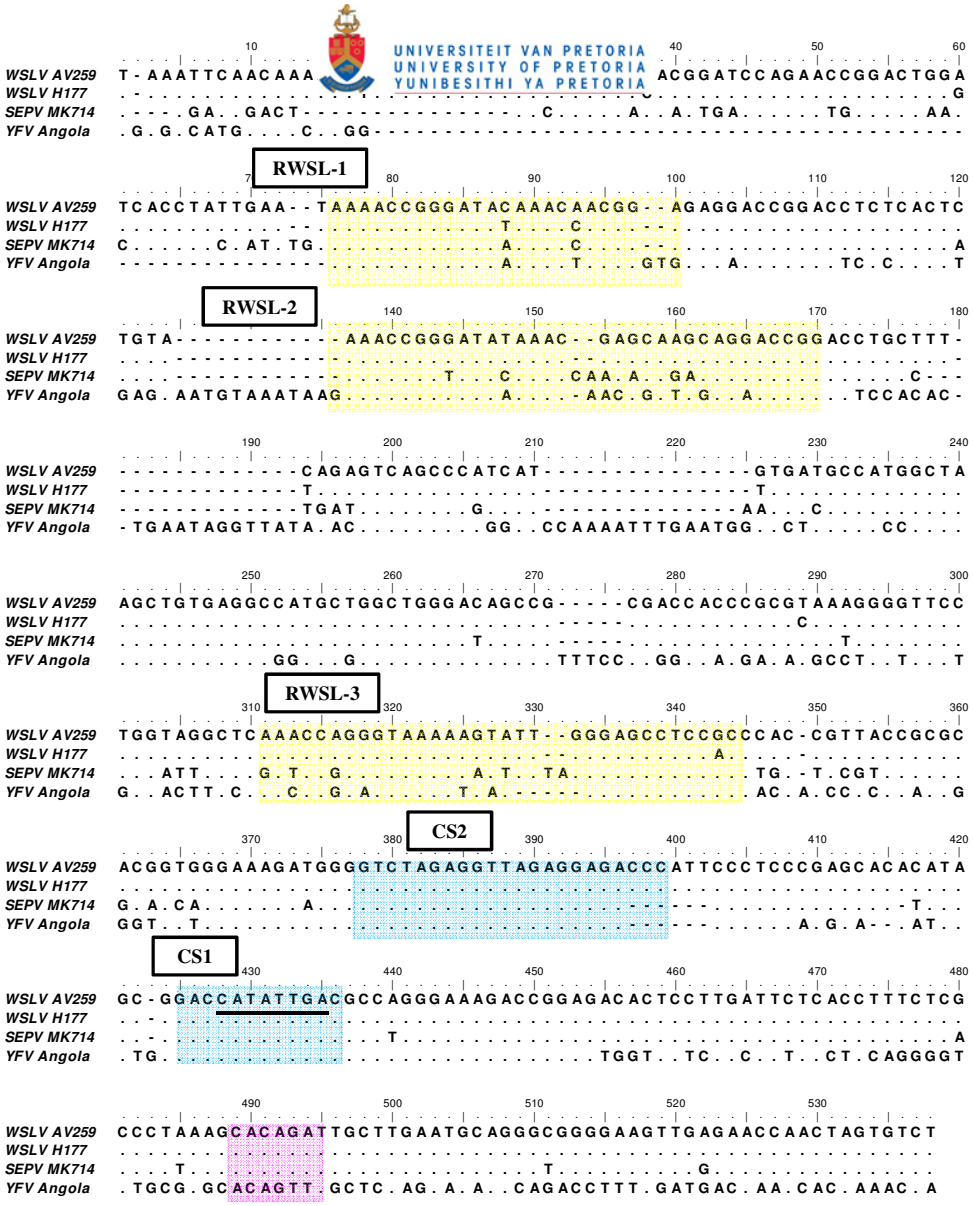

Figure 3.5c: A multiple sequence alignment representing a portion of the 3' UTR of AV259, H177, SEPV MK7148 and YFV Angola. Conserved tandem repeats (RYF) are highlighted by yellow boxes (RWSL1 – 3) followed by the conserved cyclization sequences CS2 and CS1 which are highlighted in blue with the conserved *Flavivirus* pentapeptide shown in pink. The 3' cyclization (3'-CYC) sequence falls within CS1 and is underlined.

3.3.8.3 *Genome cyclization*

The 5' and 3' CYC sequences, 3' LSH and 5' UTR of AV259 are involved in genome cyclization, forming a "pan-handle" structure (Figure 3.5d) (Hahn *et al.*, 1987; Khromykh *et al.*, 2001a). Minor differences are observed between AV259 and SEPV MK7148, which is in agreement with previous studies, demonstrating structural homology within the *Flaviviridae* (Khromykh *et al.*, 2001a). Genome cyclization secondary structures formed by H177 were almost identical to AV259 (Figure 3.5d, Appendix 2).

3.3.9. AV259 Phylogenetic Analysis

ML trees were generated from the amino acid sequences of the full coding sequence, as well as the structural and non-structural proteins of AV259.

3.3.9.1. *Full coding sequence of AV259*

Phylogenetic analysis of the full coding sequence of AV259 is represented by Figure 3.6. WSLV strain AV259 falls into the YFV group within the *Aedes* mosquito-borne subgroup and clusters most closely with SEPV. Similar to Figure 3.1 and Figure 3.4, two monophyletic groups formed within the WSLV cluster, with H177 and AV259 falling into two different groups.

All trees constructed for the structural and non-structural proteins show that WSLV clusters most closely with SEPV, both of which group with YFV as seen in a previous study (Grard *et al.*, 2010). In comparison, the analysis in Figure 3.1 groups WSLV most closely with YFV.

AV259 and H177 were 94% identical at the nucleotide level and 99% identical on an amino acid level in the coding polyprotein. When compared to SEPV, the most closely related of the flaviviruses, AV259 shared only 76% similarity on the nucleotide level and 87% on the amino acid level, confirming that WSLV is a separate species (Table 3.7) (Grard *et al.*, 2010). In comparison, YFV Angola-71 shared only 60% and 62% homology on the amino acid and nucleotide level respectively (Figure 3.6). An overall average divergence of 23% was calculated on the amino acid level. The full genome sequence with indicated protein placement is represented in Figure 3.7a, Appendix 2. Figures 3.7b and 3.7c are snap-shots of the NS3 and E proteins of AV259 relative to H177 and the rest of the YFV and unassigned group within the *Flavivirus* genus (Appendix 2).

Figure 3.6: **A Maximum-likelihood tree of the full amino acid coding sequence of the encephalitic WSLV strain AV259 with representative sequences from flaviviruses of the mosquito-borne, tick-borne and no-known-vector groups (see next page).** The tree was constructed using the HKY codon position substitution model using PhyML (Guindon *et al.*, 2009) and drawn to scale with the bar indicating 0.3 nucleotide substitutions per site. Estimates were based on 100 bootstrap replicates and only values >70 are shown. The tree was rooted with CFAV (M91671). The WSLV isolate AV259 sequenced in this study is indicated by a triangle (▲). The WSLV group is highlighted in blue within the *Aedes*-transmitted mosquito-borne viruses. Banzi virus (BANV) is highlighted with an asterisk as it is transmitted by *Culex*-species mosquitoes. Additional virus divisions are shown on the right hand side of the phylogram. GenBank accession numbers of F*lavivirus* strains used in this analysis are shown in Table 3.6a and Table 3.6b of Appendix 2.

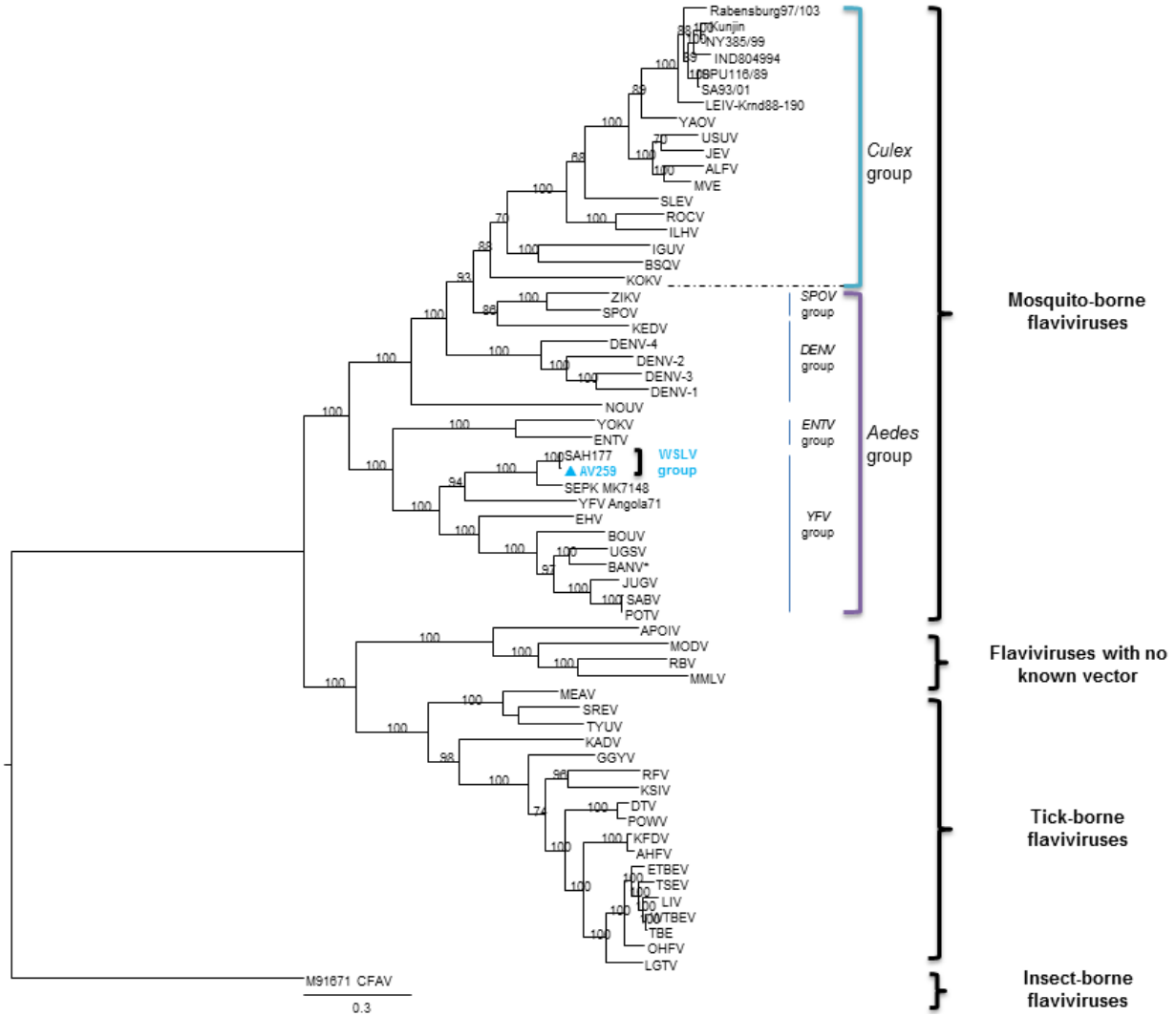

Figure 3.6: **A maximum-likelihood tree representing the coding sequence of AV259 is compared to flaviviruses from mosquito-borne, tick-borne and no-known vector groups within the *Flavivirus* genus.** See previous page for caption.

3.3.9.2. *The structural proteins of AV259*

ML tress representing the core (C), membrane (M) and envelope (E) proteins are shown in Figures 3.8 A – C. Amino acid and nucleotide similarity (for each protein) between AV259, H177, SEPV and YFV is summarized in Table 3.7.

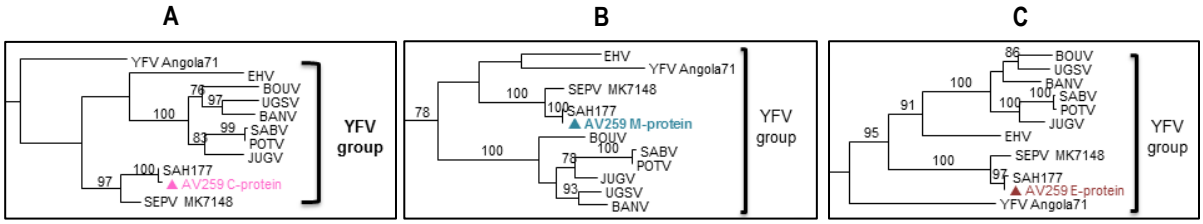

Figure 3.8: Maximum-likelihood comparison of the full coding sequences of the A) C-protein, B) M-protein and C) E protein for WSLV strain AV259. The trees were constructed as described for Figure 3.6. The YFV and unassigned viruses are represented with AV259 proteins indicated as follows: C-protein▲, M-protein▲ and E-protein▲.

3.3.9.3. *The non-structural proteins of AV259*

ML trees representing the non-structural (NS1-NS5) are shown in Figure 3.9A – G. Amino acid and nucleotide similarity (for each protein) between AV259, H177, SEPV and YFV is summarized in Table 3.7.

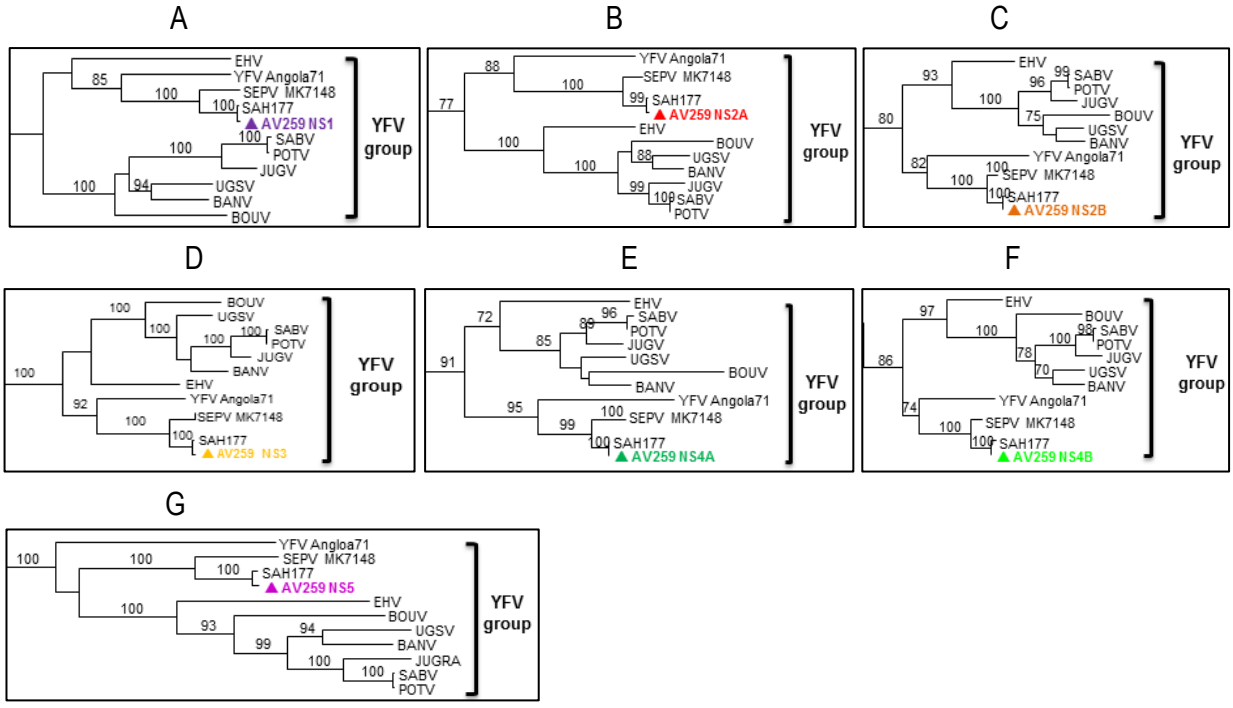

Figure 3.9: Maximum-likelihood comparison of the full coding sequences of the A)NS1, B)NS2A, C)NS2B, D)NS3, E)NS4A, F)NS4B and G)NS5 proteins of WSLV strain AV259. The trees were constructed as described for Figure 3.6. The YFV and unassigned viruses are represented with AV259 proteins indicated as follows: NS1▲, NS2A▲, NS2B▲, NS3▲, NS4A▲, NS4B▲ and NS5▲.

3.4. DISCUSSION

Many viral diseases may go mis- or undiagnosed each year due to a lack of surveillance or knowledge of their disease potential and medical presentation, or are scientifically neglected (Weissenbock *et al.*, 2010). In SA, equine mortality is largely due to African horse sickness (AHS) (Bremer & Viljoen, 1998) while neurological disease is associated with equine encephalosis (EE), equine herpes virus (EHV) (Kirisawa *et al.*, 1993) and WNV, as demonstrated in Chapter 2. However neurological disease as a result of EEV infection remains controversial (Howell *et al.*, 2008; MacLachlan & Guthrie, 2010). As AHS is predominant in SA (Bremer & Viljoen, 1998), it the first aetiological agent thought of in severe cases of infection and often undiagnosable cases are disregarded as AHS (Venter *et al.*, 2009a).

For this reason, a wide range of potential pathogens that may cause similar symptoms, escape diagnosis. Particularly due to the dogmatic approach to equine disease diagnosis in SA; leading to further disease dissemination principally as preventative measures, or possible treatment plans, would not have been established. As such, flaviviruses are often not considered by veterinarians when diagnosing neurological disease in horses. This study aimed to determine whether unexplained neurological or hepatic disease or fever cases in horses, due to flaviviruses other than WNV, were being missed. The identification of WSLV in horses with neurological disease prompted a molecular epidemiological investigation to compare these clinical strains to ones identified in humans (with neurological or febrile disease), mosquitoes and sheep.

Although isolated form mosquitoes (Kokernot *et al.*, 1958) and being a known pathogen of animals, birds and humans (Weissenbock *et al.*, 2010), WSLV has never been diagnosed in horses. Experimental infection of two horses with the original van Tonder strain resulted in mild febrile reactions in these animals (Weiss *et al.*, 1956), and was therefore not considered an important equine pathogen. The limitation of such an experiment however, is that disease presentation may differ dramatically between individual animals and individual virus strains, as with many other flaviviruses. WNV infection in horses for example, induces symptoms in roughly 20% of cases, 90% of which display neurological disease.

During *Flavivirus* screening, 2 WSLV PCR positive cases (SAE118/08 and SAE122/08) were identified (Table 3.5) in horses displaying severe neurological symptoms including ataxia and paresis. Cases originated from different regions of the country at different times of the year; one in February (SAE122/08) and the other in May (SAE118/08) which is the beginning of autumn; and the arbovirus season in SA (McIntosh, 1986). While the disease presented similarly in both animals, strain SAE118/08 was amplified from plasma, suggesting the viraemic stage of the disease. In contrast, SAE122/08 was amplified from brain tissue, indicating end-stage disease, such as is seen in WNV infections (Castillo-Olivares & Wood, 2004).

The favourable outcome of SAE118/08 is possibly due to early diagnosis and adequate supportive treatment; however the recovery period was extensive, lasting a year post-infection. Euthanasia was elected for SAE122/08 as this horse's condition progressed too rapidly for effective treatment and histological analysis on brain tissue revealed viral encephalitis. *Flavivirus* entry into the CNS during infection reduces the chance of a full recovery substantially. Viral replication in the CNS takes place in foci (Castillo-Olivares & Wood, 2004) and therefore screening different tissue sections is imperative during diagnosis. Correct sample type, collection and storage is critical for *Flavivirus* diagnosis (Specter *et al.*, 2002) by PCR-based assays, which can be negative if the sample is taken too late in the infection. This is due to the limited viraemic period which often precedes disease manifestation. Serology is therefore the preferred method of diagnosis, although early infections may be missed.

ML analysis of the 200 bp NS5 fragment amplified during clinical sample diagnosis, grouped SA WSLV strains together on their own branch, but most closely to YFV and Yokose virus (Figure 3.1). P-distance analysis revealed a closer relationship to SEPV, rather than YFV, which was confirmed by additional amplification and phylogenetic analysis of a section of the E-protein and NS5/3'UTR for clinical strains (Figure 3.2 and 3.3, Appendix 2). Phylogenetic analysis revealed a high degree of conservancy in the NS5/3'UTR region as both clinical isolates were identical to one another as well as to strains H177 and AR2209, which were also identical to one another in this region. Identical isolates of WNV have originated from different sources, isolation years or regions in some instances (Burt *et al.*, 2002), which suggests that this may occur for

WSLV. However, E-protein analysis for strain SAE122/08 showed a divergence from the two isolates H177 and AR2209 (Figure 3.3, Appendix 2). Analysis of a 940 nt E-protein fragment amplified for WSLV isolates confirmed that WSLV and SEPV are more closely related (Figure 3.2) as described previously (Grard *et al.*, 2010), in contrast to the diagnostic NS5 gene analysis (Figure 3.1).

To investigate the genome composition and differences between encephalitic and febrile WSLV strains, the full genome of strain AV259, isolated from a patient with non-fatal encephalitis (Jupp & Kemp, 1998), was sequenced and compared to a previously sequenced strain, H177 (EU707555), which caused febrile disease in a human (Smithburn *et al.*, 1957).

The genomic organisation of AV259 shares the pattern found in other flaviviruses (Lindenbach *et al.*, 2007) and is identical to H177. Full genome sequence comparisons between AV259 and H177 revealed 99% amino acid and 94% nucleotide identity (Table 3.7; Figure 3.7a-c, Appendix 2). As expected, the cysteine residue distribution in the prM, E and NS1 proteins (6, 12 and 12) of AV259 is similar to the pattern observed in other flaviviruses because of the critical importance of correct protein folding afforded by disulphide bridges (McMinn, 1997). Although glycosylation has been implicated in replication, neurovirulence in mice, virion maturation and release of viral-like particles (Goto *et al.*, 2005; Li *et al.*, 2006; Muylaert *et al.*, 1996), no difference in glycosylation was observed between AV259 and H177 (Table 3.8). These strains also share a number of conserved *Flavivirus* structural motifs in the E, NS5 and NS3 proteins (Table 3.9) (Beasley *et al.*, 2004; Kuno *et al.*, 1998; Valle & Falgout, 1998; Wu *et al.*, 2005) as well as known *Flavivirus* pathogenic determinants (Lee *et al.*, 2000).

A number of amino acid substitutions were present throughout the ORF of AV259 when compared to H177 (Table 3.10). Notably, were changes involving the structural determining amino acids in the NS2A, NS4B and NS5 proteins. There is a strong association of both the NS2A and NS4B proteins with the *Flavivirus* replication complex (NS3 and NS5 proteins) (Lindenbach *et al.*, 2007). NS2A is hypothesised to be essential for release of infectious particles (Kummerer & Rice, 2002) and biogenesis of virus-induced membranes in infected cells (Leung *et al.*, 2008). Additionally mutations

in this protein have been shown to block virus assembly (Liu *et al.*, 2003). NS4B is believed to be involved in early replication events as it co-localizes with protein NS3 and double-stranded RNA in infected cells (Miller *et al.*, 2006) and may play a role in blocking interferon signalling (Munoz-Jordan *et al.*, 2003). The NS5 protein has both methyltransferase and RNA-dependent-RNA polymerase activity, responsible for capping and replication of the viral genome (Bollati *et al.*, 2009; Lindenbach *et al.*, 2007). Mutation of this protein in YFV 17D has led to a reduced virulence in mice (Xie *et al.*, 1998) and has been implicated in reduced secretion of infectious Kunjin virions (Khromykh *et al.*, 2001b). As these proteins are involved in *Flavivirus* replication, changes in amino acids may have a direct impact on viral replication, infectivity and therefore pathogenesis and virulence. This suggests that AV259's virulence phenotype is partially controlled by structural determinants in at least three non-structural proteins (Table 3.10).

The ML tree topology generated for the full amino acid coding sequence (Figure 3.6), as well as the structural (C, E and M) (Figures 3.8 A – C) and non-structural (NS1 – NS5) proteins (Figures 3.9 A - G) confirm the results generated by phylogenetic analysis of clinical strains (Figure 3.1). Additionally, an amino acid difference of 12.6% between AV259 and SEPV was calculated, confirming that the viruses are two separate species. These findings are in agreement with previous studies (Grard *et al.*, 2010; Kuno & Chang, 2006). SEPV, a *Flavivirus* that was isolated in Papua New Guinea (Kuno & Chang, 2006), has only been associated with disease in one human patient and a sheep in Australia (Grard *et al.*, 2010). Similar to Figures 3.1 and 3.4, WSLV isolates form two monophyletic groups; one containing AV259 and the other, H177. Grard *et al* proposed that the YFV group should be limited to WSLV, SEPV and YFV, which is supported by the morphology of the ML trees generated in this study.

Flavivirus UTRs maintain secondary and tertiary structure homology (Figure 3.5a and Figure 3.5b) (Proutski *et al.*, 1997) to ensure successful replication (Hahn *et al.*, 1987), translation and genome cyclization (Khromykh *et al.*, 2001a; Simmonds & Smith, 1999). Sequence variation within these base-paired regions is hypothesised to be minimal as any substitution/deletion would require simultaneous mutations in the nucleotides on both sides of potential loops structures to maintain correct base-pairing

(Simmonds & Smith, 1999). This is reflected in the nucleotide similarities in both the 5' and 3' UTRs (98%) of AV259 and H177. In comparison, SEPV MK4871 shared 86% and 89% nucleotide similarity with AV259 in these regions, while YFV Angola-71 shared only 54% and 65% similarity to AV259. It is postulated that sequence differences in the variable region of the 3' UTR is associated with virus replication in mosquito vectors in viruses such as YFV and DENV (Wang *et al.*, 1996).

Genome "stacking" (Hahn *et al.*, 1987) occurs during viral replication due to conserved cyclization (CYC) sequences in the 5' and 3' UTR as demonstrated by Figure 3.5c. In AV259, the 5' CYC is found in the C-protein rather than the 5' UTR, as is seen in Kunjin virus (Khromykh *et al.*, 2001a). Viruses AV259, H177 and SEPV-MK4871 form similar structures during genome cyclization (Figure 3.5d, Appendix 2) particularly the 3' LSH which is conserved in the tick-borne and mosquito-borne viruses. Cross-linking studies have demonstrated binding of the NS3 gene, NS5 gene and certain host factors to terminal stem-loop (SL) structures, indicating their possible role in replication (Khromykh *et al.*, 2001a).

Horses are ideal to use as sentinel animals for *Flavivirus* surveillance as they are highly susceptible to these infections, as demonstrated in Chapter 2, and are closely monitored by their owners which increases the likelihood of veterinary involvement during illness. These 2 neurological WSLV cases suggest that severe disease may occur in these animals, however, the percentage of horses that display symptoms following WSLV infection remains unclear. It is, however, known that new-borne lambs are highly susceptible to the disease (Coetzer & Barnard, 1977; Coetzer *et al.*, 1978) and may therefore be a more sensitive measure of WSLV activity.

The use of sentinel horses to screen for flaviviruses in general will aid in providing a better understanding of the clinical manifestation of these diseases as well as the identification and prevention of possible outbreaks. This study suggests that WSLV should be considered in differential diagnosis of neurological disease in horses especially in WSLV endemic countries during the arbovirus season when the mosquito vectors are most active. The development of neurological disease in humans and horses suggests that WSLV, although from the *Aedes*-clade of mosquito-borne viruses which are associated more with haemorrhagic-type diseases (Gaunt *et al.*, 2001), is able to

cause encephalitic disease in dead-end-hosts such as horses. This is supported by WSLV crossing the blood-brain-barrier which confirms the likely causative pathogenesis of WSLV in these cases. To our knowledge WSLV has never before been identified in acute neurological cases in horses by RT-PCR of CNS tissue. This is the first study to document the clinical presentation of natural WSLV infection in these animals.

3.5 CONCLUSION

The identification of WSLV in severe cases of neurological disease in equines is important as it illustrates the lack of knowledge and awareness of WSLV and its disease association in animals other than livestock. This finding also highlights that WSLV diagnosis in horses could potentially have been missed in the past. Full-genome sequencing suggested possible virulence differences between strains of WSLV in proteins associated with replication efficiency and provides information on genes best suited to phylogenetic analysis and diagnostic surveillance. P-distance analysis revealed that the NS3 and NS5 proteins of the replication complex had the least divergence between WSLV, SEPV and YFV. This suggests that these targets would be best for PCR-based diagnostic assays, while the surface C- and E-proteins would be best suited to phylogenetic analysis as these proteins had a P-distance greater divergence. Important changes in the structural amino acids of the non-structural proteins essential for viral replication, as seen in AV259, could explain differences in pathogenicity between WSLV strains circulating in SA

CHAPTER 4

ALPHAVIRUSES AS PATHOGENS OF HORSES IN SOUTH AFRICA

4.1. INTRODUCTION

Alphaviruses, in the *Togaviridae* family, are vector-borne pathogens of medical and veterinary importance, are globally widespread and infect a number of terrestrial animals, fish, and insects also being a major cause of morbidity and mortality in humans (Attoui *et al.*, 2007; Gould *et al.*, 2009; Powers *et al.*, 2001). Geographic locations, reservoir host and vector play an important role in the ecology of the virus (Poidinger *et al.*, 1997; Powers *et al.*, 2001; Sanchez-Seco *et al.*, 2001). Of the two main groupings within the alphaviruses, Old World strains result in a self-limiting febrile, arthritic-type disease in the small joints occasionally accompanied by a rash (Kokernot *et al.*, 1957; Laine *et al.*, 2004; Poidinger *et al.*, 1997; Simpson *et al.*, 1996), rarely causing encephalitis or death (Lewthwaite *et al.*, 2009). New World viruses may result in fatal neurological disease (Powers *et al.*, 2001; Sanchez-Seco *et al.*, 2001).

The alphaviruses are a family of single-stranded, positive-sense RNA viruses are approximately 11kb in length (Norder *et al.*, 1996). The 5' two-thirds of the genome encode non-structural proteins (nsp1 – nsp4) required for replication while the 3' end encodes the structural envelope (E1 and E2) and capsid (C) proteins (Strauss & Strauss, 1994) derived from a 26S RNA molecule. Approximately 40 recognised species exist within this genus which is divided into 7 complexes (Kuhn, 2007).

In South Africa (SA), *Alphavirus* infections are primarily due to two Old World viruses, Sindbis virus (SINV) and Middelburg virus (MIDV). These are transmitted by *Culex* and *Aedes* mosquitoes (Kokernot *et al.*, 1960; Weinbrein *et al.*, 1956) respectively, also the main transmission vectors of West Nile virus (WNV) and Wesselsbron virus (WSLV) (McIntosh, 1986). SINV and WNV are extensively distributed on the Highveld of SA according to serological surveys carried out in the early1960's on both human and animal sera (McIntosh *et al.*, 1962; McIntosh *et al.*, 1964). They also have similar ecologies, being endemic to the Highveld and Karoo regions where they cause sporadic outbreaks (Jupp, 2005; McIntosh *et al.*, 1962; Weinbrein *et al.*, 1956). SINV and MIDV are maintained in nature through cyclic transmission between infected mosquito vectors and susceptible hosts: wild birds in the case of SINV (McIntosh *et al.*, 1976), while

sheep are thought to play a role in MIDV amplification, however, this remains unclear (Kokernot *et al.*, 1957).

Until the 1974 outbreaks in the Karoo region of SA (Jupp *et al.*, 1986b), SINV was considered medically insignificant (Laine *et al.*, 2004; Sammels *et al.*, 1999). In 1983/84 an epizootic occurred in the Witwatersrand/Pretoria regions of SA, where hundreds of human cases were reported (Jupp *et al.*, 1986b); most cases in the Gauteng province were due to febrile illness. Two Sindbis-like virus isolates from SA, SA-AR86 and Girdwood, have been associated with patients suffering from febrile illness. Girdwood has the distinction of having been derived directly from a human case (Malherbe *et al.*, 1963), whereas SA-AR86 was isolated from a pool of *Culex* mosquitoes (Weinbrein *et al.*, 1956). In 1962 a serological study in SA showed a high prevalence of MIDV in livestock with an absence in humans, and it was concluded that man is not susceptible to the disease (McIntosh *et al.*, 1962). This was confirmed by the isolation of MIDV from mosquito populations in Kwa-Zulu Natal with little to no antibodies present in humans resident in the area. No reports of MIDV as an animal pathogen were documented until it was isolated from the spleen of a horse in Zimbabwe that died of symptoms clinically similar to African horse sickness (AHS) (Attoui *et al.*, 2007). High prevalence's of both SINV and MIDV on the Highveld, suggests a relatively high percentage of infection and virus activity in animals (Dickinson *et al.*, 1965).

To determine whether *Alphavirus* infection may be associated with disease in equine populations in southern Africa, horses displaying unexplained neurological disease or fevers were screened with *Alphavirus* family-specific RT-PCR.

4.2. MATERIALS AND METHODS

Please refer to 2.2.1. for clinical sample collection and 2.2.2. for sample preparation and RNA extraction.

4.2.1. Diagnostic *Alphavirus* RT-PCR Screening

An *Alphavirus* family-specific RT-PCR targeting the nsP4 gene was used to screen clinical specimens using degenerate primers Alpha1+ and Alpha 1- (Sanchez-Seco *et al.*, 2001) (Table 4.1) with the Titan One Tube RT-PCR kit (Roche, Mannheim, Germany) according to manufacturers' instructions. Each reaction contained 10µl

template RNA and 0.8µM of each primer in a final volume of 50µl. Cycling began with 50°C for 30 min, 94°C for 2 min and 35 cycles of 94°C for 30s, 52°C for 1 min and 68°C for 30s with a final extension of 68°C for 7 min. The Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) was used in a nested PCR, according to manufacturers' instructions. Each PCR reaction contained 2µl of first round template and 0.8µM of each primer (Alpha2+ and Alpha2-) (Sanchez-Seco *et al.*, 2001) in a final volume of 50µl. Cycling commenced at 94°C for 2 min and 35 cycles of 94°C for 30s, 52°C for 1 min and 72°C for 15s with a final elongation step of 72°C for 5min. Positive RT-PCR products were purified as described in 2.2.8, cloned as described in 4.2.3, confirmed by sequencing and phylogenetically analysed as described 2.2.9. To confirm results, each positive specimen was re-extracted from starting material (serum/plasma/brain tissue) and re-amplified with the diagnostic nsP4 PCR, purified, cloned and sequenced as described above.

4.2.2. *Alphavirus* E-protein Amplification

E-protein RT-PCR was carried out on SINV and MIDV positive specimens. Complementary DNA (cDNA) of each positive specimen was made as described in 3.2.4.2, followed by SINV-specific and MIDV-specific E-protein PCR's. PCR products were purified, confirmed by sequencing and phylogenetically analysed as described in 2.2.8 and 2.2.9.

4.2.2.1. *SINV E-protein RT-PCR*

The Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) was used in a first round PCR using 0.8µM of each first round primer SIN8136EF and A2 (Lundstrom & Pfeffer, 2010) (Table 4.1), 5µl Q-solution (Qiagen, Valencia, CA) and 5µl cDNA in a final volume of 50µl. PCR amplification began at 94°C for 2 min and 40 cycles of 94°C for 10s, 52°C for 30s and 72°C for 1 min with a final elongation step of 72°C for 7 min. A semi-nested reaction was carried out using 5µl of first round product and primers SIN8136EF and SIN8787ER (Table 4.1) using the Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) according to manufacturers' instructions. Cycling commenced at 94°C for 2 min and 10 cycles of 94°C for 30s, 52°C for 30s and 72°C for 1min; 25 cycles of 94°C for 30s, 52°C for 30s and 72°C for 1min+10s/cycle with a final elongation step of 72°C for 7 min.

4.2.2.2. *MIDV E-protein RT-PCR*

Briefly, 0.8µM of each primer MID10475E+ and MID11045E- (Table 4.1) and 5ul cDNA was added in a final volume of 50µl using the Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany) in a first round reaction. Cycling began at 94°C for 2 min and 40 cycles of 94°C for 30s, 48°C for 45s and 72°C for 1 min with a final extension of 72°C for 7min. A nested PCR, using the Expand High Fidelity^{PLUS} PCR system (Roche, Mannheim, Germany), was carried out using 2µl of the first round template, 5µl Q-solution (Qiagen, Valencia, CA) and primers MID10543EN+ and MID10911EN-, according to the manufacturer's recommendation's. Cycling commenced at 94°C for 2 min and 40 cycles of 94°C for 30s, 50°C for 45s and 72°C for 1 min with a final elongation step of 72°C for 7 min.

4.2.3. Cloning

4.2.3.1. *Luria broth (LB) plates*

LB plates were prepared by mixing 5g Bacto-tryptone (Merck, Darmstadt, Germany) 2.5g yeast extract (Merck, Darmstadt, Germany), 5g NaCl (Merck, Darmstadt, Germany) and 7.5g agar (Merck, Darmstadt, Germany) up to 500ml with distilled water. Agar was autoclaved and once cooled, supplemented with 1µl/ml ampicillin (Lucigen, Middleton, WI). Plates were stored at 4°C until use.

4.2.3.2. *Cloning*

Positive nsP4 PCR products were cloned using the CloneJet PCR cloning kit (Fermentas, Canada, ON) in combination with *E. cloni* 10G chemically competent cells (Lucigen, Middleton, WI) according to manufacturers' recommendations.

4.2.3.3. *Colony PCR*

Recombinant clones were screened with the Expand High Fidelity^{PLUS} system PCR kit (Roche, Mannheim, Germany) using 0.3µM of each primer pJet-F and pJet-R (Table 4.2) and 1µl recombinant bacteria in a total volume of 50µl according to suppliers instructions. PCR cycling commenced at 94°C for 2 min and 35 cycles of 94°C for 30, 55°C for 30s and 72°C for 1min with a final extension of 72°C for 7 min.

Table 4.1: Primers used to amplify clinical specimens

PCR	Primer Name	Orientation	Sequence (5' – 3')	Primer Tm (°C)	PCR Tm (°C)	Target virus	Target gene	Fragment size (nt)	Genome position	Reference
First	Alpha+1	Sense	GAY GCI TAY YTI GAY ATG GTI GAI GG	-	52	*Alphavirus* family-specific	nsP4	481	6216 - 6697*	Sanchez-Seco *et al*, 2001
	Alpha -1	Antisense	KYT CYT CIG TRT GYT TIG TIC CIG G	-						
Nested	Alpha+2	Sense	GIA AYT GYA AYG TIA CIC ARA TG	-	52			195	6394 - 6589*	
	Alpha -2	Antisense	GCR AAI ARI GCI GCI GCY TYI GGI CC	-						
First	SIN8136EF	Sense	TCG TCA GCA TAC GAC ATG GAG	54	52	SINV	E2	1328	8136 – 9464*	This study
	A2	Antisense	TGG GCA ACA GGG ACC ATG CA	56						Lundstrom & Pfeffer, 2010
Semi-nested	SIN8787ER	Antisense	GTA TCC AAA CTG GGC GGA AGT	54	52			671	8136 – 8807*	This study
First	MID10475E+	Sense	GGT GCA CGT TCC ATA TAC CC	54	48	MIDV	E1	589	10475 – 11064#	This study
	MID1104E5-	Antisense	TCC CAA TAG CAA TCA CCA CA	50						
Nested	MID10543EN+	Sense	TGA ACC ACA AGG CTC CTT TC	52	50			387	10543 – 10930#	
	MID10911EN-	Antisense	CAC TTT GCT GTG CAA GTG GT	52						

***** Genome positions for SINV/*Alphavirus* primers are based on strain SA-AR86 (accession number U38305); **#** Primer positions for MIDV are based on strain MIDV857 (accession number EF536323)

Table 4.2: CloneJET PCR primer set

Primer name	Orientation	Sequence (5' – 3')	Reference
pJET-f	Sense	CGA CTC ACT ATA GGG AGA GCG GC	Fermentas, Canada, ON
pJet-R	Antisense	AAG AAC ATC GAT TTT CCA TGG CAG	Fermentas, Canada, ON

4.3. RESULTS

4.3.1. Specimen Screening and Disease Description

Of the 261 clinical specimens collected between January 2008 to December 2010; 79 in 2008, 47 in 2009 and 141 in 2010, a total of 17 *Alphavirus* RT-PCR positive cases were identified by the Zoonosis unit, Department of Medical Virology, University of Pretoria.

4.3.1.1. *Sindbis virus (SINV)*

Sequence confirmation of the family-specific nsP4 PCR products identified SINV in 6/17 *Alphavirus* positive cases; 3/6 from plasma, 1/6 from serum and 2/6 from brain tissue (Table 4.5). Signs recorded for single SINV infections (n = 2) included fever and mild neurological disease (lame hind limbs) for both animals; additional signs included mild colic and pale mucous membranes. EEV and AHS co-infections were identified by RT-PCR in 2 cases, both of which made full recoveries. SINV-EEV co-infection resulted in fever only; while SINV-AHSV co-infection resulted in a febrile disease and jaundice. AHSV was isolated from the plasma of this horse; however, the AHSV vaccine had been administered to this animal prior to it falling ill. The only horses with severe neurological disease (n = 2/6) had West Nile virus (WNV) co-infections and both horses were euthanized for humane reasons (Table 4.3, Appendix 3). E-protein amplification for SINV cases was unsuccessful. Horse ages ranged between 2 to 14 years (Table 4.5). No SINV infections were detected in horses during 2010.

4.3.1.2. *Middelburg virus (MIDV)*

MIDV was identified in 11/17 *Alphavirus* positive cases. Fever occurred in 3/11 animals; while neurological symptoms were identified in 8/11 cases (Table 4.4, Appendix 3). Two horses succumbed to the disease (either died or were euthanized) after displaying neurological symptoms of ataxia, dog-sitting, partial paralysis, lateral recumbency and paddling, similar to WNV cases (Table 4.4, Appendix 3). Co-infections detected by RT-PCR included EEV and Shuni virus (SHUV); however these animals recovered (Table 4.5). E-protein amplification was successful for samples SAE68/09, SAE25/10, SAE29/10 and SAE31/10 (Figure 4.4). Horse ages ranged from 6 months to 20 years of age. No MIDV infections were identified in 2008. In 2009 both MIDV and SINV were detected, while 2010 positive cases were made up of only MIDV infections.

4.3.1.2.1. *MIDV outbreak in the Underberg region of the Southern Drakensberg*

A draft farm located in the Underberg region of the Southern Drakensberg in the Kwa-Zulu Natal Province experienced an outbreak of unknown aetiology during the months of March and April of 2010. The outbreak progressed from the wetter low lying region of the farm consisting of vlei's and dams to the higher lying northern area of the farm. Due to the high altitude and cool temperatures in this region, cases of AHSV and EEV had not been documented up till April of 2010. In total, 5 groups of animals were involved in the outbreak however, only samples from the group at the northern end of the farm were submitted to the Medical Virology laboratory for testing. This group consisted of approximately twenty 18-month-old colts, of which only 4 plasma samples were received. Two were RT-PCR positive for MIDV (Table 4.4, Appendix 3), and one horse succumbed to the illness after displaying ataxia, muscle twitching, increased heart and respiratory rates and fever. *Alphavirus* RT-PCR on the brain tissue of this horse was negative. The other MIDV case displayed acute fever (39.9°C) and recovered. The fourth horse displayed only fever and was negative for all viruses. The syndrome displayed by these horses had never before been documented in the region according to the veterinarian and animal caretakers and was likened to "three-day-stiffness" syndrome which is a viral disease usually seen in cattle and water buffalo (Nandi & Negi, 1999). Other arboviruses identified in this outbreak included WNV in a horse with fever (39.1°C), stiffness and ataxia.

4.3.2. Seasonality and Distribution

4.3.2.1. *SINV*

SINV cases were identified in the late summer - autumn between the months of March and May; the rainy season in the central and northern parts of SA (Figure 4.1). SINV-WNV co-infections were detected in April and early May. Although widely distributed throughout SA, SINV infections predominated in the Gauteng province (Figure 4.2).

4.3.2.2. *MIDV*

MIDV cases were identified between March and June (Figure 4.1). The 2010 season started earlier than in 2009 and tapered off in May. MIDV infections were widely dispersed throughout SA (Figure 4.2).

Table 4.5: Summary of symptoms, ages and co-infections of *Alphavirus* positive specimens identified from 2008 - 2010

Clinical detail	**SINV**	**SINV-WNV**	**SINV-EEV**	**SINV-AHSV**	**MIDV**	**MID-EEV**	**MIDV-SHUV**
Total number PCR positives	**N = 2**	**N = 2**	**N = 1**	**N=1**	**N = 9**	**N = 1**	**N = 1**
Type specimens							
Serum	1						
Plasma	1		1	1	9	1	1
Brain		2					
Symptoms							
Fever	2		1	1	7	1	1
Collapse		1					
Muscle twitching					2		
Lame hind legs	2	2			2		
Ataxia		1			5		
Recumbency		2			1	1	
In coordination					3		
Dog sitting		1			1		
Paddling					1		
Weak facial reflexes		1					
Jaundice/icterus				1	2		
Poor tail tone		1					
Abdominal pain/colic	1						
Poor appetite							1
Perennial laxity		1					
Diahorrea					1		
Died	0	2	0	0	2	0	0
Age							
< 1					1		
1 - 10	2	1	1	1	6	1	1
10 >		1			2		

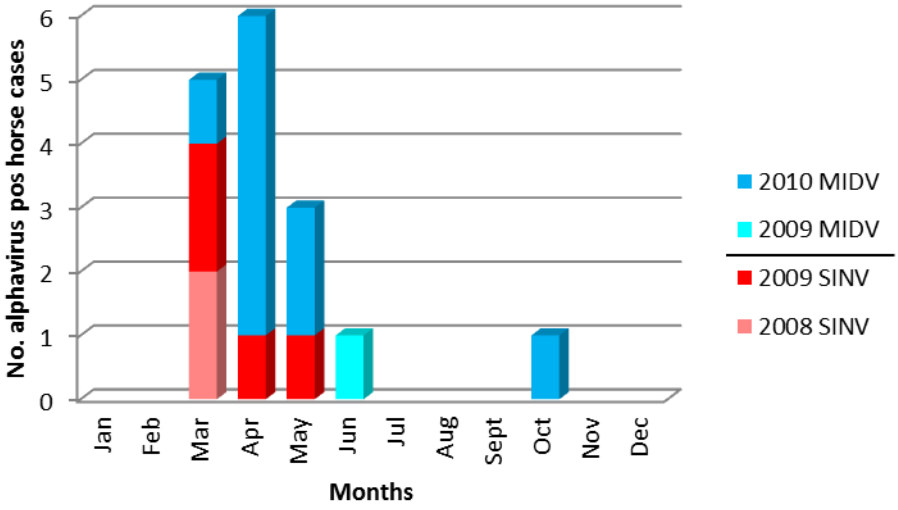

Figure 4.1: ***Alphavirus*** **seasonality in South Africa**. Viruses and years are indicated in the legend on the right hand side of the curve. SINV indicated in shades of red, MIDV indicated in shades of blue.

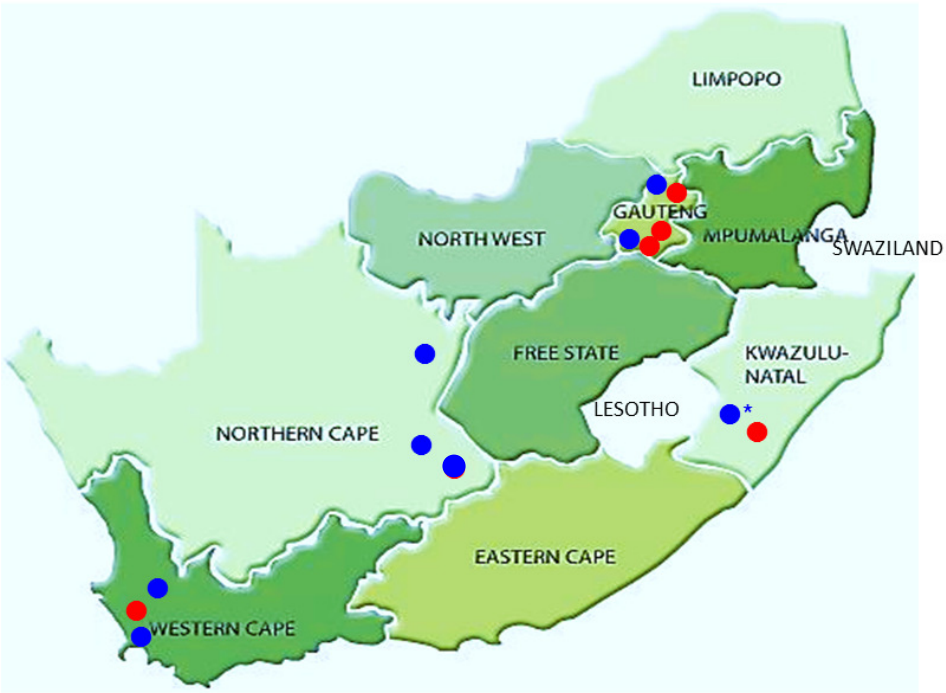

Figure 4.2: **Geographic distribution of *Alphavirus* positive cases in South Africa. SINV (indicated by red dots)**: Gauteng (Midrand, Vereeneging, Boschkop), Kwa-Zulu Natal (Pietermartizburg) & the Western Cape (Tulbagh); **MIDV (indicated by blue dots)**: Gauteng (Bronkhorstspruit, Tarlton and Pretoria), Northern Cape (Colesburg and Kimberly), Kwa-Zulu Natal (Underberg)* and the Western Cape (Tulbagh and Paarl). The asterisks* indicates the area where the MIDV outbreak in horses occurred (2010).

4.3.3. Phylogenetic Analysis

4.3.3.1. *Diagnostic nsP4 PCR*

All positive *Alphavirus* nsP4 PCR samples were re-extracted and re-amplified using the nsP4 diagnostic RT-PCR assay (Sanchez-Seco *et al.*, 2001) and confirmed through sequencing.

4.3.3.1.1. *SINV strains*

SINV strains identified in horses during this study cluster with one another and grouped closely with SA-AR86 and YN78448, strains previously isolated from febrile patients in SA and China (Liang *et al.*, 2000; Simpson *et al.*, 1996), with 0% - 0.7% nucleotide divergence (Figure 4.3). Five clinical strains were identical to one another in this region; three strains clustered geographically, being identified in horses from the Gauteng province; SAE93/08 and SAE110/08 from Midrand and SAE55/09 from Pretoria. Strains SAE30/09 and SAE31/09 which were also identical, originated from Pietermaritzburg in Kwa-Zulu Natal and Tulbagh in the Western Cape respectively. SAE40/09 was the only strain to form a separate branch and was identified in a horse from Vereeneging, Gauteng, that had a WNV co-infection (Figure 4.3). This strain showed a 0.7% nucleotide divergence to the remaining clinical strains as well as to SAAR86 and YN78448; while being 2% different to strain Girdwood SA (Figure 4.3). Clinical SINV strains were more closely related to Ockelbo virus (6.8% - 7.5% divergence), a SINV-like strain isolated in Sweden (Shirako *et al.*, 1991), than to the prototype SINV (8.2% - 8.8% divergence). This is supported by previous studies (Simpson *et al.*, 1996). The average nucleotide divergence between clinical SINV strains was 0.2%.

4.3.3.1.2. *MIDV strains*

MIDV strains identified in this study formed two separate clusters (Figure 4.3) and had an average divergence of 2.1% and 1.7% on a nucleotide and amino acid level respectively. SAE68/09, SAE29/10 and SAE31/10 (cluster 2) MIDV strains from 2009 and 2010 clustered separately (Figure 4.3). Strain SAE68/09, from Bronkhorstspruit in Gauteng, formed its own branch and differed by 1.4% to SAE29/10 and SAE31/10 and between 4.1% - 4.8% to cluster 1 clinical strains. SAE29/10 and SAE31/10 are strains identified in the MIDV outbreak in the Underberg and are identical. Nucleotide divergence between cluster 2 strains and the

previously sequenced AR749 prototype MIDV strain isolated in SA (Kokernot *et al.*, 1957) and strain MIDV857 isolated in Zimbabwe (Attoui *et al.*, 2007), ranged 1.4% - 4.8%.

Cluster 1 strains were more closely related to one another and to strain MIDV857 from Zimbabwe (0.7% – 1.4%). In this cluster, SAE64/10 and SAE129/10 formed their own branches and differed from one another by 1.4% while showing only a 0.7% difference to the remaining strains (Figure 4.3). These strains were identified in horses from Paarl and Pretoria, respectively. Six MIDV strains were identical to one another in this region. Geographical clustering was seen with strains SAE25/10 and SAE33/10 from Colesburg in the Northern Cape and strains SAE47/10 and SAE69/10 from Tulbagh in the Western Cape. However, SAE38/10 and SAE41/10, which were also identical, were identified in horses from Kimberly and Gauteng respectively. Comparisons between cluster 1 clinical strains and MIDV857 ranged from 0.7% – 1.4% and differed by 3.4% – 4.1% to MIDV AR749.

4.3.3.2. *MIDV E-protein phylogenetic analysis*

Phylogenetic analysis of a region within the E-protein of 4 MIDV strains that could be amplified in this study showed a slight divergence to isolates MIDV857 and the prototype strain AR749 (Figure 4.4). As with the nsP4 analysis, strains SAE29/10 and SAE31/10 were identical to one another. An average nucleotide and amino acid divergence of 1.1% and 0.09% was calculated between clinical strains identified in this study and by 1.4% - 2% to strains AR749 and MIDV857 on the nucleotide level.

Figure 4.3: Maximum-likelihood comparison of a partial section of the nsP4 region amplified for clinical *Alphavirus* positive specimens with representative old and new world mosquito-borne *Alphavirus* sequences (see next page). The tree was constructed using the HKY codon position substitution model using PhyML (Guindon *et al.*, 2009) and drawn to scale with the bar indicating 0.2 nucleotide substitutions per site. Estimates were based on bootstrap values carried out with 100 replicates. The tree was midpoint rooted. Clinical strains identified in this study are indicated: ■ 2008, ♦ 2009 and ✶2010. The SINV and MIDV groups are indicted as well as the two major groupings of the alphaviruses: the Old and New World viruses. Accession numbers and origin of reference strains can be viewed in Table 4.6 in Appendix 3.

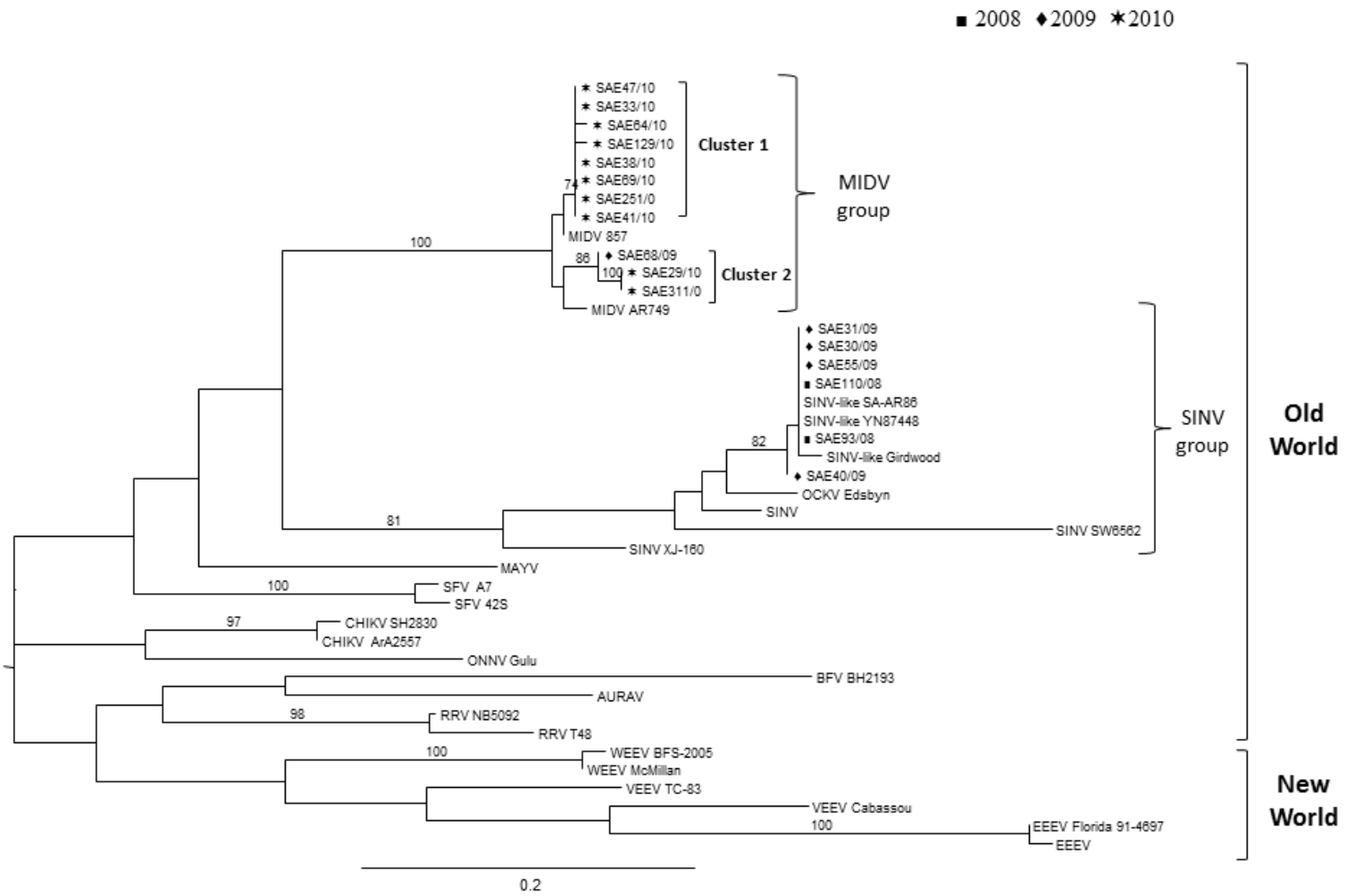

Figure 4.3: *Alphavirus* cases identified in horses in South Africa over three consecutive years. See previous page for caption.

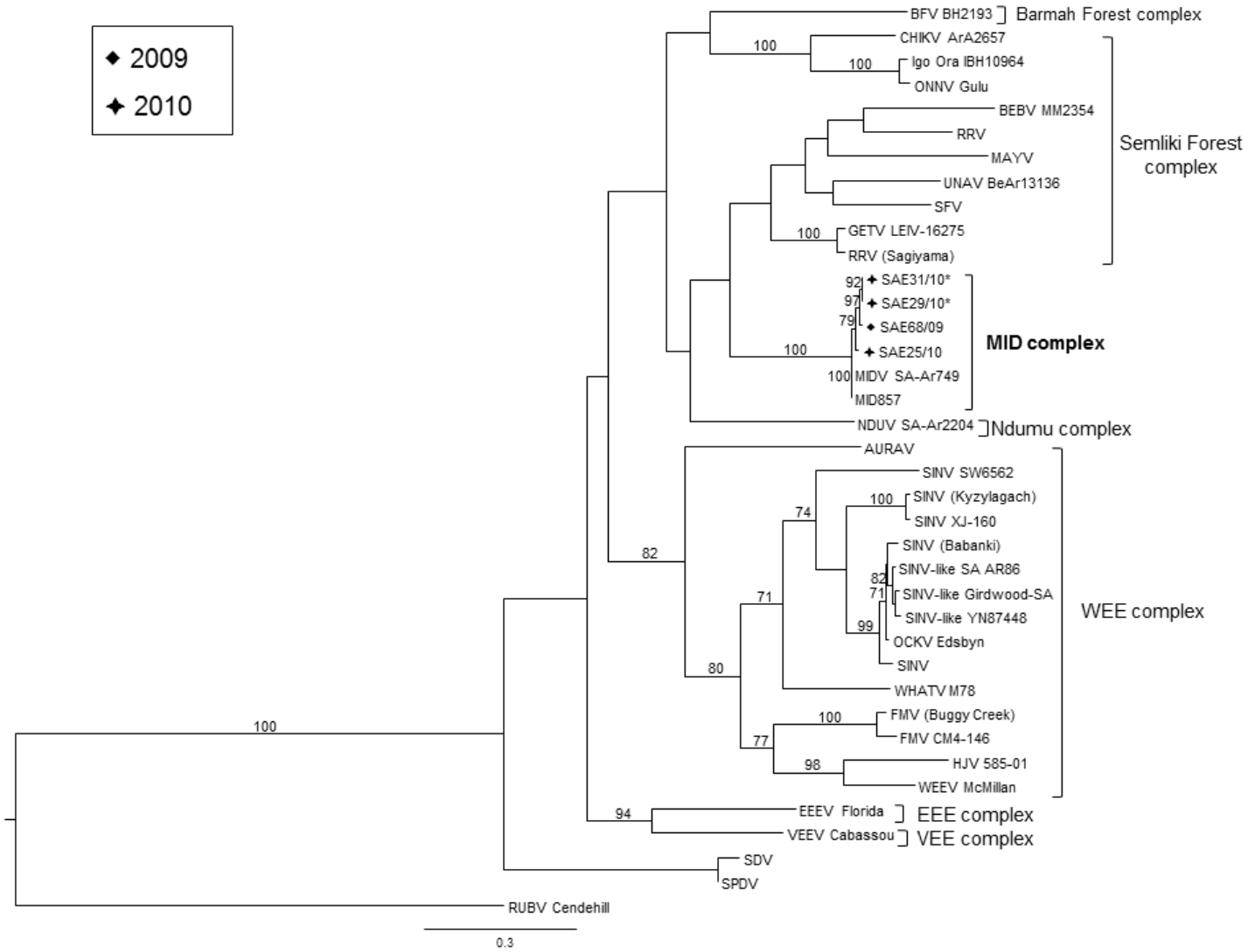

Figure 4.4: Phylogenetic relationship between MIDV cases identified in horses in SA, using a partial region of the E-protein. See following page for caption.

Figure 4.4: Maximum-likelihood comparison of a partial section of the E-protein for strain MIDV strains identified in this study. See previous page. The tree was constructed as described for Figure 4.3. The scale bar indicates 0.3 nucleotide substitutions per site and the tree was rooted using RUBV strain Cendehill. Clinical strains identified in this study are indicated: ♦ 2009 and ✦ 2010. The seven *Alphavirus* sero-complexes are indicated on the right hand side of the phylogram, the MID complex has been bolded. Accession numbers and origin of reference strains can be viewed in Table 4.6 of Appendix 3. Accession numbers for clinical samples amplified can be found in Table 4.7 in Appendix 3.

4.4. DISCUSSION

Identification of disease causing agents is key to establishing protective measures in terms of handling infected animals and preventing the spread of disease. Annually, a high percentage of unexplained neurological disease and fever in horses may go mis- and/or undiagnosed because a considerable number of potential pathogens are excluded from routine diagnostic testing. In Africa these diseases may often be disregarded as African horse sickness (AHS) or equine encephalosis (EE), the most common viral pathogens of horses (MacLachlan & Guthrie, 2010).

Our results show that the alphaviruses should be considered in differential diagnosis of unexplained fevers and neurological disease. Over three consecutive seasons, 17 *Alphavirus* RT-PCR positive cases were confirmed by DNA sequencing; 11/17 of which were MIDV and 6/17 SINV (Figure 4.3). RT-PCR is limited to detect positive cases during the viraemic period (~5 days), therefore, serological assays such as IgM ELISAs and serum neutralization should be developed in order to detect and confirm recent infections that may have passed the viraemic "window-period".

Most cases were identified during late summer - autumn, which is the rainfall season in the central parts of SA. MIDV and SINV virus activity in SA peaked in March/April and tapered off in May. Few positive cases extended into the colder months on the Highveld, possibly due to unusually high rainfall and warm winters in the last two years, enabling vector persistence and extension of the arbovirus season (Figure 4.1). Both SINV and MIDV cases were widely distributed throughout SA (Figure 4.2). Previous serological studies have suggested the highest distribution to be on the inland plateau (Highveld, Karoo and Northern Cape) due to the presence of their mosquito vectors in these areas (Kokernot *et al.*, 1960; McIntosh, 1986).

Severe neurological disease in SINV cases was only reported when WNV co-infections were identified, both viruses being detected in the brain tissue of these horses (SAE40/09 and SAE55/09) (Table 4.3, Appendix 3). This is an interesting finding, as SINV has only been recorded as a febrile or arthralgic disease (Malherbe *et al.*, 1963). WNV-associated encephalitis may disrupt the blood-brain barrier (BBB) which induces pleiocytosis, microglial activation and inflammation and eventual neuron death (Verma *et al.*, 2009). Although BBB disruption plays a major role in the eventual production of encephalitis in a patient, another route of viral dissemination into the central nervous system (CNS) may be via cell-free virus crossing the BBB without compromising its integrity. This occurs from the infection of the microvascular endothelial cells of the BBB (Persidsky *et al.*, 2006; Verma *et al.*, 2009). In addition, activation of tight junction proteins and cell adhesion molecules may help in trafficking infected immune cells into the CNS, leading to the development of encephalitis. This could possibly explain why SINV was found in the CNS tissue of horses with SINV-WNV co-infections and suggests that SINV requires the presence of a neurotropic virus such as WNV in order to cross the BBB. Horses with single SINV infections or SINV-EEV co-infections were able to recover after suffering a febrile illness, which is consistent with human disease (McIntosh *et al.*, 1976; McIntosh *et al.*, 1964). Both horses with single SINV infections also experienced hind-limb lameness, suggesting that neurological disease may occur, although these animals recovered (Table 4.3, Appendix 3; Table 4.5).

While neurological disease was unexpected in horses with single SINV infections, it is well documented that SINV causes encephalomyelitis in mice (Griffin, 1998). This is dependent on virus strain and mouse age; causing fatal disease in 1-day-old mice but being avirulent in weanling mice (Reinarz *et al.*, 1971; Taylor *et al.*, 1955). Whereas infection with most SINV isolates leads to avirulent disease in older mice, the SA SIN-like virus strain SA-AR86, is virulent in both adult and neonate mice (Russell *et al.*, 1989). When compared to the avirulent AR339 strain from Egypt, a 5.57% nucleotide divergence was observed, characterised by a number of insertions in the 3' half of the nsP3 gene. This region is not well conserved among the alphaviruses (Strauss & Strauss, 1994). Additionally, a 54 nt deletion was also found in the nsP3 gene. In comparison, the avirulent Girdwood strain did not have the 54 nt deletion, but did contain the remaining insertions. Intracerebral inoculation of Girdwood, SA-AR86 and Ockelbo virus in 3-6 week old CID-1 mice resulted in neurological disease developing only in those infected with SA-AR86 (Simpson *et al.*, 1996), as demonstrated previously by Russel *et al* (Russell *et al.*, 1989). Further investigations are

necessary to determine whether any variation exists between circulating SA SINV strains which could possibly explain the range of disease presentation in horses. SINV strains identified in this study were most closely related to strain SA-AR86, which has also been isolated in China (strain YN87448) (Zhou *et al.*, 1999), suggesting a wide distribution for this strain. Overall, clinical SINV strains were more closely related to previously identified SINV-like strains from SA and Ockelbo virus, a SINV-like virus discovered in Sweden that is antigenically related to SINV and has been serologically associated with human disease (Niklasson *et al.*, 1984); than to SINV strains isolated in China and Australasia (Figure 4.3) (Simpson *et al.*, 1996). This is in agreement with other studies (Simpson *et al.*, 1996). Horses of all ages were affected; however factors such as virus strain variation, vector load (and therefore virus titre) may contribute to disease progression. Previous exposure to these viruses may also account for differences in disease severity in animals; however, it is unclear whether previous exposure leads to sterilising immunity.

MIDV, although primarily neurological, (Table 4.4, Appendix 3), resulted in only 2 fatalities. Horses with EEV or SHUV co-infections were able to recover from the illness, suggesting that co-infections did not seem to worsen the disease presentation. This is the only description of MIDV-associated neurological disease in horses. The single other description of MIDV as a pathogen was reported in a study by Attoui and others (Attoui *et al.*, 2007) which characterized the genome sequence of a MIDV strain isolated from the spleen of a horse displaying clinical symptoms similar to AHSV infection. The clinical description of this case was, however, limited.

MIDV was identified as the aetiological agent in an outbreak on a draft farm in the Underberg region of Kwa-Zulu Natal, strains SAE29/10 and SAE31/10 (Figure 4.3). Symptoms displayed by these horses (ataxia, fever, stiffness and refusal to move) had never before been documented by the veterinarian and animal caretakers, who likened the disease to "three-day-stiffness" or bovine ephemeral fever (BEF). BEF is a short-lived, arthropod-borne viral disease of cattle and water buffalo characterised by fever, stiffness and unwillingness to move. However, recovery is common (Nandi & Negi, 1999). WNV infections were also detected in this outbreak, emphasising the co-circulation of alpha- and flaviviruses in SA and the need to test for both.

E-protein phylogenetic analysis (Figure 4.4) of 4 MIDV clinical strains showed differences to MIDV857, isolated in Zimbabwe, and MIDV AR749 the prototype strain isolated from *Aedes*

mosquitos in SA (Kokernot *et al.*, 1957). All MIDV-infected horses displayed a degree of neurological disease; however, mortalities (2/11) were only recorded in horses displaying symptoms similar to severe WNV disease. Previous exposure and other host factors may play a role in disease severity.

Serological surveys done on the Highveld in 1961 reported a significant exposure of horses to both MIDV and SINV (McIntosh *et al.*, 1962). It is unclear whether cross-protection occurs or whether previous exposure leads to sterilising immunity or increases disease severity. This seems unlikely when evaluating the genetic differences in the MIDV envelope proteins. Age may play a role, however, as with SINV, no age-associated pattern was observed in this study.

Although *C. univittatus* and *A. caballus* are weakly anthropophilic (Jupp, 1973) infected mosquitoes have the potential to cause large outbreaks, as observed in the 1974 outbreak in the Karoo and Northern Cape Province (Jupp *et al.*, 1986a). This poses a potential threat to a wide range of domesticated and wild animals, livestock and humans that may be infected during conditions favouring mosquito vector breeding (McIntosh, 1986). As the diseases mentioned here are transmitted by the same mosquito vector (McIntosh, 1986), *Aedes* (MIDV and WSLV) and *Culex* (WNV, SINV and SHUV), inclusion of these viruses in diagnostic screening would be beneficial.

4.5. CONCLUSION

Detection of an increase in virus activity using horses as sentinel animals could potentially prevent epizootic outbreaks caused by alphaviruses in humans and other animals in Africa. While both diseases were always thought of as benign, this study suggests that severe neurological signs, similar to WNV disease, can develop as a result of MIDV infection. Further investigation is needed to determine whether any association exists between human and animal disease in this region. Previously, cases in animals may have been under diagnosed or missed until now which may also reflect the situation in humans.

CHAPTER 5
CONCLUDING REMARKS

Arboviral diseases are among the most important of the emerging infectious diseases worldwide (Gubler, 2001). In SA, important arboviruses include members from the *Flaviviridae* (WNV and WSLV) and *Togaviridae* (SINV and MIDV). Although having been responsible for large outbreaks and epizootics (WNV and SINV), disease in livestock and may have been responsible for zoonotic infection (WSLV) (van der Riet *et al.*, 1985), the epidemiology of these viruses as well as their disease description as animal pathogens remains poorly described in SA.

The primary objectives of this study were to determine whether these arboviruses were being missed due to a lack of surveillance and knowledge of disease presentation in dead-end hosts such as horses in SA, and if detected, whether or not they contributed to unexplained neurological disease or fever in these animals.

In this study, 261 samples (serum/plasma and post-mortem CNS tissue) from horses displaying unexplained neurological disease or fever were screened over three consecutive years (January 2008 – December 2010) using *Alphavirus* and *Flavivirus* family-specific PCR assays. Additionally, WNV infections were detected by FRET probe analysis which distinguishes between the WNV lineages (Zaayman *et al.*, 2009), as well as WNV IgM and serum neutralizations. These screening methods were shown to be effective in detecting *Flavivirus* (and WNV-specific) and *Alphavirus* cases in horses with the above mentioned symptoms. The seasonal distribution of arbovirus infections could be well defined, particularly in the case of WNV, SINV and MIDV. WSLV infections did however fall within the early part of the arbovirus season in SA. There was no correlation between age and arbovirus disease in horses

Identification of these positive samples facilitated the epidemiological investigation of these viruses in a current day setting as well as describe the disease presentation of WNV, WSLV, SINV and MIDV infections in these dead-end hosts. WNV and WSLV infections were primarily neurological in nature, establishing our hypothesis that these flaviviruses cause neurological disease in equines in SA, and confirms that L2 WNV strains are pathogenic in horses. Co-infections of AHSV and SINV were detected in a few WNV cases, however this

was expected as SINV shares the same transmission vector (*Culex* species mosquitoes) while AHSV is usually transmitted at the same time of year as WNV, as the rainy season favours the breeding of the AHSV *Culicoides* transmission vector. The mortality rate for WNV infections was calculated at 44%, which in concurrence with studies described for L1 infections in the USA and Italy (Cantile *et al.*, 2000; Ostlund *et al.*, 2001; Ward *et al.*, 2006). This study has also shown that milder clinical disease characterized by fever, which was detected in a few cases, can also be displayed in L2 WNV positive horses. The detection of L1 WNV in South Africa, a lineage which had previously been found exclusively in Northern hemisphere shows the sensitivity of horses as sentinels of WNV and other emerging zoonoses (Hubalek *et al.*, 1998). This study emphasises the potential of WNV to spread to new geographic localities by infected migratory birds and highlights the need for WNV and MIDV vaccinations for horses in southern Africa.

WSLV was identified in 2 cases of severe neurological disease. This is the first description of WSLV-associated disease in horses in SA. These cases highlight the lack of knowledge of the range of dead-end animals that may be affected by these viruses as well as the need for this kind of surveillance program. Genome characterization of an encephalitic WSLV strain showed changes in the structural determining amino acids in a number of non-structural proteins, required for efficient *Flavivirus* replication. This may affect the virulence of this strain and suggests that, like WNV, a number of highly and less virulent viruses are in circulation in SA. However, further investigation is required to establish this.

Screening for co-circulating alphaviruses identified a number of SINV and MIDV cases in horses. Although described as primarily causing fever and arthralgia in humans, SINV symptoms in horses included fever as well as mild neurological disease; while MIDV which was postulated as non-pathogenic in humans and animals presented as a mainly neurological disease in horses. Most MIDV-infected horses were able to recover from the disease even when co-infections with EEV and SHUV were present. Severe neurological disease in SINV infections was only detected when co-infections with WNV were present, and both viruses were detected in the brain tissue of the horses. This highlights that SINV requires a neurotropic virus to breach the BBB in order to cause encephalitis in the host. Additional investigation into the genome sequences of circulating SINV and MIDV cases is SA, may

elucidate whether pathogenic differences exist for each of these viruses and ultimately then how they will present as disease in dead-end hosts.

Additionally the disease description of naturally acquired L2 WNV in horses as well as WSLV and the *Alphavirus* infections (SINV and MIDV) could be accurately described. This leads to an increased awareness of the disease potential of this virus by both veterinarians and horse owners. The value of using horses as sentinels was revealed in the timely diagnosis of L2 WNV in a horse that was treated in the correct manner and managed to survive the disease. Increased knowledge and awareness of when this disease may occur could possibly decrease the amount of WNV fatalities as well as increase the correct diagnosis of this disease in SA, leading to further advantages such as virus activity "hot spots".

These results indicate that arbovirus (WNV, WSLV, MIDV and SINV) activity is being missed in horses in SA due to lack of surveillance rather than infections being non-existent. It also proves that using horses as sentinels for these diseases is: 1) feasible due to their susceptibility to the above mentioned viruses and 2) essential in outbreak identification and prevention (Ward & Scheurmann, 2008) as WNV outbreaks may occur after periods of heavy rainfall, even in countries in which the disease in endemic (Jupp, 2001; McIntosh, 1986). Additionally, a better understanding can be gained from this study regarding the clinical disease presentation of each virus in dead-end-hosts such as horses, which could then lead to identification of effective supportive treatment for these animals. Currently no preventative treatment or vaccinations are available for any of these diseases in SA. WSLV infections were detected much less frequently than WNV in these animals.

Both the alpha- and flaviviruses that form the centre of this thesis are excluded from routine diagnostic or differential diagnosis testing for disease characterised by unexplained neurological disease or fever in both humans and animals in SA. Identification of these viruses in horses has created awareness of these pathogens and their disease potential. Active presentation of the results from this study at veterinary conferences as well as publications in veterinary newsletters has been used to create awareness. Veterinarians enquiring about our diagnostic service expanded significantly between 2008 and 2010, increasing the amount of samples received and therefore the number of cases detected each year. This shows a change in mind-set of how SA veterinarians define equine disease, submitting samples for

differential diagnosis of these arboviruses which enables them to make informed decisions of how best to treat infected horses. This increases the prognosis of infected animals substantially.

As the viraemia of *Flavivirus* infections is so short and virus levels low in horses (Bunning *et al.*, 2002; Castillo-Olivares & Wood, 2004), PCR based assays can easily miss virus in the blood of infected animals. Therefore a limitation to this study was that the time of sample collection relative to the disease onset, in many cases was usually after symptoms had started. In these cases, it would be preferential to diagnose cases serologically through the use of IgM ELISAs and serum neutralization methods specific to each virus. This would increase the number of cases detected and more accurately determine virus distribution. A surveillance study such as this has allowed this to document certain disease presentation characteristics in horses and has enabled us to make the veterinarians and horse owners throughout SA aware of this disease.

To conclude:

This study has indicated the need for arbovirus surveillance programs in SA. These arbovirus pathogens have now been identified as potential causes of neurological disease or fever in horses which indicates the need for their inclusion as differential diagnoses of these symptoms. The value of horses as sentinels for arbovirus disease in SA has been confirmed through the rapid identification of L1 WNV for the first time in SA. Ecologically and economically, these viruses have the potential to induce losses in animals in SA such as horses, livestock and wildlife.

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APPENDIX 1

Table 2.4: WNV positive specimens identified in horses in South Africa, 2008

	UP case code	Date received	Location	Specimen	Disease	Outcome	Final Diagnosis	Results of differential diagnosis
WNV RT PCR +ve	**HS101/08**	2008/04/15	Pretoria, Gauteng	Brain	Neurological	Euthanised	WNV L2 PCR +ve, WNV isolate	AHSV PCR-ve, Alpha PCR-ve, WSLV IMP +ve, EEV IMP -ve, EHV IMP-ve, Rabies FAT -ve
	HS123/08	2008/05/08	Midrand, Gauteng	Brain	Neurological	Euthanised	WNV L2 PCR +ve	AHSV PCR –ve, Alpha PCR-ve, EEV sero-ve, EHV sero -ve
	HS125/08	2008/05/26	Pretoria, Gauteng	Brain	Neurological	Euthanised (shot by owner)	WNV L2 PCR +ve	AHSV PCR +ve, AHSV isolate, AHSV IMP +ve
	SAE126/08	2008/03/07	Midrand, Gauteng	Brain	Neurological	Died suddenly	WNV L2 PCR +ve	AHSV PCR +ve, Alpha PCR-ve, AHSV IMP +ve, EEV IMP –ve, WNV neut –ve
	SAE134/08	2008/07/18	Potchefstroom, North West	Serum	Neurological	Recovered	WNV L2 PCR +ve, WNV IgM +ve	Alpha PCR-ve, AHSV sero -ve, EEV sero -ve, EHV-1 weak sero +ve
	SAE139/08	2008/09/02	Tanzania - ***Singita Grumeti Reserves***	Brain	Neurological	Died	WNV L2 PCR +ve	*Trypanosoma brucei* PCR +ve
WNV IgM +ve	**SAE12/07**	2007/04/23	Johannesburg, Gauteng	Serum	Neurological	Recovered	WNV IgM +ve, WNV IgG +ve, WNV neut +ve	AHSV PCR-ve; EEV PCR –ve
	SAE75/08	2008/02/26	Colesburg, Karoo	Plasma	Neurological	uk	WNV IgM +ve, WNV neut +ve	WNV PCR -ve, Flavi PCR -ve, Alpha PCR -ve, EEV PCR -ve, WSLV-HI +ve, Flavi-HI +ve; AHSV sero +ve; EEV sero +ve
	SAE89/08	2008/03/03	Colesburg, Karoo	Plasma	Neurological	Euthanised (after a week on fluid therapy)	WNV IgM +ve, WNV neut +ve	WNV PCR –ve, Flavi PCR-ve, alpha PCR-ve, EEV PCR-ve, WNV HI+ve , AHSV IFA -ve; EEV sero -ve, SIN sero +ve,
	SAE112/08	2008/04/14	Midrand, Gauteng	Plasma	Neurological	Recovered	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR -ve, EEV PCR –ve, WNV-HI +ve, WSLV-HI +ve, AHS IFA weak +ve
	SAE113/08	2008/04/14	Midrand, Gauteng	Plasma, serum	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, AHSV IFA +ve
	SAE156/08	2008/12/09	Gauteng	Serum	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WSLV-HI +ve
	SAE159/08	2008/12/09	Gauteng	Serum	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV HI +ve

Table 2.5: WNV positive specimens identified in horses in South Africa, 2009

	UP case code	Date received	Location	Specimen	Disease	Outcome	Final diagnosis	Results of differential diagnosis
WNV RT PCR +ve	**SAE36/09**	2009/04/03	Midrand, Gauteng	Brain	Neurological	Euthanised	WNV L2 PCR +ve, WNV neut +ve	Alpha PCR -ve
	SAE40/09	2009/04/14	Vereeniging, Gauteng	Brain	Neurological	Euthanised	WNV L2 PCR +ve , SINV PCR +ve	WSLB IMP +ve
	SAE55/09	2009/05/06	Pretoria, Gauteng	Brain and spinal cord	Neurological	Euthanised	WNV L2 PCR +ve , SINV PCR +ve,	EEV PCR -ve, EHV PCR -ve, EHV-1 HI +ve, EHV-4 HI –ve, WNV HI +ve
WNV IgM +ve	**SAE35/09**	2009/03/30	Mooi River, Kwa-Zulu Natal	Plasma	Neurological	Recovered	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, WNV-HI+ve
	SAE54/09	2009/05/04	Midrand, Gauteng	Serum, plasma	Neurological	Recovered	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, WNV-HI +ve
	SAE65/09	2009/05/22	Mooi River, Kwa-Zulu Natal	Serum	Neurological	Unknown	WNV IgM +ve, WNV IgG +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, WSLV-HI +ve

Table 2.6a: WNV positive specimens identified in horses in South Africa by RT-PCR, 2010

	UP case code	Date received	Location	Specimen	Disease	Outcome	Final diagnosis	Results of differential diagnosis
Lineage 2 SA WNV strains	**SAE18/10**	2010/03/15	Midrand, Gauteng	Brain, spinal cord	Neurological	Died	WNV L2 PCR +ve	Alpha PCR -ve, EEV PCR -ve
	SAE22/10	2010/03/23	Midrand, Gauteng	Spinal cord, brain	Neurological	Euthanised	L2 WNV FRET probe +ve	Alpha PCR -ve, EEV PCR-ve, SHUV PCR -ve
	SAE28/10	2010/04/13	Underberg, Kwa-Zulu Natal	Plasma, serum	Neurological	Recovered	L2 WNV FRET probe +ve	Alpha PCR -ve, EEV PCR -ve, SHUV PCR -ve, WNV neut +ve
	SAE51/10	2010/04/26	Pretoria, Gauteng	Brain & spinal cord	Neurological	Euthanised	WNV L2 PCR +ve	Alpha PCR -ve, EEV PCR -ve, SHUV PCR -ve
	SAE81/10	2010/06/10	Pietermaritzburg, Kwa-Zulu Natal	Plasma	Fever	Recovered	WNV L2 PCR +ve	Alpha PCR -ve, EEV PCR -ve, SHUV PCR -ve
Lineage 1 SA WNV strains	**SAE75/10**	2010/06/02	Ceres, Western Cape	Brain	Neurological	Died	WNV L1 PCR +ve,	Alpha PCR -ve, EEV PCR -ve, SHUV PCR -ve, EHV PCR-ve,
	SAE76/10	2010/06/02	Ceres, Western Cape	Brain	Neurological	Died (foetus aborted day before mare died)	WNV L1 FRET probe +ve	Alpha PCR -ve, EEV PCR -ve, SHUV PCR -ve

Table 2.6b: WNV positive specimens identified in horses in South Africa by WNV IgM, 2010

UP case code	Date received	Location	Specimen	Disease	Outcome	Final diagnosis	Results of differential diagnosis
SAE10/10	2010/03/08	Bronkhorstspruit, Gauteng	Serum, brain	Neurological	Euthanised	WNV IgM +ve, WNV IgG +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, SHUV PCR -ve, RVF PCR -ve, Rabies FAT -ve
SAE11/10	2010/03/09	Bethlehem, Free State	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV IgG +ve, WNV neut +ve, EHV PCR +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, SHUV PCR-ve, RVF PCR-ve
SAE12/10	2010/03/12	Hertzogville, Free State	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, EHV PCR-ve
SAE34/10	2010/04/13	Colesburg, Karoo, Northern Cape	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, SHUV PCR-ve
SAE39/10	2010/04/14	Kimberly, Northern Cape	Serum, plasma	Fever	Alive	WNV IgM +ve, WNV neut +ve, SHUV PCR +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve
SAE53/10	2010/05/03	Colesburg, Karoo, Northern Cape	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, SHUV PCR-ve
SAE56/10	2010/05/04	Balmoral, Kwa-Zulu Natal	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, SHUV PCR-ve
SAE84/10	2010/06/23	Bethlehem, Free State	Serum, plasma	Neurological	Alive	WNV IgM +ve, WNV neut +ve	WNV PCR-ve, Flavi PCR-ve, Alpha PCR-ve, EEV PCR-ve, SHUV PCR-ve

Table 2.11a: Strains used to generate maximum-likelihood trees for phylogenetic analysis of WNV strains identified in horses in South Africa (2008 – 2010)

Strain name	Virus	Accession number	Reference
SPU116/89	West Nile virus	EF429197	Botha et al, 2008
SA93/01	West Nile virus	EF429198	Botha et al, 2008
SA381/00	West Nile virus	EF429199	Botha et al, 2008
H442	West Nile virus	EF429200	Botha et al, 2008
Goshawk-Hung/04	West Nile virus	DQ116961	Bakonyi et al, 2006
Sparrowhawk-Hung/05	West Nile virus	EF116943	Erdelyi et al, 2007
Goshawk-Austria-361	West Nile virus	HM015884	Wodak et al, 2011
B956D117B3	West Nile virus	M12294	Castle et al, 1985
B956	West Nile virus	AY532665	Yamshchikov et al, 2004
ArD76104	West Nile virus	DQ318019	Borisevich, et al, unpublished
ArB310/67	West Nile virus	GQ851608	May et al, 2011
ArB3573/82	West Nile virus	DQ318020	Borisevich, et al, unpublished
Sarafend	West Nile virus	AY688948	Chu et al, 2005
AnMg798	West Nile virus	DQ176636	Keller et al, 2006
MgAn-786/6/1995	West Nile virus	AY839589	Nordstrom et al, 2005
IND 804994	West Nile virus	DQ256376	Bondre et al, unpublished
LEIV-Krnd88-190	West Nile virus	AY277251	Lvov et al, 2004
Rabensburg 97-103	West Nile virus	AY765264	Hubalek et al, 1998
Kunjin MRM61C	West Nile virus	D00256	Coia et al, 1988
Goose-Hungary/03	West Nile virus	DQ118127	Bakonyi et al, 2006
Mexico TM171/03	West Nile virus	AY660002	Beasley et al, 2004
NY385/99 CLONE TYP9376	West Nile virus	AY848697	Ding et al, 2005
NY382/99 FLAM	West Nile virus	AF196835	Lanciotti et al, 1999
WNV TX 2002 02	West Nile virus	DQ164205	Davis et al, 2005
NY385_99	West Nile virus	DQ211652	Borisevich et al, 2006
Egypt101	West Nile virus	AF260968	Bowen, unublished
WNV Ast02-2-25	West Nile virus	DQ374653	Lyapina et al, unpublished
Ast02-2-692	West Nile virus	DQ411035	Sadykova et al, unpublished
Ast01-187	West Nile virus	DQ411031	Sadykova et al, unpublished
WNV Italy/98 EQ	West Nile virus	AF404757	Lanciotti et al, 2002
LEIV-Vlg00-27924	West Nile virus	AY278442	Sadykova unpublished
WNFCG	West Nile virus	M12294	Castle et al, 1986
REB-VLG/07-H	West Nile virus	FJ425721	Platonov et al, unpublished
WNV 04-218-CO	West Nile virus	DQ431703	Herring et al, 2007
WNV IS-98 STD	West Nile virus	AF481864	Malkinson et al, 2002
PaH001	West Nile virus	AY268133	Charrel 2003
PaAn001	West Nile virus	AY268132	Charrel 2003
Morocco 96-11	West Nile virus	AY701412	Schuffenecker et al, 2005
WNV RO9750	West Nile virus	AF260969	Savage 1999
KN3829	West Nile virus	AY262283	Charrel, 2003
57-VLG/07-M	West Nile virus	FJ425729	Platanov et al, unpublished
WNV Wengler	West Nile virus	AF196531	Scherret unpublished
IBAN7019	West Nile virus	GQ851607	May et al 2011
JEV JaOAr5982	Japanese encephalitis	M18370	Sumiyoshi et al, 1987

Table2.11b: Accession numbers for WNV strains identified in horses in South Africa (2008 – 2010)

	WNV strain	Diagnostic NS5 region	E-protein	NS5/3'UTR
2008 WNV strains	HS123/08	FJ464376	FJ464381	JN226826
	HS125/08	FJ464377	-	-
	HS101/08	FJ464378	-	-
	SAE126/08	FJ464379	-	-
	SAE134/08	FJ464380	-	-
	SAE139/08	FJ464382	-	-
2009 WNV strains	SAE36/09	-	JN226820	JN226827
	SAE40/09	-	JN226821	JN226828
	SAE55/09	-	JN226822	JN226829
2010 L2 WNV strains	SAE18/10	-	JN226823	JN226830
	SAE22/10	-	JN226824	-
	SAE28/10	-	-	-
	SAE51/10	-	JN226825	JN226831
	SAE81/10	-	-	JN226832
2010 L1 WNV strains	SAE75/10	HQ594467	HQ594469	HQ594470
	SAE76/10	HQ594468	-	-

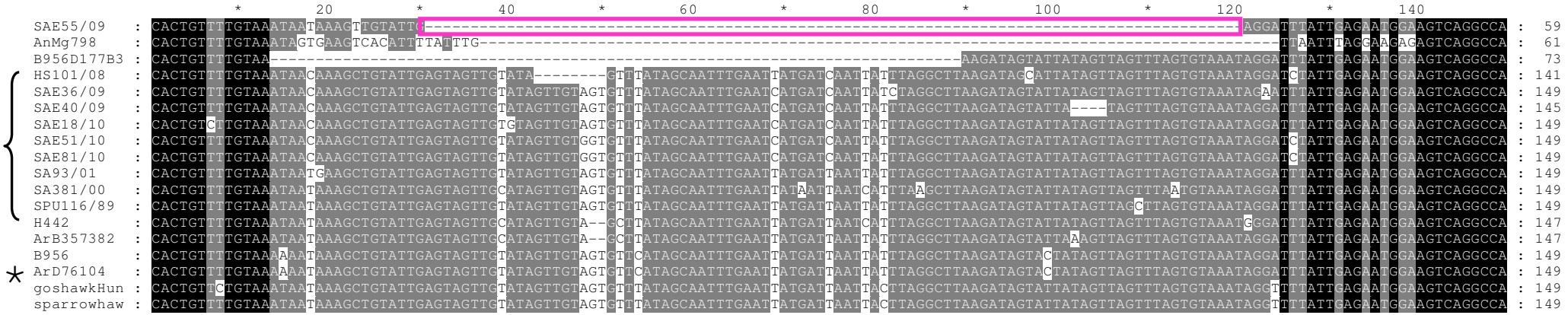

Figure 2.6: A GeneDoc multiple sequence alignment representing the NS5/3' UTR of L2 WNV positive horses identified in South Africa (2008 – 2010) (Nicholas & Nicholas, 1997). WNV strain SAE55/09 identified in a horse stabled in Pretoria is highlighted by the pink rectangle. A large portion of the 3' UTR fragment amplified is deleted in this strain as compared to other strains isolated in horses and humans in South Africa (bracketed). This architecture is similar to older strains, AnMg798 and B956D177B3 that have comparable deletions in this region. In comparison, other L2 WNV strains identified in horses in SA had similar deletions to L2 WNV strains previously identified in humans in SA (bracketed) and birds in Europe (⋆).

APPENDIX 2

Table 3.6a: Accession numbers and virus strains used to generate maximum-likelihood trees for Wesselsbron virus phylogenetic analysis

Strain name	Virus	Accession number	Reference
SA H177	Wesselsbron	EU707555	Volk et al, 2009
SEPV MK7148	Sepik	DQ387642	Kuno et al, 2006
YFV Angola71	Yellow Fever	AY968064	von Lindern et al, unpublished
NY385-99	West Nile L1a	EF571854	Rayner et al, unpublished
Kunjin MRM61C	West Nile L1b	D00246	Coia et al, 1988
SPU116/89	West Nile L2	EF429197	Botha et al, 2008
SA93/01	West Nile L2	EF429198	Botha et al,2008
Rabensburg 97-103	West Nile L3	AY765264	Bakonyi et al, 2005
LEIV-Krnd88-190	West Nile L4	AY277251	Lvov et al, 2004
IND804994	West Nile L5	DQ256376	Bondre et al, unpublished
DENV-1	Dengue	FJ384655	Carvalho et al, unpublished
DENV-2	Dengue	M20558	Deubel et al, 1988
DENV-3	Dengue	DQ675533	King et al, 2008
DENV-4	Dengue	FJ639764	Henn et al, unpublished
YAOV	Yaounde	EU082199	Moureau et al, unpublished
USUV	Usutu Budapest	EF206350	Bakonyi et al, 2007
JEV	Japanese Encephalitis	M18370	Sumiyoshi et al, 1987
ALFV	Alfuy	AY898809	May et al, 2006
MVE	Murray Valley Encephalitis	NC_000943	Hurrelbrink et al, 1999
SLEV	St Louis Encephalitis	EU566860	Blaney et al, 2008
ROCV	Rocio	AY632542	Kuno & Chang, 2005
ILHV	Ilheus	NC_009028	Kuno & Chang, 2005
IGUV	Iguape	AY632538	Kuno & Chang, 2005
BSQV	Bussuquara	AY632536	Kuno & Chang, 2005
KOKV	Kokobera	NC_009029	Kuno & Chang, 2005
ZIKV	Zika	DQ859059	Grard et al, 2010
ZIKV	Zika	AY632535	Kuno & Chang, 2007
SPOV	Spondweni	DQ859064	Grard et al, 2010
KEDV	Kedougou	DQ859061	Grard et al, 2010
NOUV	Nounane	FJ711167	Junglen et al, 2009
YOKV	Yokose	AB114858	Tajima et al, 2005
ENTV	Entebbe Bat	DQ537641	Kuno and Chang, 2006
EHV	Edge Hill	DQ859060	Grard et al, 2010
BOUV	Bouboui	DQ859057	Grard et al, 2010
UGSV	Uganda S	DQ859065	Grard et al, 2010
BANV	Banzi	DQ859056	Grard et al, 2010
JUGV	Jugra	DQ859066	Grard et al, 2010
SABV	Saboya	DQ859062	Grard et al, 2010
POTV	Potiskum	DQ859067	Grard et al, 2010
APOIV	Apoi	AF160193	Kuno & Chang, 2005
MODV	Modoc	AJ242984	Leyssen et al, 2002
RBV	Rio Bravo	AF144692	Billoir et al, 2000
MMLV	Montana myotis leukoencephalitis	AJ299445	Charlier et al, 2002
MEAV	Meaban	DQ235144	Grard et al, 2007
SREV	Saumarez Reef	DQ235150	Grard et al, 2007
TYUV	Tyuleniy	DQ235148	Grard et al, 2007
KADV	Kadam	DQ235146	Grard et al, 2007
GGYV	Gadgets Gulley	DQ235145	Grard et al, 2007
RFV	Royal Farm	DQ235149	Grard et al, 2007
KSIV	Karshi	DQ235147	Grard et al, 2007
DTV	Deer-Tick	AF311056	Kuno et al, 2001
POWV	Powassan	HM440563	Pesko et al, 2010
KFDV	Kyasanur Forest Disease	HM055369	Cook et al, unpublished
AHFV	Alkhurma	AF331718	Charrel et al, 2001
ETBEV	Eastern Tick-borne, Sofjin	AB062064	Goto et al, 2001
TSEV	Turkish sheep encephalitis	DQ235151	Grard et al, 2007
LIV	Louping Ill	Y07863	Gritsun et al, 1997
WTBEV	Western Tick-borne	U27495	Mandl et al, 1998
TBE	Tick-borne encephalitis, Salem	FJ572210	Suss et al, 2007
OHFV	Omsk Haemorrhagic Fever, Kubrin	AY438626	Li et al, 2004
LGTV	Langat, TP21	EU790644	Mitzel et al, 2008
CFAV	Cell fusing agent	M91671	Kuno & Chang, 2005

Table 3.6b: Accession numbers for WSLV strains identified in horses during 2008 and previously isolated SA WSLV strains used in this study

Protein	Clinical strains		WSLV strains previously isolated in South Africa					
	SAE118/08	**SAE122/08**	**TAR100**	**AR778**	**AR2209**	**AR11189**	**H177**	**AN16210**
NS5	JN226797	JN226798	JN226799	JN226800	JN226801	JN226802	JN226803	JN226804
Envelope	-	JN226805	JN226806	JN226807	JN226808	JN226809	JN226810	JN226811
NS5/3'UTR	JN226812	JN226813	JN226814	JN226815	JN226816	JN226817	JN226818	JN226819

Figure 3.2

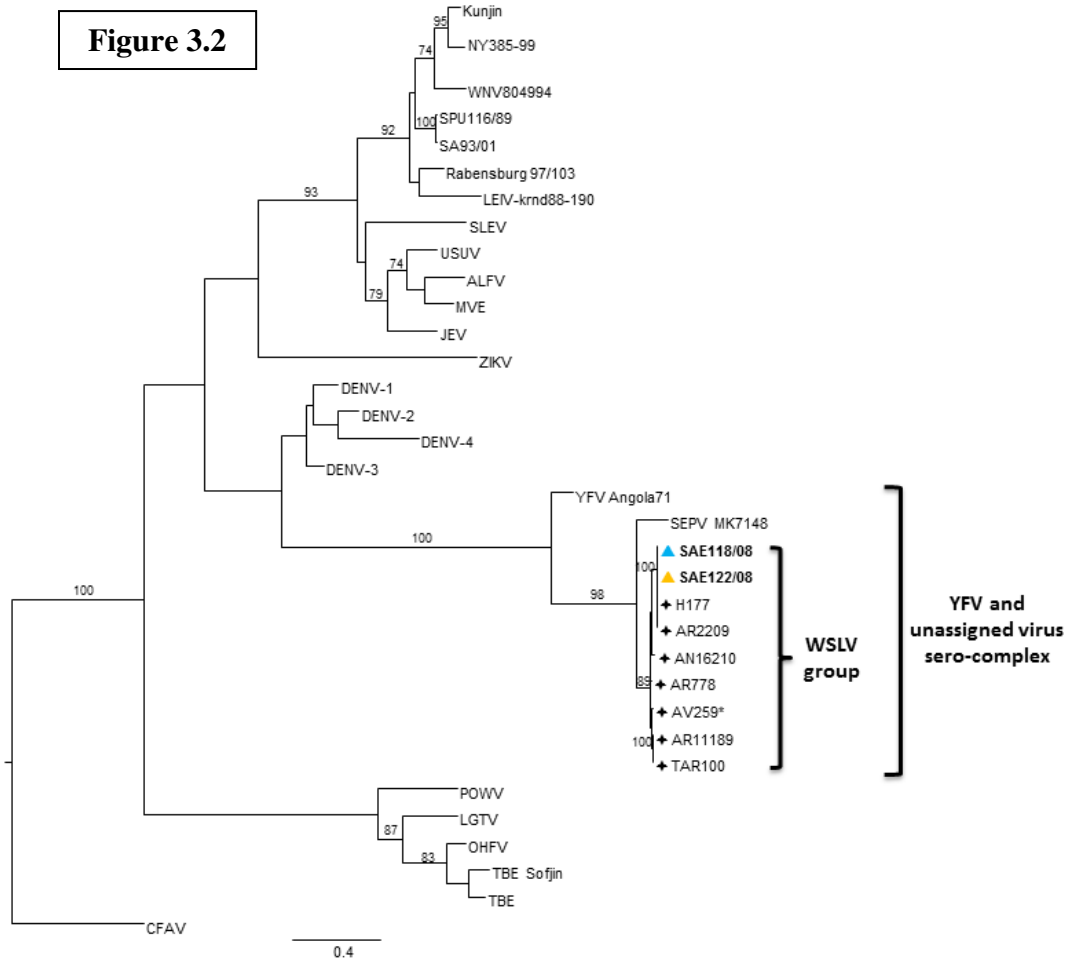

Figure 3.3

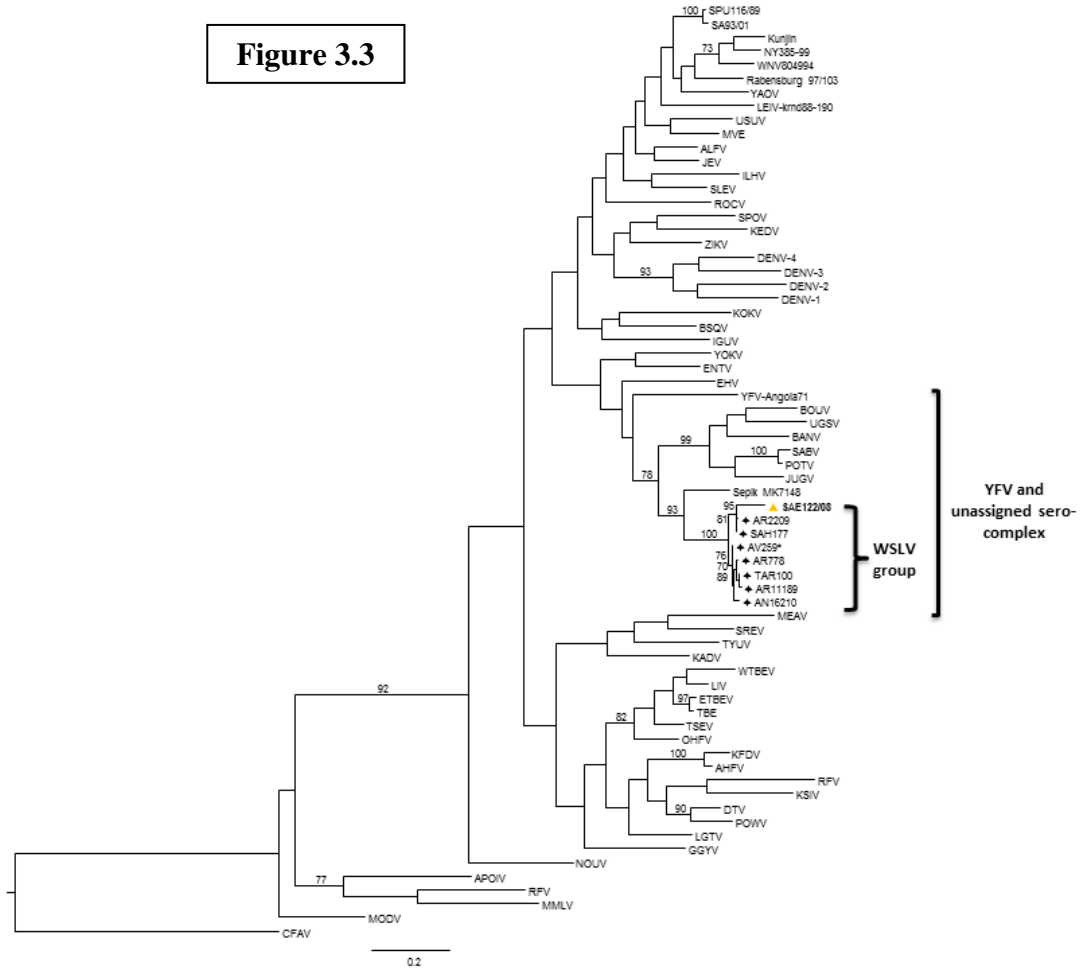

Figure 3.2: ML comparison of a partial region of the NS5/3'UTR junction region, and Figure 3.3 ML comparison of a partial region of the E-protein amplified for clinical WSLV strains. The tree was constructed as described in Figure 3.1 and the scale bars shown. Strain are indicated: SAE118/08 (▲) and SAE122/08 (▲), WSLV isolates (✦). AV259 is highlighted by an astericks. The WSLV and YFV and unassigned virus serocomplex groups are shown. Accession numbers can be found in Table 3.6a and Table 3.6b.

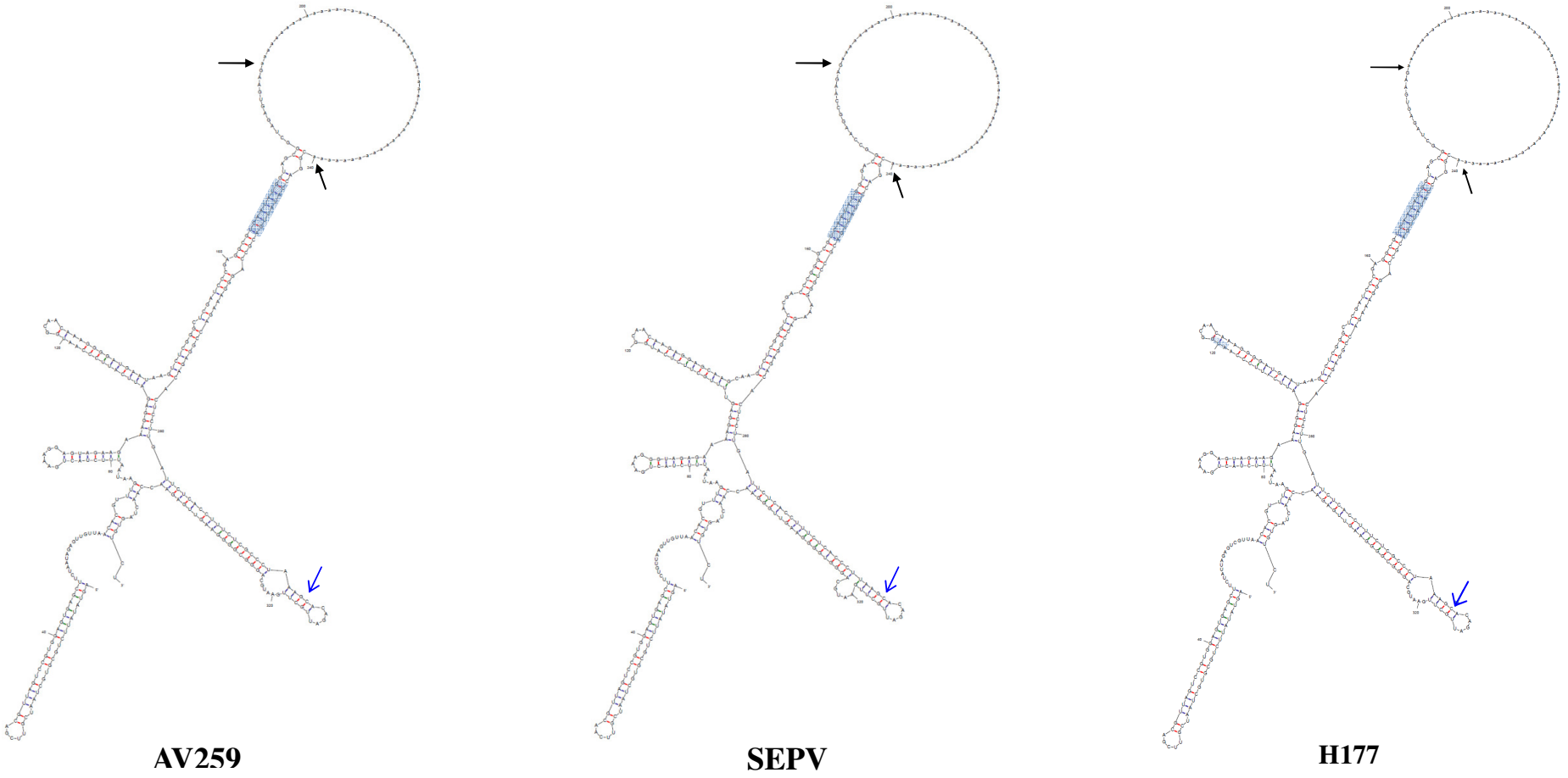

Figure 3.5d: Comparison of AV259, SEPV MK7148 and WASL H177 secondary structures formed during genome cyclization. M-fold (Zuker, 2003) was used to generate the structures using the first 190 nt at the 5' end and the last 115 nt of the 3'UTR. A poly-A spacer was used to represent the virus polyprotein (indicated by black arrows). The cyclization sequences (CYC) are indicated by a grey box. The conserved pentanucleotide sequence (CACAGA) in the loop structure of the 3'LSH is indicated by a blue arrow.


C-protein
prM-protein
M-protein
E-protein
NS1 protein
NS2A protein
NS2B protein
NS3 protein
WSLV AV259

Figure 3.7a: Complete coding sequence of AV259 indicating individual proteins. Proteins are highlighted in different colours: C-protein (blue), prM-protein (orange), M-protein (orange), E-protein (purple), NS1 (yellow), NS2A (dark blue), NS2B (dark green), NS3 (red), NS4A (light blue), 2K (light green), NS4B (royal blue) and NS5 (pink).

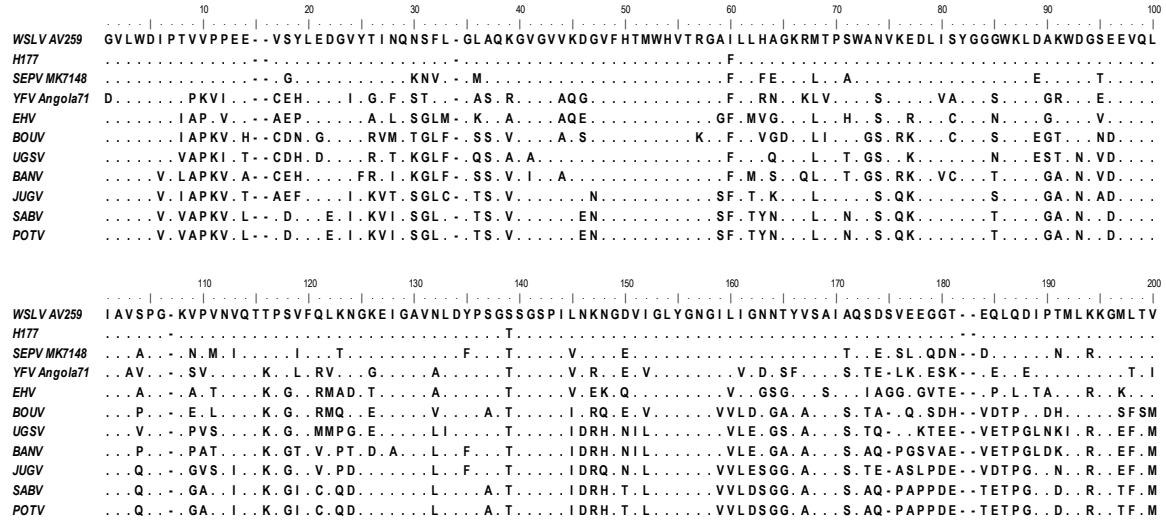

Figure 3.7b: Multiple sequence alignment representing a partial region of the NS3 coding sequence of AV259 relative to the YFV and unassigned serocomplex viruses.

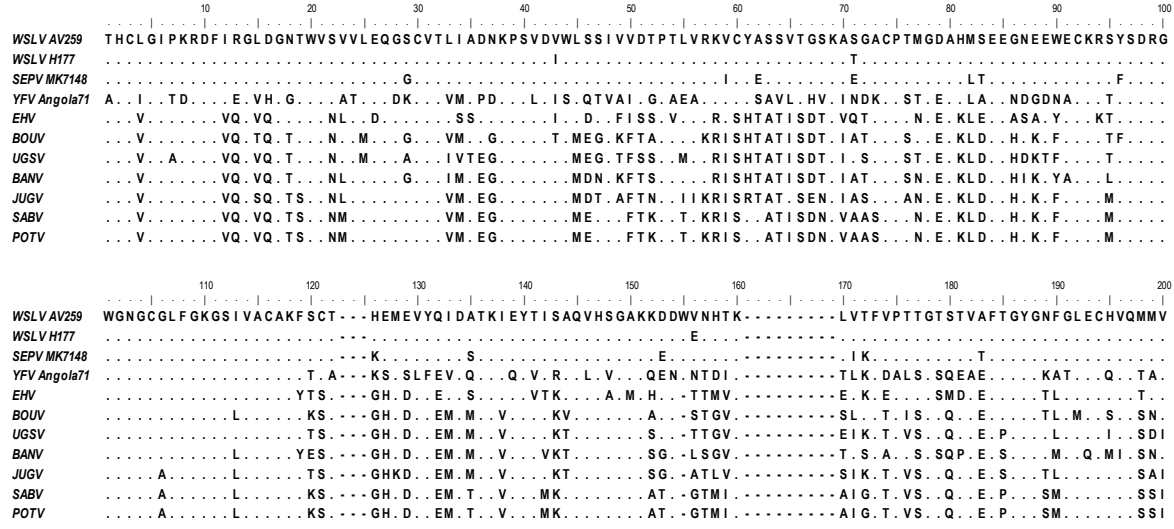

Figure 3.7c: Multiple sequence alignment representing a partial region of AV259's E-protein relative to the rest of the YFV and unassigned serocomplex viruses.

APPENDIX 3

Table 4.3 Sindbis virus positive cases identified in horses in South Africa, 2008 – 2009

UP case code	Date received	Location	Outcome	Specimen	Final PCR result	Clinical symptoms	Results for other viral tests	Age
SAE93/08	13/03/2008	Midrand, Gauteng	Alive	Plasma	SINV and EEV	**Febrile** (42°C).	Weak AHS IFA +ve, WNV neut -ve, WNV PCR-ve, Flavi PCR-ve	6y
SAE110/08	18/03/2008	Midrand, Gauteng	Alive	Serum	SINV	**Acute Fever**. Mild neurological disease (lame hind limbs).	AHS IFA +ve, WNV neut -ve, WNV PCR-ve, Flavi PCR -ve, EEV PCR-ve	uk
SAE30/09	04/03/2009	Pietermaritzburg, Kwa-Zulu Natal	Alive	Plasma	SINV and AHSV	**Febrile**. Jaundice (treated for biliary)	WNV neut-ve, WNV PCR -ve, Flavi PCR -ve	5y
SAE31/09	05/03/2009	Tulbagh, Western Cape	Alive	Plasma	SINV	**Febrile** (37.7°C). Abdominal pain/colic, pale mucous membranes and lame hind legs.	WNV neut +ve, WNV PCR-ve, Flavi PCR -ve	2y
SAE40/09	14/04/2009	Vereeneging, Gauteng	Euthanized	Brain	SINV and WNV	**Neurological**. Collapsed in stable, severe ataxia, weak facial reflexes, recumbency and dog sitting.	WSLV IMP +ve	3y
SAE55/09	06/05/2009	Pretoria, Gauteng	Euthanized	Brain	SINV and WNV	**Neurological**. Rigged in a sling, weak hindquarters, recumbent, poor tail tone and perennial laxity. Couldn't gain weight after weaning her foal.	WNV HI +ve, EEV PCR -ve, EHV PCR -ve, EHV-1 HI +ve, EHV-4 HI -ve	14y

+ve, positive; -ve, negative; y, year; uk, unknown; neut, neutralization; PCR, polymerase chain reaction; IFA, immunofluorescence assay; IMP, immunoperoxidase; HI, haemagglutination inhibition; AHS, African horse sickness; WNV, West Nile virus; EEV, equine encephalosis virus; WSLV, Wesselsbron virus, flavi; flavivirus

Table 4.4: Middelburg virus positive horse cases identified in South Africa, 2009 - 2010

UP case code	Date received	Location	Outcome	Specimen	Final PCR result	Clinical symptoms	Results for other viral tests	Age
SAE68/09	03/06/2009	Bronkhorstspruit, Gauteng	Alive	Plasma	MIDV	**Neurological** - Severe ataxia, tremors in hind limbs, fever (39.5C), slight icterus	WNV PCR -ve, Flavi PCR -ve	10y
SAE25/10	29/03/2010	Colesburg, Karoo	Alive	Plasma	MIDV	**Neurological** - Fever (41°C), ataxic, eating and drinking normally. No response to antibiotics	WNV neut -ve, WNV PCR -ve, Flavi PCR -ve, EEV PCR -ve, SHUV PCR -ve	1y
SAE29/10	13/04/2010	Underberg, Natal	Died	Plasma	MIDV	**Neurological** - severe headache, muscle twitching, ataxic, fever (38.7°C)	WNV neut -ve, WNV PCR -ve, Flavi PCR -ve, EEV PCR -ve, SHUV PCR -ve	18m
SAE31/10	13/04/2010	Underberg, Natal	Alive	Plasma	MIDV	**Febrile** (39.9°C)	WNV neut -ve, WNV PCR-ve, flavi PCR-ve, EEV PCR-ve, SHUV PCR-ve	18m
SAE33/10	13/04/2010	Colesburg, Karoo	Alive	Plasma	MIDV	**Neurological** - Ataxia, incoordination, fever (40°C)	WNV neut toxic, WNV PCR-ve, flavi PCR-ve, EEV PCR-ve, SHUV PCR-ve	1y
SAE38/10	14/04/2010	Kimberly, Northern Cape	Alive	Plasma	MIDV and SHUV	**Febrile -** poor appetite, not working well, leucopoenia	WN neut +ve, WNV PCR -ve, Flavi PCR -ve, EEV PCR-ve	uk
SAE41/10	15/04/2010	Tarlton, Gauteng	Euthanised	Plasma	MIDV	**Neurological** - Ataxia, dog sitting, partial paralysis, lateral recumbency, paddling.	WN neut +ve, WNV PCR -ve, Flavi PCR -ve	uk
SAE47/10	23/04/2010	Tulbagh, Western Cape	Alive	Plasma	MIDV and EEV	**Neurological** - listless, fever (39.5°C) and recumbent, slightly dehydrated, raised RR	WNV neut +ve, WNV PCR -ve, Flavi PCR -ve	7-9y
SAE64/10	13/05/2010	Paarl, Western Cape	uk	Total NA (plasma)	MIDV	**Neurological**	WNV PCR-ve, Flavi PCR -ve	20y
SAE69/10	24/05/2010	Tulbagh, Western Cape	Alive	Plasma	MIDV	**Febrile** - fluctuating temperature (40°C)	WNV PCR -ve, Flavi PCR -ve, EEV PCR -ve, SHUV PCR-ve	6m
SAE129/10	11/10/2010	Pretoria, Gauteng	Alive	Plasma	MIDV	**Neurological** - Hind limb paresis, icterus, petechial haemorrhages on MM, fever (38.1°C), raised RR and HR, diarrhoea	WNV PCR -ve, Flavi PCR –ve, EEV PCR -ve, SHUV PCR -ve	15y

+ve, positive; -ve, negative; y, year; m, month; uk, unknown; neut, neutralization; PCR, polymerase chain reaction; IFA, immunofluorescence assay; IMP, immunoperoxidase; HI, haemagglutination inhibition; RR, respiratory rate; HR, heart rate; WNV, West Nile virus; flavi; flavivirus; EEV, Equine encephalosis virus; SHUV, Shuni virus

Table 4.6 Alphavirus strains used to generate maximum-likelihood trees for phylogenetic analysis of alphavirus positive horses in South Africa (2008 – 2010)

Strain name	Virus	Accession number	Reference
Eastern Equine Encephalitis	EEEV-Florida91-4691	AY705241	Platteborz et al, 2005
Eastern Equine Encephalitis	EEEV	X63135	Volchkov et al, 1991
Venezuelan Equine Encephalitis	VEEV Cabassou CaAr508	AF075259	Kinney et al, 1998
Western Equine Encephalitis	WEEV McMillan	GQ287640	Keene et al, 2009
Western Equine Encephalitis	WEEV BFS2005	GQ287644	Logue et al, 2009
Highlands J	HJV 585-01	FJ827631	Allison A.B, 2009
Chikungunya	CHIKV ArA 2657	HM045818	Volk et al, 2010
Chikungunya	CHIKV SH2830	HM045798	Volk et al, 2010
Igo Ora	Igo Ora IBH10964	AF079457	Lanciotti et al, 1998
O nyong Nyong	ONNV gULU	M20303	Strauss et al, 1988
Mayaro	MAYV	AF237947	Netto et al, unpublished
Semliki Forest	SFV-42S	X04129	Garoff et al, 1980
Semliki Forest	SFV-A7	Y147615	Tuittila et al, 2000
Una	UNAV	AF339481	Kinney and Pfeffer, unpublished
Bebaru	BEBV	AF339480	Kinney and Pfeffer, unpublished
Ross River	RRV NB5092	M20162	Faragher et al, 1988
Ross River	RRV T48	GQ433359	Jones et al, 2010
Ross River	RRV (Sagiyama)	AB032553	Shirako. and Yamaguchi 2000
Getah	GETV LEIV 16275	EF631998	Guriev and Prilipov, unpublished
Middelburg	MIDV Ar749	AF339486	Kinney and Pfeffer, unpublished
Middelburg	MIDV857	EF536323	Attoui et al, 2007
Barmah Forest	BFV BH2193	U73745	Lee et al, unpublished
Aura	AURAV	AF126284	Rumenapf et al, 1995
Sindbis	SINV SW6562	AF429428	Saleh et al, 2003
Sindbis	SINV Kyzylagach LEIV 65A	AF339478	Kinney and Pfeffer, unpublished
Sindbis	SINV XJ-160	AF103728	Liang et al, 2000
Sindbis	SINV (Babanki DakAry 251	AF339477	Kinney and Pfeffer, unpublished
Sindbis	SINV-like SAAR86	ACU38305	Simpson, et al, 1996
Sindbis	SINV-like Girdwood	ACU38304	Simpson et al, 1996
Sindbis	SINV-like YN87448	AF103734	Zhou et al, 2001
Sindbis	SINV	J02363	Ou et al, 1981
Ockelbo	OCKV Edsbyn	M69205	Shirako et al, 2003
Whataroa	WHATV M78	AF339479	Kinney and Pfeffer, unpublished
Fort Morgan	FMV (Buggy Creek) 81V8122	AF339474	Kinney and Pfeffer, unpublished
Fort Morgan	FMV CM4-146	GQ281603	Allison A.B., 2009
Ndumu	NDUV SaAr 2204	AF339487	Kinney and Pfeffer, unpublished
Sleeping disease	SDV	AJ316246	Weston J, unpublished
Salmon Pancreatic disease	SPDV F93125	AJ316244	Weston et al, 2002
Rubella	RUBV cendehill	AF188704	Lund and Chantler, 2000

Table 4.7: GenBank accession numbers of SINV and MIDV strains identified in horses (2008 – 2010)

MIDV POSITIVE	
Strain	**E-protein**
SAE68/09	JN226792
SAE25/10	JN226793
SAE29/10	JN226794
SAE31/10	JN226795

APPENDIX 4

Part of the work from this thesis has been published and presented at conferences:

REFEREED JOURNAL PUBLICATIONS

- Venter M, **Human S**, van Niekerk S, Williams J, van Eeden C and Freeman F. Fatal neurological disease and abortion in mare infected with lineage 1 WNV, South Africa. **2011**. Emerging Infectious Diseases, 17(8) 1534 - 1536. (Impact factor 6.8)
- Venter M, Steyl J, **Human S**, Parker R ,Weyer J, Zaayman D, Blumberg L, Leman P, Paweska J and Swanepoel R. **2010**. Transmission of West Nile virus during horse autopsy. Emerging Infectious Diseases, 16(3) 573 - 575. (Impact factor 6.8)
- Webber LM and **Human S.** Viruses affecting the musculoskeletal system: selected South African arboviral examples. **2010**. SA Rheumatology Journal, Feb/Mar 22 – 26.
- Venter M, **Human S**, Zaayman D, Gerdes T, Williams J, Steyl J, Leman P, Paweska J, Setzkorn H, Rous G, Murray S, Parker R , Donnellan C and Swanepoel R. **2009**. Lineage 2 West Nile Virus as a cause of fatal neurological disease in horses in South Africa. Emerging Infectious Diseases, 15(6) 877 - 884. (Impact factor 6.8)
- Zaayman D, **Human S** and Venter M. A highly sensitive method for the detection and genotyping of West Nile virus by Real-Time PCR. **2009**. Journal of Virological Methods, 157(2) 155 - 160. (Impact factor 2.1)

REFEREED CONFERENCE CONTRIBUTIONS

National

- Naicker K, **Human S**, and Durbach S. *Characterization of a soil microbe with anti-mycobacterial activities* [Poster]. Bio-08 Combined National Conference, Grahamstown, South Africa; 21 – 25 January 2008.
- **Human S**, Leman P, Kemp A, Paweska J. and Venter M. *Molecular characterization of Wesselsbron virus from animals and humans in South Africa*. [Oral presentation]. Proceedings of the 8th Annual Conference of the South African Society for Veterinary Epidemiology and Preventive Medicine (SASVEPM), Gauteng, South Africa; 20 – 22 August 2008.

- Venter M, **Human S**, Gerdes T, Williams J, Leman P, Kemp A, Paweska J, Setzkorn H, Rous G, Stroebel JC, Steyl, J Murray S, Parker R and Donnellan, C. *Investigation of the pathogenesis of West Nile virus and other zoonotic Flavi and alpha viruses in humans and horses in South Africa* [Oral presentation]. Proceedings of the 8th Annual Conference of the South African Society of Veterinary Epidemiology and Preventative Medicine (SASVEPM), Gauteng, South Africa; 20 – 22 August 2008.

- Venter M, **Human S**, Zaayman D, Gerdes T, Williams J, Steyl J, Leman P, Paweska J, Setzkorn H, Rous G, Stroebel JC, Murray S, Parker R , Donnellan C, Swanepoel R . *Lineage 2 West Nile virus as a cause of fatal neurological disease in horses in South Africa* [Oral presentation]. Conference proceedings paper, South African Equine Veterinary Association Congress, Kwa-Zulu Natal, South Africa; 15-19 February 2009
- **Human S**, Steyl J, Williams J, Last R, Van Niekerk S and Venter M. *Sindbis and Middelburg virus as a cause of disease in animals in South Africa: the molecular epidemiology* [Oral presentation]. Proceedings of the South African Society for Veterinary Epidemiology and Preventative Medicine (SASVEPM) conference, Pretoria, South Africa; 18 - 20 August 2010.
- Venter M, **Human S**, van Eeden C, van Niekerk S, Williams J, Steyl J, Gerdes T and Swanepoel R. *The role of zoonotic vector borne viruses as neurological pathogens in horses and wildlife in South Africa* [Oral presentation]. Proceeding of the Veterinary Epidemiology and Preventative Medicine (SASVEPM) conference, Pretoria, South Africa; 18 – 20 August 2010.
- Van Eeden C, **Human S**, Van Niekerk S, Williams J, Gerdes T, Van Wilpe E, Odendaal L, Viljoen A, Swanepoel R and Venter M. *Molecular epidemiology of Shuni virus, a cause for encephalitic disease in horses in South Africa* [Oral presentation]. Proceedings of the Veterinary Epidemiology and Preventative Medicine (SASVEPM) conference, Pretoria, South Africa; 18 - 20 August 2010.
- Williams JH, **Human S**, Van Eeden C, Gerdes GT, Van Wilpe E, Meiring T, Odendaal L, Donnellan C, Viljoen A, Page P, Sanz M and Venter M. *Pathology of recently diagnosed fatal neurotropic arboviral infections in horses in South Africa* [Oral presentation]. Proceedings of the Veterinary Epidemiology and Preventative Medicine (SASVEPM) conference, Pretoria, South Africa; 18 – 20 August 2010.

International:

- **Human S**, Gerdes T, Williams J, Leman P, Kemp A, Paweska J, Steyl J, Stroebel J and Venter M. *Molecular epidemiology and characterization of Wesselsbron virus and co-circulating alphaviruses in sentinel animals in South Africa* [Oral presentation]. Proceedings of the 12TH International Symposium on Veterinary Epidemiology and Economics (ISVEE XII), Durban, South Africa; 10 – 14 August 2009.
- Venter M, **Human S**, Zaayman D, Gerdes T, Williams J, Steyl J, Leman P, Paweska J, Donnelan C and Swanepoel R. *West Nile virus Lineage 2 as a cause of zoonotic neurological disease in humans and horses in Southern Africa: importance and potential treatments* [Oral presentation]. Proceedings of the 12TH International Symposium on Veterinary Epidemiology and Economics (ISVEE XII), Durban, South Africa; 10 – 14 August 2009.
- Venter M, Steyl J, **Human S**, van Eeden C, Smit S and Williams J. *Zoonotic arboviruses threatening wildlife in Southern Africa* [Oral presentation]. International Meeting on infectious Diseases and Surveillance (IMED), Vienna, Austria; 4 - 7 February 2011.

APPENDIX 5

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19 May 2010

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Dear Dr Venter

RE-APPROVAL for 2010

H017-09: Characterization of zoonotic flavi and alphaviruses in sentinel animals in South Africa *(S Human)*

Re-approval was granted for the period May 2010-May 2011.

Best regards

E Mostert

Elmarie Mostert

AUCC Co-ordinator