Investigation of viral causes of undiagnosed neurological disease in animals and their zoonotic risk to humans in South Africa

by

Charmaine van Eeden

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Supervisor: Prof. M. Venter

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I certify that the thesis hereby submitted to the University of Pretoria for the degree PhD (Medical Virology) has not been previously submitted by me in respect of a degree at any other university.

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Charmaine van Eeden

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ETHICS

This study has been approved by both the Animal use and care committee protocol number H016/09 and the Faculty of Health Sciences Research Ethics Committee protocol number 28/2011, University of Pretoria.

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SUMMARY

Every year thousands of cases of neurological disease go undiagnosed largely due to the vast number of potential causes, especially neglected are those thought to be of viral origin. Arboviruses in particular play a significant role particularly due to their zoonotic potential, being that they are easily transmitted between animals and human through various vector species. Animals have for centuries been used as sentinels for human disease, horses in particular are sensitive to many arboviruses and their commercial and emotional value and close association with their human handlers makes them valuable study subjects. The purpose of this study was to investigate the potentially novel causes of undiagnosed neurological disease in horses. Firstly, through the development of a virus discovery technique that would allow for the amplification of unknown agents and secondly, through the investigation of the agents thereby identified.

An arbitrarily primed PCR was developed which allowed for the identification of both viral and bacterial agents from cell culture and as well as tissue specimens. The efficacy and reproducibility of this assay led to its incorporation into our diagnostic compliment. Shuni virus (SHUV) was identified in a cell culture isolate from a horse that had displayed severe neurological signs and had histopathology suggestive of meningoencephalitis. This little known orthobunyavirus, a member of the Simbu serogroup, had not been well studied since its discovery in the 1960's, although two cases of neurological disease in horses had

been documented. Thus the focus became to further elucidate the role SHUV may play in neurological disease in South Africa.

Two SHUV specific assays, a conventional and a real-time nested PCR were developed and employed in a five year epidemiologic study. From January 2008 to December 2012, 497 horses and 143 other animals submitted to our surveillance program with febrile and neurological disease were screened for the presence of SHUV. 13 SHUV cases were identified, nine in horses and four in wildlife species. In horses symptoms ranged from mild febrile illness to severe neurological disease, where 45% of animals either succumbed due to the severity of symptoms or were euthanized on humane grounds. All wildlife cases presented with paralysis and all proved fatal, 50% due to humane euthanasia. Two cases of co-infection with the alphavirus, Middelburg virus were identified.

Increased interest following these diagnoses made analysing the full genome of SHUV essential, allowing for the molecular characterisation of this pathogen.

The genome was amplified and characterised and SHUV's (SAE 18/09) relationship to the prototype SHUV (An 10107) isolate and the Simbu serogroup fully clarified. Results obtained correlated well with previous genomic analyses, and all conserved features and domains identified for the Simbu serogroup were also maintained for SHUV. No evidence for reassortment was found. Of significance was the finding that the original SHUV isolate's (An 10107) sequence differed from SAE 18/09 at one of the M segment cleavage sites, such changes are known to affect pathogenicity, and will have to be investigated further.

Finally due to the zoonotic potential of SHUV, a serological survey was conducted on veterinarians, to determine whether they may be at increased level of exposure due to occupational risk. Both a SHUV and a WNV neutralization assay were developed. WNV was used in comparison as zoonotic transmission of this virus during animal autopsy had been documented and multiple studies conducted to analyse its sero-prevalance. 12.5% of veterinarians were found to have neutralizing antibodies to WNV and 4% to SHUV, these values correlate with what is seen in equine studies (WNV 8.7% - SHUV 1.9%) and highlights the zoonotic potential of these pathogens.

PUBLICATIONS AND COMMUNICATIONS

Publications

- Charmaine van Eeden, June Williams, Truuske Gerdes, Erna van Wilpe, Adrienne Viljoen, Robert Swanepoel and Marietjie Venter. Shuni Virus as a Cause of Neurological Disease in Horses. 2012. Emerging Infectious Diseases. 18 (2).
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LIST OF ABBREVIATIONS

A	Alanine
aa	Amino acid
A, C, G, T	Adenine, cytocine, guanine, thymine
AHSV	African horse sickness virus
AINOV	Aino virus
AKAV	Akabane virus
AS-PCR	Asymmetrical PCR
BANV	Banzi virus
BSL2	Bio-Safety Level 2
BWA	Burrows-Wheeler aligner
C	Cysteine
cDNA	Complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
cm	Centimetres
CNS	Central nervous system
cRNA	Complementary ribooxynucleic acid
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxy nucleotide triphosphate
DOP	Degenerate oligonucleotide primer
DSS	Dengue shock syndrome
DTT	Dithiothreitol
DVBID	Division of vector-borne infectious diseases
EDTA	Ethylene diamine tetraacetic acid
EEV	Equine encephalosis virus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscope
EtOH	Ethanol
FRET	Fluorescein resonance energy probe
G	Glycine
GBV-A	GB virus A
GBV-C	GB virus C
Gc	Glycoprotein c

Gn	Glycoprotein n
HE	Haematoxylin and eosin
HKY	Hasegawa, Kishino and Yano
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JATV	Jatobal virus
JEV	Japanese encephalitis virus
JTT	Jones-Taylor-Thornton
KAIV	Kaikular virus
KUNV	Kunjin virus
L	L segment
M	M segment
MAQ	Mapping and assembling qualities
$MgCl_2$	Magnesium chloride
MIDV	Middelburg virus
Min	Minute
mM	Micromoles
MVA	Molecular virology animal
N	Nucleoprotein
NaCl	Sodium chloride
NaOAc	Sodium acetate
NGS	Next generation sequencing
nm	Nanometre
nt.	Nucleotide
NSs	Non-structural protein s
NSm	Non-structural protein m
ORF	Open reading frame
OROV	Oropouche virus
ºC	Degrees Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEAV	Peaton virus
PEG	Polyethylene glycol
pH	is a measure of the activity of the (solvated) hydrogen ion
PHYML	Phylogenetic estimation using maximum likelihood
R	Arginine

Rc	Reverse complement
RDA	Representational difference analysis
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Revolutions per minute
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
RVFV	Rift valley fever virus
S	S segment
SA	South Africa
SAE	South African equine
SATV	Sathuperi virus
SBV	Schmallenberg virus
SHAV	Shamonda virus
SHUV	Shuni virus
SISPA	Sequence independent single primer amplification
SOAP	Short oligonucleotide alignment program
ssp	Subspecies
TINV	Tinaroo virus
U	Units
UCHGOP	University College Hospital General Outpatients Clinic
UP	University of Pretoria
USA	United States of America
UTR	Untranslated region
VIDISCA	Virus discovery cDNA AFLP
vRNA	Virion-sense RNA
WHO	World Health Organization
WNV	West Nile virus
WNV1	West Nile virus lineage 1
WNV2	West Nile virus lineage 2
Y	Tyrosine
YFV	Yellow fever virus
µg	Microgram
µl	Microliter
µm	Micromolar

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CHAPTER 1
Literature Review

1.1 INTRODUCTION

Arboviruses are among the most important emerging pathogens facing the world today and play an important role in encephalitic disease (Dobler, 1996). Epidemiological studies in the USA suggest that the incidence of viral encephalitis is approximately 3.5-7.4 per 100 000 individuals. In studies where the specific cause has been investigated arboviruses have shown to be the primary cause of acute disease, in up to 50% of cases however, no specific cause can be found (WorldHealthOrganization, 2006). In Africa, a lack of infrastructure and resources suggests these values may be even higher. Arboviruses are viruses which are maintained in nature between haematophagous arthropods such as mosquitoes, ticks, culicoid midges and sand-flies, and susceptible vertebrate hosts (Gould, 2011). According to the Division of vector-borne infectious diseases of the Centres for disease control and prevention (CDC), there are now more than 537 arbo- and related viruses recognized worldwide (www.cdc.gov/ncidod/dvbid/misc/organiz.htm). Though only around 125 are known to cause illness in humans and livestock with less than 50 being considered as important human and veterinary pathogens (Calisher *et al.*, 1999). In recent years several arboviruses have emerged from Africa as new pathogens in previously unaffected regions and caused major epidemics and epizootics, including West Nile virus; Rift Valley fever and Chikungunya virus (Hollidge *et al.*, 2010).

Arboviruses produce a large range of illnesses from asymptomatic fever to severe systemic disease with haemorrhagic complications and/or encephalitis (Gould, 2011). A similar range of illness is also observed in horses (Castillo-Olivares & Wood, 2004). Many of these viruses have a zoonotic component in their life cycles, where zoonoses are defined as ‘diseases and infections which are naturally transmitted between vertebrate animals and man’ (W.H.O., 1959; WorldHealthOrganization, 1959), in this instance via arthropod vectors. The recent escalation of air travel, climate change and urbanization have not only increased their geographical range, but also the opportunity for both human and domestic animal contact with these vectors, greatly increasing the opportunity for disease (Denizot *et al.*, 2012; Gould & Higgs, 2009).

Several arboviruses in the families *Flavi-*, *Bunya-* and *Togaviridae* have emerged from Africa as new pathogens in previously unaffected regions and have caused major epidemics and epizootics, including West Nile virus (WNV); Rift Valley fever virus (RVFV) and Chikungunya virus (CHIKV) (Hollidge *et al.*, 2010). Horses in particular are highly sensitive to some of these viruses, in particular WNV which can lead to the development of severe neurological disease. Horses have thus been targeted as sentinel animals in the identification of zoonotic arboviruses associated with neurological disease in South Africa (Venter & Swanepoel, 2010).

Clinical laboratories are rapidly adopting viral species-specific assays for virus identification, thereby increasing sensitivity and reducing the time needed for diagnosis. Although widely successful these methods are limited when it comes to the detection of divergent or novel viruses due to their high specificity. In recent years much effort has been invested in the development of methods for the discovery of novel viruses. Rotavirus, Astrovirus, Bovine parvovirus and parvovirus 4 were discovered using sequence-independent single primer amplification (SISPA) (Lambden *et al.,* 1992, Matsui *et al*., 1993, Allander *et al.,* 2001, Allander *et al.,* 2005, Jones *et al*., 2005). Simpler methods such as random and degenerate oligonucleotide primer (DOP) polymerase chain reactions (PCR) have also been used to detect both DNA and RNA viruses (Nanda *et al*., 2008, Ambrose and Clewley, 2006). The next generation sequencing (NGS) revolution has further improved on the efficiency and turnover of new pathogen discovery (Rothberg & Leamon, 2008). Despite these advances, the longstanding and well established technique of electron microscopy still remains an important tool in virus discovery, a significant example being the discovery of Nipah virus in 1999 (Chua, 2004).

This project specifically focused on gaining an increased understanding of the contribution of zoonotic arboviruses as causal agents of neurological disease in South Africa through investigation into sentinel animals with unsolved neurological disease. This was achieved through the establishment of virus discovery techniques to screen horses with neurological disease that tested negative for known viruses to determine if other pathogens were present. This was done with the aim of detecting potentially undescribed causes of encephalitis in animals which could pose a risk to humans. We identified Shuni virus, an uncharacterised orthobunyavirus in a horse with fatal encephalitis. We characterised this virus through full genome analysis and developed specific assays to screen animals with

neurological signs to define its epidemiology. Due to the zoonotic component of the arboviruses, we also investigated the potential threat of this virus to humans through a seroprevalence study in individuals whom have an occupational risk to exposure and compared it to the seroprevalence of West Nile virus (WNV) in this same group.

The subsequent literature review provides a description of the advances in virus discovery techniques and an overview of the arboviruses that are associated with neurological disease. This is followed by an in-depth summary of the *Orthobunyavirus* genus, including the molecular biology and disease association of member viruses, with particular focus on those present in Africa.

1.2 THE ARBOVIRUSES

The name *arbovirus* is an acronym for **AR**thropod-**BO**rne viruses and was coined in part by Dr. William C. Reeves (1916-2004) to connote the class of viruses carried by arthropods, responsible for diseases like Dengue fever and West Nile fever (Lenzer, 2004). The vast majority of arboviral diseases are zoonotic [The word is derived from the Greek words *zoon* (animal) and *nosos* (disease)], with primary, enzootic transmission cycles involving wild animals with humans and domestic animals representing tangential or dead-end infections that do not influence the long-term evolution of the pathogen. Human infection by zoonotic arboviruses can follow one of three cycles (Figure 1.1): in the epidemic cycle humans directly amplify the virus which can lead to rapid spread and extensive infection of many individuals in an area, resulting in massive outbreaks; the enzootic cycle involves avian, rodent or non-human primates as reservoir or amplification hosts where humans become infected via direct spillover; lastly, there may be secondary amplification of the virus involving domestic animals, in the epizootic cycle, high rates of infection and increased circulation around humans, greatly increases the likelihood of spillover infection (Figure 1.1) (Weaver & Barrett, 2004).

Most medically important arboviruses belong to the *Flaviviridae, Bunyaviridae* or *Togaviridae* families, but a small number are members of the *Reoviridae*, and *Orthomyxoviridae* (www.cdc.gov/ncidod/dvbid/misc/organiz.htm).

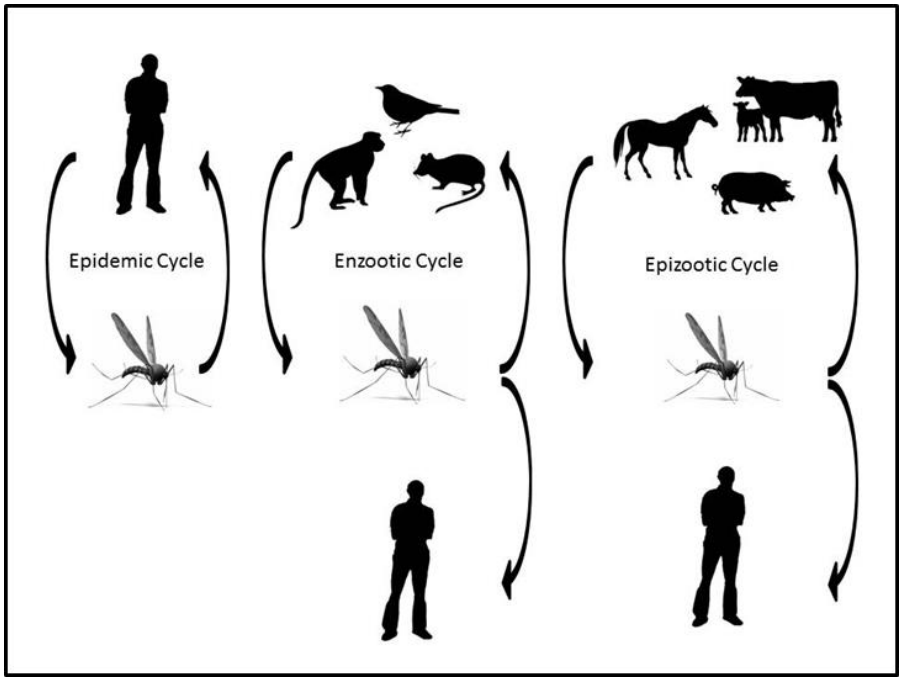


Figure 1.1 Mechanisms of human infection by zoonotic arboviruses (Authors own, based on figure by Weaver & Barrett, 2004). Epidemic cycle, humans directly amplify viruses. Enzootic cycle, involving avian, rodent, or non-human primates as reservoir and/or amplification hosts where humans become infected via direct spillover. Epizootic cycle, secondary amplification involving domestic animals can increase circulation around humans, increasing the chance of infection via spillover.

1.2.1 *Flaviviridae*

Over a century ago the first human virus, yellow fever, was discovered by Walter Reed who demonstrated that yellow fever could be experimentally transferred via the filtered serum of an infected individual, and that this infectious agent was transmitted to humans by mosquitoes (Strode, 1951). Yellow fever virus (YFV) is now but one representative of a large family of related positive-strand RNA viruses, the *Flaviviridae* (from the Latin *flavus*, "yellow"). This family currently consists of three genera (Figure 1.2): *Flavivirus*, *Pestivirus* (from the Latin *pestis*, "plague"), and *Hepacivirus* (from the Greek *hepatos*, "liver"). The *pesti-* and *hepaci-* genera are not considered to be arthropod-borne. Only the *Flavivirus* genus holds arboviruses, some of which are the most clinically important arboviruses world-wide, like dengue, yellow fever, Japanese encephalitis and West Nile viruses (Gould & Solomon, 2008).

The *Flavivirus* genus consists of more than 70 viruses and cause a variety of diseases, including fevers, encephalitis, and haemorrhagic fevers. Entities of major global concern include dengue virus (DENV) with its associated dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), Japanese encephalitis virus (JEV) the most important

cause of epidemic encephalitis worldwide (Solomon & Whitley, 2004), West Nile virus (WNV), and YFV (Schoub & Blackburn, 2004). On average less than 20% of flavivirus infections are thought to result in clinical symptoms, with complications occurring in an even smaller proportion of West Nile cases (www.cdc.gov/westnile). YFV is the exception as up to 50% of infected persons develop clinical symptoms (Cleton *et al.*, 2012).

1.2.2 *Bunyaviridae*

The family *Bunyaviridae* was formally established in 1975 and now contains four genera of animal-infecting viruses (*Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, and *Hantavirus* genera) and one genus of plant-infecting viruses (*Tospovirus*) (Fauquet & Fargette, 2005) (Figure 1.2). The *Bunyaviridae* comprise over 300 viruses, making up for over half of all known arboviruses. Members of the *Orthobunyavirus, Nairovirus, Phlebovirus* and *Tospovirus* genera are arthropod borne, whereas *Hantavirus* is rodent-borne (Elliott, 1990). Viruses in the genus *Phlebovirus* are of considerable public health importance, as they can cause a variety of clinical syndromes ranging from a brief, self-limiting febrile illness to retinitis, encephalitis, meningoencephalitis and fatal haemorrhagic fever, Toscana virus and Rift Valley fever virus are of significance (Tesh, 1988). The most clinically significant serogroups within the *Orthobunyavirus* genus are the California encephalitis and Simbu serogroups, within the *Nairovirus* genus only Crimean-Congo haemorrhagic fever virus is considered to be of clinical importance (Solomon & Whitley, 2004). The clinical infection rate for bunyaviruses is not well defined and most infections are thought to be asymptomatic. Exceptions are Crimean-Congo haemorrhagic fever which causes clinical disease in 25% of cases and Oropouche virus in 30-60% of cases (Cleton *et al.*, 2012).

1.2.3 *Togaviridae*

The Togaviruses are enveloped, positive strand RNA viruses that are spherical in appearance. The name Togavirus is derived from the Latin *'toga'*, meaning a Roman cloak and refers to the virus particles' morphology. This family currently comprises three genera; *Alphavirus*, *Rubivirus* and the unassigned viruses (Fauquet & Fargette, 2005) (Figure 1.2). Arboviruses are found in the *Alphavirus* genus which has 29 recognized species, which are responsible for a variety of human and animal diseases, involving encephalitis, arthritis, fever, rash, and arthralgia, and are transmitted primarily by

arthropod vectors. About 50% of the alphaviruses cause disease in humans (Powers *et al.*, 2001). The *Togaviridae* family is less known for its arthropod-borne infectivity than for the single member of the *Rubivirus* genus, Rubella, the causal agent of German "measles". Togaviruses have a high clinical attack rate with 50-85% of infections resulting in clinical manifestations; exceptions are Sindbis, Western equine encephalitis and Eastern equine encephalitis viruses where symptomatic infections occur in only 0.1-5% of cases (Cleton *et al.*, 2012).

1.3 ARBOVIRUSES THAT CAUSE NEUROLOGICAL DISEASE

Arboviruses classically cause three disease patterns in humans: fever-arthralgia-rash syndromes, viral haemorrhagic fevers and neurologic disorders. In disease affecting the central nervous system, manifestations such as myelitis, meningitis and/or encephalitis can occur, with associations such as behavioural changes, paralysis, paresis, convulsions and incoordination (Solomon *et al.*, 2007; Solomon & Whitley, 2004).

Despite intensive efforts and investigations on the pathways leading to infections of the CNS by arboviral families the exact mechanism remains to be defined. There are however multiple routes that can be considered; a) direct viral spread from the periphery to the CNS particularly for arboviruses involved in brain infections (Myint *et al.*, 2007), b) Murray valley encephalitis (MVEV), St Louis encephalitis (SLEV), and Japanese encephalitis (JEV) viruses were speculated to enter the CNS via the olfactory pathway (Monath *et al.*, 1983), c) while transcytosis across cerebral capillary endothelial cells was reported in JE (Liou & Hsu, 1998) and d) virion-budding on the parenchymal cells after replication at the blood-brain barrier may also occur (McMinn, 1997).

The most important neurological arboviruses of the world appear to be WNV, Ilheus virus (ILHV) Japanese encephalitis virus (JEV), Murray valley encephalitis virus (MVEV), Rift Valley fever virus (RVFV), Sindbis virus (SINV) and Tahyna virus (TAHV) (Cleton *et al.*, 2012; Digoutte & Adam, 2005; Dobler, 1996; McIntosh, 1980; Smithburn, 1958; Solomon & Whitley, 2004; Swanepoel & Burt, 2009). Of these, WNV and JEV have caused some of the most recognised, as well as some of the largest outbreaks of disease. WNV quickly established itself in North America after its recognition in New York City in 1999.

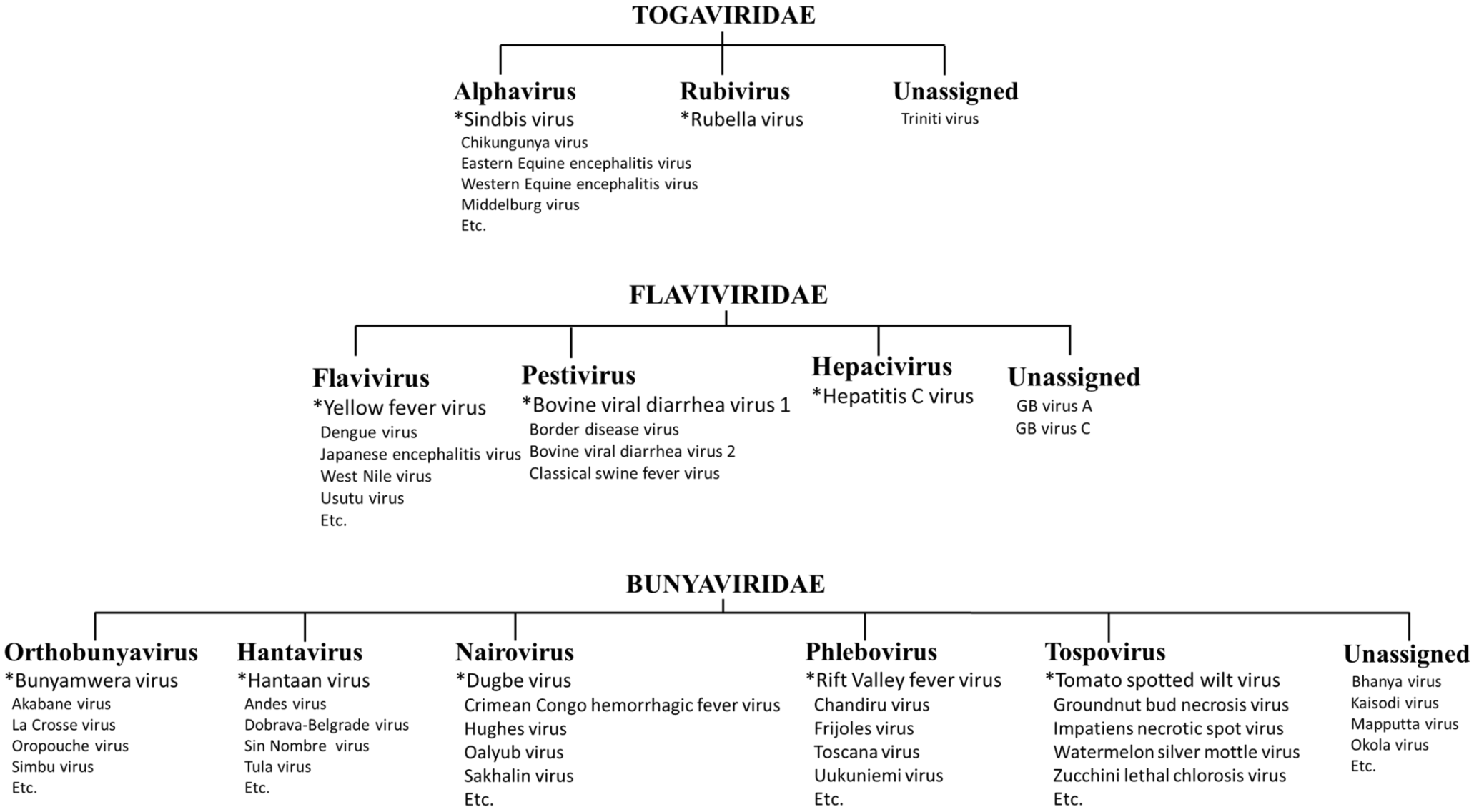


Figure 1.2 Schematic indicating the genera and type viruses (*) of the *Toga*-, *Flavi*- and *Bunyaviridae*. Based on ICTV records (Fauquet and Fargette, 2005).

Historically WNV has been associated with dispersed outbreaks of mild febrile illness (Bernkopf *et al.*, 1953; Hubalek & Halouzka, 1999), more recently outbreaks have become more frequent and illness more severe (Campbell *et al.*, 2001; Platonov *et al.*, 2001). The largest outbreak of WNV meningoencephalitis ever reported occurred North America in 2002, 4156 cases were documented, 2354 of which had meningoencephalitis and 284 resulting in death (CDC, 2002). An estimated 3 billion people live in countries where JEV is endemic and the annual incidence of disease is 30 000-50 000 cases, the annual number of deaths is 10 000-15 000 where infection can cause irreversible neurological damage (Monath & Heinz, 1996; Solomon & Vaughn, 2002). In a study focusing on travel associated diseases, Cleton et al. (2012), summarized the available information for the most important arboviruses; visually summarising their geographic distribution in relation to symptoms and taxonomy, the neuroinvasive arboviruses are shown in Figure 1.3.

1.3.1 Arboviruses causing disease in Southern Africa

Of the 537 arboviruses listed in the International Catalogue of Arboviruses by the Division of Vector-Borne and Infectious Diseases (DVBID) (DVBID, 2009), only 35 have been isolated in South Africa. Only those viruses which have caused widespread epidemics in humans or animals have been studied extensively. The largest investigation into the epidemiology of arboviruses in southern Africa was conducted by McIntosh. (1980), the study covered 22 years (1957-1979) and led to the identification of 33 arboviruses in South Africa. Of these 25 were from the three major arboviral families; *Togaviridae* (5), *Flaviviridae* (6) and *Bunyaviridae* (14).

Several of these viruses have been identified in human and/or domestic animals; Chikungunya virus (CHIKV), Sindbis virus (SINV), Banzi virus (BANV), Spondweni virus (SPOV), West Nile virus (WNV), Wesselsbron virus (WESS), Bunyamwera virus (BUNV), Middelburg virus (MIDV), Semliki forest virus (SFV), Germiston virus (GERV), Witwatersrand virus (WITV), Rift valley fever virus (RVFV), Shuni virus (SHUV), Ndumu virus (NDUV), Tahyna virus (TAHV) and Simbu virus (SIMV) (McIntosh, 1980). By 1961 most of the currently known, important human pathogens had been identified and so more emphasis was placed on the epidemiology of Chikungunya (CHIK), Sindbis (SIN), West Nile (WN) and Rift valley fever (RVF) viruses (McIntosh, 1980), which were apparently the four most important human arboviral pathogens. Some of the more prominent viruses (Table 1.1) are discussed below.

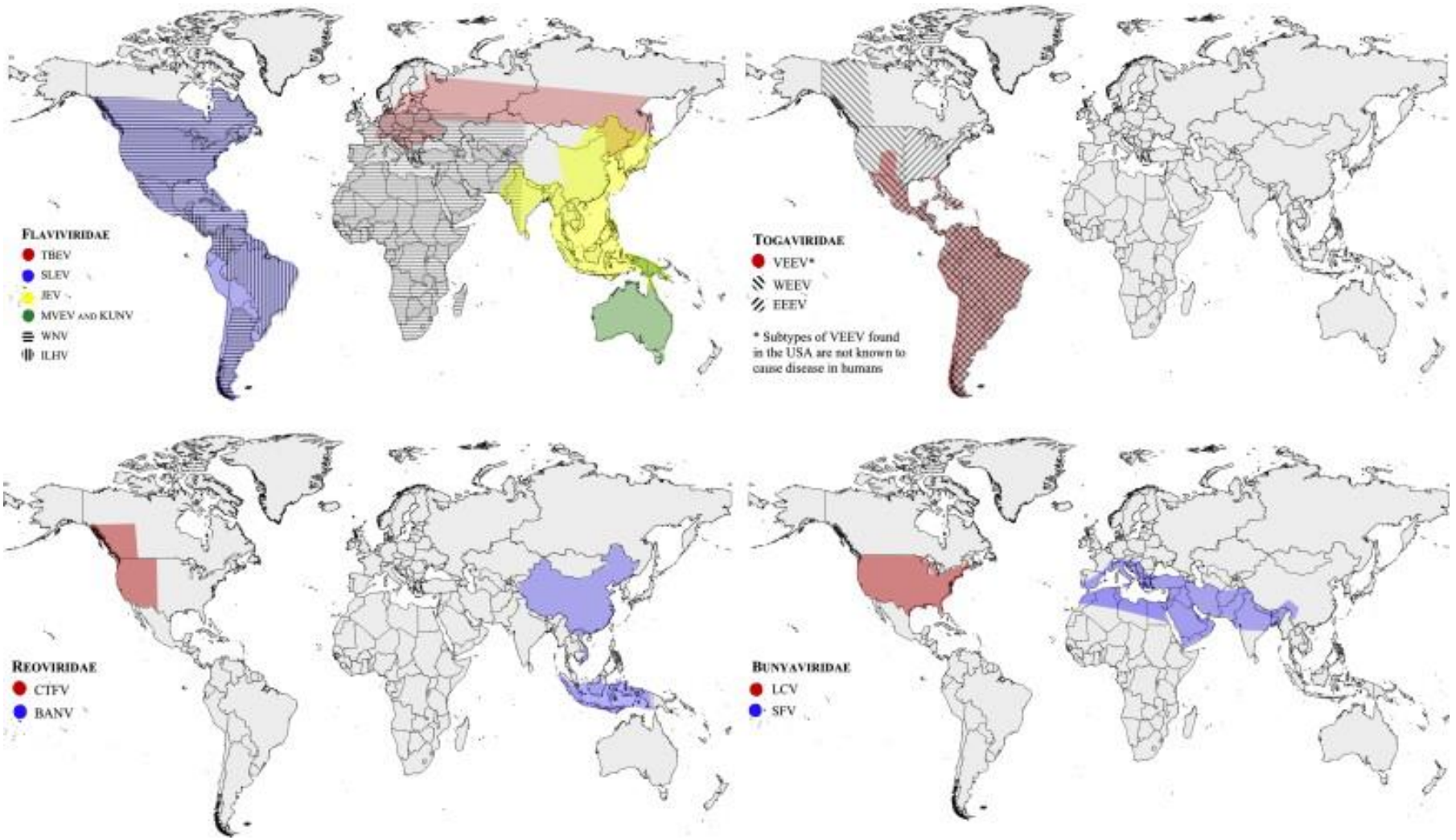


Figure 1.3 Map indicating the general geographic incidence of medically important neuroinvasive arboviruses (Cleton et al., 2012).

Table 1.1 Summary of the most common arboviral causes of disease in South Africa

Virus	First identification in South Africa	Hosts	Human infection	Vectors	Disease association	References
Chikungunya	1956	N/A	Yes	*Aedes ssp.*	Febrile illness Severe joint pain	(Gear & Reid, 1957; Smithburn *et al.*, 1959a)
Sindbis	1954	Livestock, birds	Yes	*Culex ssp.* *Mansonia spp.*	Febrile illness	(Kokernot *et al.*, 1956; Weinbren *et al.*, 1956)
Middelburg	1957	Livestock, horses	Antibodies	*Aedes ssp.*	Febrile illness Neurological disease	(Human, 2011; Kokernot *et al.*, 1957a)
West Nile	1955	Birds, horses	Yes	*Culex ssp.*	Febrile illness Neurological disease	(Kokernot & McIntosh, 1959; Venter & Swanepoel, 2010; Weinbren, 1955)
Wesselsbron	1955	Livestock, horses	Yes	*Aedes ssp.*	Abortion, Febrile illness, Neurological disease	(Human, 2011; Smithburn *et al.*, 1959a; Weiss *et al.*, 1956)
Banzi	1956	Livestock, rodents	Yes	*Culex ssp.*	Febrile illness	(McIntosh *et al.*, 1976c; Smithburn *et al.*, 1959b)
Rift valley fever	1950	Livestock	Yes	*Aedes ssp.*	Abortion, Febrile illness, Neurological disease	(Gear *et al.*, 1955; Gear *et al.*, 1951; van Velden *et al.*, 1977)
Bunyamwera	1955	N/A	Yes	*Aedes ssp.*	Febrile illness	(Kokernot *et al.*, 1957b; Kokernot *et al.*, 1958)
Shuni	1957	Livestock, horses	Yes	*Culex ssp.* *Culicoides ssp.*	Febrile illness Neurological disease	(Coetzer & Howell, 1998; McIntosh *et al.*, 1972)

1.3.1.1 *Togaviridae*

1.3.1.1.1 Chikungunya virus (CHIKV)

First isolated during an epidemic in Tanzania in 1952 and 1953, where it was isolated from *Aedes aegypti* and human beings (Ross, 1956). The second recognised outbreak and first isolation in South Africa occurred in the Transvaal Lowveld, in 1956 (Gear & Reid, 1957). Early indications of CHIKV infection in humans in South Africa was obtained during serosurveys before the 1956 outbreak. Smithburn et al. (1959) collected sera in the eastern Transvaal and northern Natal regions, where 10.6% of adults and 0.9% of children had neutralizing antibodies. Human beings from several areas of Southern Africa were tested for antibodies to CHIKV during the period 1957 to 1973. In South Africa, antibodies were only found in the Lowveld, where 0.9% of children and 3.3% of adults were positive (McIntosh, 1980). The only other recorded outbreaks occurred during 1975 and 1976 in the Transvaal Lowlands (McIntosh *et al.*, 1977).

1.3.1.1.2 Sindbis virus (SINV)

First isolated in 1952 in the Sindbis district of the Nile Delta, Egypt (Taylor *et al.*, 1955). During the studies it was isolated on several occasions from *Culex univittatus* and once from a crow. Antibody to the virus was found in human beings, birds and several species of domestic animal (Kokernot *et al.*, 1956). The existence of SINV in South Africa was established in 1954 when three isolations were made from Culex mosquitoes (Weinbren *et al.*, 1956). A few years later three isolation were made from *Cx. Neavei* and one from *Mansonia uniformis* in Natal (Worth *et al.*, 1961). Antibody surveys in human beings, birds and domestic animals showed infection in man had occurred throughout South Africa with the highest incidence being in the Orange Free State (Kokernot *et al.*, 1956). Apart from human infections of an epidemic scale recorded in 1974, SINV was not recognized as a significant health problem in South Africa (McIntosh *et al.*, 1976a). In 1983/84 however, another outbreak occurred and hundreds of febrile human cases were reported (Jupp *et al.*, 1986). Sindbis fever is still reported annually in South Africa (Storm *et al.*, 2012).

1.3.1.1.3 Middelburg virus (MIDV)

First isolated in 1957 from *Aedes* mosquitoes during an outbreak of illness with mortality in the Middelburg district, South Africa (Kokernot *et al.*, 1957a). Antibody surveys in Natal, found MIDV antibodies in humans, cattle, sheep and goats (Kokernot *et al.*, 1961; Smithburn *et al.*, 1959a). No reports of MIDV as a natural animal pathogen were

documented until 1993, where it was isolated from the spleen of a horse in Zimbabwe, which had symptoms similar to that of African horse sickness (AHS) (Attoui *et al.*, 2007). MIDV has also been identified in several cases of neurological disease in horses in South Africa (Human, 2011).

1.3.1.2 *Flaviviridae*

1.3.1.2.1 West Nile virus (WNV)

First isolated in 1937 from the blood of a febrile human being in Uganda (Smithburn *et al.*, 1940) and in 1950 three isolations were reported from children in Egypt (Melnick *et al.*, 1951). Antibodies to WNV as reported by Weinbren (1955), were widespread in humans throughout South Africa but at low rates (1-4.5%) with the exception of the Orange Free State (Free State) where 20.8% of sera were positive. These findings of low rates in the Natal region and high rates on the plateau were confirmed by Kokernot *et al.* (1956) (Kokernot *et al.*, 1956) and Smithburn *et al.*, (1959) (Smithburn *et al.*, 1959a). The original isolation of WNV in South Africa was from a human being in Natal in 1958 (Kokernot & McIntosh, 1959). Later surveys (1962-1969) confirmed a high presence of antibodies in humans in the Highveld at Olifantsvlei (38%) as well as a significant prevalence in the Cape (13.5%) and Orange Free State (Free State) (16.1%). The high rate of infection at Olifantsvlei, was suggested to be linked to the artificially created moist conditions at a sewage disposal plant. Here treated effluent was used to irrigate pasture which provided favourable conditions for both birds and vector species (McIntosh, 1980).

In the years that followed only sporadic cases were identified, until 1974 when an epidemic occurred in the Karoo and Northern Cape province (McIntosh *et al.*, 1976a). Several identifications of the virus were made in human sera during the epidemic, where thousands of clinical cases were observed. During this time isolations were also made from several *Culex* species (McIntosh *et al.*, 1976a). Antibody surveys which followed the epidemic showed high levels of antibodies in humans (55%) and horses (53%) (McIntosh *et al.*, 1976a). An epizootic of WNV and SINV occurred between 1983-1984 in the Witwatersrand (Gauteng) area of the Highveld were increased levels of infection were also observed (Jupp *et al.*, 1986). More recently WNV was identified as a causal agent of neurological disease in horses (Venter *et al.*, 2009; Venter & Swanepoel, 2010). In 2010, lineage 1 WNV was isolated in a mare and her aborted foetus; this was the first time this lineage was seen in South Africa (Venter *et al.*, 2011).

1.3.1.2.2 Wesselsbron virus (WESS)

First isolated in 1955 from a dead lamb during an outbreak of disease with abortion and high mortality rate in sheep in the Orange Free State, South Africa (Weiss *et al.*, 1956). A month later it was isolated from a boy suffering from fever and body aches (Smithburn *et al.*, 1957). WESS has also been isolated from *Ae. Circumluteolus* in 1955 (Muspratt *et al.*, 1957) and *Ae. Caballus* in 1957 (Kokernot *et al.*, 1960). A high incidence of infection was revealed in Natal by antibody surveys in the 1950s. Neutralizing antibodies were found in 32.1% of adult human beings (Smithburn *et al.*, 1959a) and in 46.8% of cattle, sheep and goats (Kokernot *et al.*, 1961). In 2008, two horses with severe neurological disease tested positive for WESS by DNA sequence analysis (Human, 2011).

1.3.1.2.3 Banzi virus (BANV)

BANV was first isolated from the blood of a febrile child in South Africa in 1956, neutralizing antibodies were also found in 21.4% of humans from the region (Smithburn *et al.*, 1959a; Smithburn *et al.*, 1959b). This virus has also been isolated from mosquitoes, cattle and rodents in South Africa (McIntosh *et al.*, 1976c).

1.3.1.3 *Bunyaviridae*

1.3.1.3.1 Rift valley fever virus (RVFV)

In southern Africa, RVF has usually appeared in extensive outbreaks in sheep and cattle following heavy rains and human infection readily occurs through contact with infected animals. RVF was first isolated in 1930 from a sheep during an epidemic accompanied by abortion in sheep and cattle in the Rift Valley in Kenya, this was accompanied by illness in man (Daubney *et al.*, 1931). From 1950 to 1956, several severe epidemics in sheep and cattle, accompanied by human infection were recognized in South Africa and several isolations were made from mosquitoes (Gear *et al.*, 1955; Gear *et al.*, 1951). In 1956 a study by Kokernot *et al.* (1957), the presence of Rift valley neutralizing antibodies was shown to average 4% amongst humans in South Africa, with the highest rates being seen in the Cape region, 4.7%. The same team conducted a survey in domestic ruminants in the Natal region in 1961, where they found antibodies in 9.7% of animals (Kokernot *et al.*, 1961).

An epizootic of RVF occurred in an agricultural community between 1974 and 1975, in which several fatal human infections were recognized (van Velden *et al.*, 1977). An antibody survey in residents was conducted a few years later (1978), to determine the extent of human infection. It was found that 14.5% of individuals had antibodies against

the virus (McIntosh *et al.*, 1980). Immune rates were shown to be higher in adult males which may be attributed to their higher risk, due to their more intimate contact with the animals. More recently a total of 242 human RVF cases with 26 deaths were confirmed in 2010, with a further 32 cases in the first half of 2011 (www.nicd.ac.za).

1.3.1.3.2 Bunyamwera virus (BUNV)

BUNV was first isolated in South Africa in 1955 from *Aedes circumluteolus* (Kokernot *et al.*, 1957b) and later from humans who had fever and mild illness (Kokernot *et al.*, 1958). Antibody surveys in humans also showed a 54% seropositivity in adults in the 1950s (Smithburn *et al.*, 1959a).

1.3.1.3.3 Shuni virus (SHUV)

First isolated in 1966 from a cow bled in Sokoto, Nigeria during a program for the surveillance and study of viral infections that was instituted by the University of Ibadan between 1964 and 1969. During the course of the survey SHUV was also isolated twice again from cattle and once from a sheep with neutralizing antibodies being found in dairy and trade cattle as well as sheep (Causey *et al.*, 1972; Causey *et al.*, 1969; Kemp *et al.*, 1973). SHUV was also isolated twice from *Culicoides ssp.* during field surveillance and from a one and a half year old child at the General Outpatients Clinic at the University College Hospital (UCHGOP) in August 1966, as part of the arbovirus surveillance activity in Nigeria (Lee, 1979; Moore *et al.*, 1975). In South Africa, SHUV was recovered twice from pools of *Culex theileri* mosquitoes caught near Johannesburg and from 7 apparently healthy cattle and a goat in Natal between the years 1957 and 1979 (McIntosh, 1980; McIntosh *et al.*, 1972). In 1977, the virus was isolated from the brains of two horses with nervous disease submitted for rabies virus examination; one from South Africa (Coetzer & Howell, 1998) and one from Zimbabwe (Coetzer & Erasmus, 1994).

1.4 MOLECULAR BIOLOGY OF THE *ORTHOBUNYAVIRUS* GENUS

The identification of Shuni virus in this project became the focus of this study and as such, the molecular biology of the *Bunyaviridae* will be discussed, with special emphasis on the Orthobunyavirus genus.

1.4.1 General characteristics of the Bunyaviridae

Bunyaviruses have spherical virus particles 80-120 nm in diameter, with a host cell derived bilipid layer envelope through which virus–coded glycoprotein spikes project (Figure 1.4). Viruses in the *Phlebovirus* genus have round, closely packed morphologic units, approximately 10 to 11 nm in diameter, with central cavities approximately 5 nm in diameter and are arranged in penton-hexon clusters (Martin *et al.*, 1985). The surface structure of viruses in the *Hantavirus* genus also are distinctly ordered and have a square grid-like appearance (White *et al.*, 1982). In contrast, virions in the *Orthobunyavirus* genus have surfaces covered with closely packed, knoblike morphologic units with no detectable order. Similarly, no obvious order was found for the small surface structures with central cavities observed on viruses in the *Nairovirus* genus (Martin *et al.*, 1985). The appearances of viruses in the *Tospovirus* genus have been likened to that of the nairoviruses (Milne & Francki, 1984).

Members of the family have a three segmented, negative sense, single stranded RNA genome and each of the segments small (S) medium (M) and large (L) are contained in individual nucleocapsids within the virion. The terminal nucleotide sequences are highly conserved among viruses within a genus, but differ from those of viruses in other genera. In addition to ribonucleocapsids containing virion-sense RNA (vRNA), certain viruses in the *Phlebovirus* and *Tospovirus* genera encapsidate small amounts of complementary sense (cRNA) (Raju & Kolakofsky, 1989). Therefore, some viruses in the family *Bunyaviridae* use only a negative-sense coding strategy and others use a combination of negative-sense and ambisense coding strategies. The virions contain 3 major structural proteins: two envelope glycoproteins G1 and G2, nucleocapsid protein N plus minor quantities of L protein which is believed to be the viral transcriptase (Figure 1.4).

1.4.2 Orthobunyavirus genome

1.4.2.1 S segment

The S segment encodes the nucleoprotein (N), which is the most abundant viral product in virions and infected cells and plays several important roles in viral replication. The S segments of viruses in the *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* genera produce NSs proteins as well as N. The NSs has a possible role in early replication processes (Schmaljohn & Nichol, 2007) and has been shown to play a key role in pathogenesis (Bridgen et al., 2001, Muller et al., 1995, Varela et al., 2013). Investigations into the full S

segment of the Simbu serogroup have shown nucleotide sequence identity between members of the Simbu serogroup is high and ranges between 65% and 99.1% (Saeed *et al.*, 2001a). Values are even higher within viruses where sequences are often almost identical (Saeed *et al.*, 2001a; van Eeden *et al.*, 2012; Yanase *et al.*, 2005). It has been shown that the NSs proteins are much less conserved than the N proteins (Saeed *et al.*, 2001a). Six conserved regions have been identified within the N protein of the Simbu serogroup viruses (residues 5-14, 43-51, 71-81, 88-103, 123-138 and 140-187) (Saeed *et al.*, 2001a) although not all are identical (Savji *et al.*, 2011). Four conserved residues involved in the formation of ribonucleoprotein complexes (P_{125}, G_{131}, Y_{158} and I_{231}) have also been identified (Eifan & Elliott, 2009).

1.4.2.2 M segment

The M segment codes for the viral envelope glycoproteins G1 (Gn) and G2 (Gc). The M segment gene processing events differ among the genera; Hantaviruses produce only G1 and G2, whereas some viruses in the *Phlebovirus* and *Orthobunyavirus* genera produce NSm from the same M segment mRNA as G1 and G2 (Figure 1.4). The viral glycoproteins are responsible for recognition of receptor sites on susceptible cells, manifestation of viral haemaglutinating ability and for inducing protective immune response in the host.

The nucleotide sequence identity of the M open reading frame (ORF) ranges between 47.9% and 56.0% between members of the Simbu serogroup. The amino acid (aa) sequences are highly variable (37.3% - 49.7%), although several features are retained. These features include that 59 of the 71 cysteine residues are conserved and the predicted signal peptide lies at the N-terminal of AKAV, Peaton virus (PEAV) and Aino virus (AINOV). Also the aa residues around the cleavage site between G2 and NSm are maintained in these viruses and follow a conserved arginine (R_{299}) residue. In comparison with the G1 and NSm proteins, the G2 proteins are relatively conserved among the Simbu serogroup viruses (46.4-63.7%) (Savji *et al.*, 2011; Yanase *et al.*, 2003). Predicted cleavage sites in the precursor of the Gn are Ser_{16} in the N terminus and residue 301 in the C terminus which occurs after the highly conserved motif KSLRxAR (Pollitt *et al.*, 2006)

1.4.2.3 L segment

The L protein functions as an RNA-dependent RNA polymerase with endonuclease activity and is also responsible for generating the capped primers needed for transcription

(Schmaljohn & Nichol, 2007). The *Bunyaviridae* use the mechanism of 'cap-snatching' for viral mRNA transcription, since polymerase does not possess capping activity. Cap-snatching involves binding of host capped mRNAs to the ribonucleoproteins (RNP), cleavage of these RNAs close to the 5' cap by viral endonuclease activity and use of short capped fragments as primers for viral mRNA transcription (Reguera *et al.*, 2010). There are four conserved areas in the *Orthobunyavirus* L protein; regions I (aa $P_{75}D$) and II (aa $R_{651}Y$) are located in the amino terminus and are conserved among all bunyaviruses (Muller *et al.*, 1994), region III (948-1239) contains the polymerase motifs that comprise the polymerase module [A (1045-1062), B (1129-1151), C (1170-1184), D (1214-1225)] (Poch *et al.*, 1989) (Figure 1.5). A pre-A (948-977) and E motif (1228-1239) were also identified in region III (Muller *et al.*, 1994). A fourth conserved region (1240-1343) was identified by Aquino et al. (2003) (Savji *et al.*, 2011). The Orthobunyaviruses also contain a restriction endonuclease with the conserved active site H…D…PD….DxK…..T, which is critical for cleaving the nucleic acid phosphodiester bond. The rest of this protein remains largely uncharacterised, partly due to its lack of sequence homology with other proteins (Reguera *et al.*, 2010).

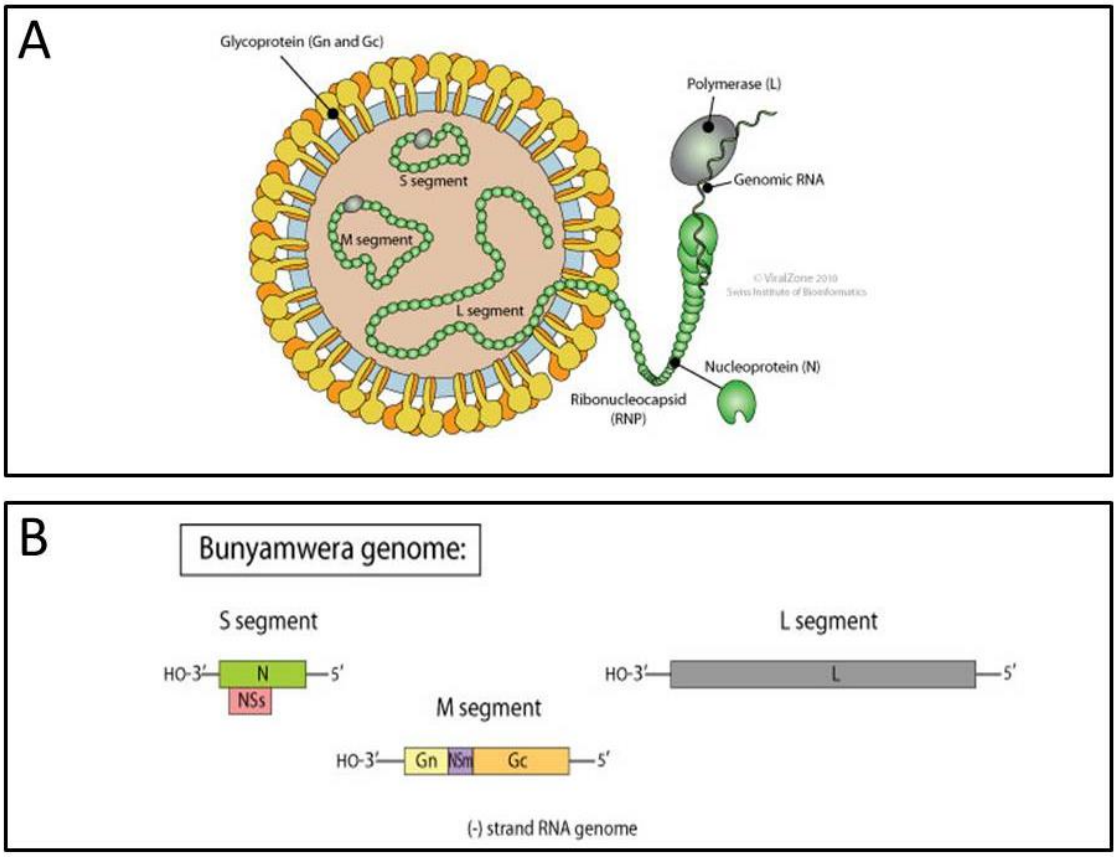


Figure 1.4 Bunyaviridae viral particle structure and Orthobunyavirus genomic structure. Adapted from Gastieger *et al.* (2003).

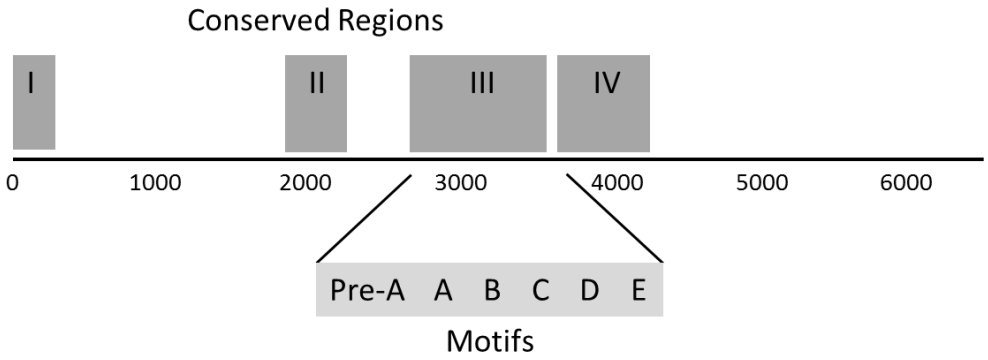


Figure 1.5 Representational diagram of the conserved regions of the Orthobunyavirus L protein (Authors own).

1.4.2.4 Genomic termini

The untranslated regions at the ends of the bunyavirus genome segments are presumed to contain signals for encapsidation, transcription regulation, replication and packaging of the RNPs into virions (Barr *et al.*, 2003; Kohl *et al.*, 2004). Members of the *Bunyaviridae* have complementary terminal sequences with one mismatch at position 9 (U and G) and are genus specific (Table 1.2) (Elliott, 1990). Analysis of the terminal sequences of members of the Simbu serogroup have shown that for Aino virus termini are similar in sequence for 25 residues with one mismatch at position 9 and a single nucleotide addition at position 10 from the 3' end (Akashi *et al.*, 1984; Akashi *et al.*, 1997b). Akabane virus AKAV has a short 3' untranslated region (UTR) (33nt) and a long 5' UTR (123nt) (Akashi *et al.*, 1997b). Oropouche virus (OROV) on the other hand has been found to have a short 3' UTR (44nt) and a very short 5' UTR (17nt) (Saeed *et al.*, 2001a).

Table 1.2 *Bunyaviridae* conserved terminal end sequences

Genus	3' Terminal sequence
Bunyavirus (Orthobunyavirus)	UCAUCACAUGA
Hantavirus	AUCAUCAUCUG
Nairovirus	AGAGAUUCU
Phlebovirus	UGUGUUUC
Tospovirus	UCUCGUUA
Uukuvirus	UGUGUUUCU

1.4.2.5 Genome reassortment

As is the case with other viruses which have segment genomes, the occurrence of genetic reassortment has been reported among orthobunyaviruses. Ngari virus (MRIV) was

generated as a result of reassortment between BUNV and Batai viruses (BATV) (Briese *et al.*, 2006; Yanase *et al.*, 2006). Jatobal (JATV) and Tinaroo (TINV) viruses were generated by reassortment of segments with OROV and AKAV, respectively (Kobayashi *et al.*, 2007; Saeed *et al.*, 2001b). Further phylogenetic analysis has revealed further reassortment within the *Orthobunyavirus* genus; in Potosi (POTV) and Main Drain (MDV) viruses (Briese *et al.*, 2007) as well as Aino (AINOV) and Peaton (PEAV) viruses (Yanase *et al.*, 2010). Most recently the newly identified Schmallenberg virus (SBV) was shown to have been the result of the reassortment of Shamonda virus (S, L segment) and Sathuperi virus (M segment) (Goller *et al.*, 2012).

1.4.3 Classification of the *Orthobunyavirus* genus

The *Orthobunyavirus* genus is the largest of the *Bunyaviridae* and contains more than 170 viruses (Calisher, 1996; Fauquet & Fargette, 2005), Bunyamwera virus (BUNV) is the prototype virus. Classification of the orthobunyaviruses is a complex issue, where the majority of viruses were placed within one of 18 serogroups (Figure 1.6), based on complement-fixation (N protein antibodies), haemaglutination and neutralization (G protein antibodies). Many viruses were however not classified into these serogroups (Calisher, 1996; Fauquet & Fargette, 2005). Serological relatedness however varies within these serogroups and due to the occurrence of reassortment amongst members of the *Orthobunyavirus* genus; viruses may be placed within different serogroups based on the assay used for classification (Calisher, 1988; Nichol, 2001). The latest report of the International Committee for the Taxonomy of Viruses separates the orthobunyaviruses into more than 44 species (Figure 1.7), based on phylogenetic relationships (Fauquet & Fargette, 2005). Such allocation needs however, to be regarded as fluid due to the scarcity of molecular characterization within this genus, where comprehensive studies have involved viruses in only four serogroups, namely, Bunyamwera, group C, California, and Simbu (Bowen *et al.*, 1995; Dunn *et al.*, 1994; Nunes *et al.*, 2005; Saeed *et al.*, 2001a).

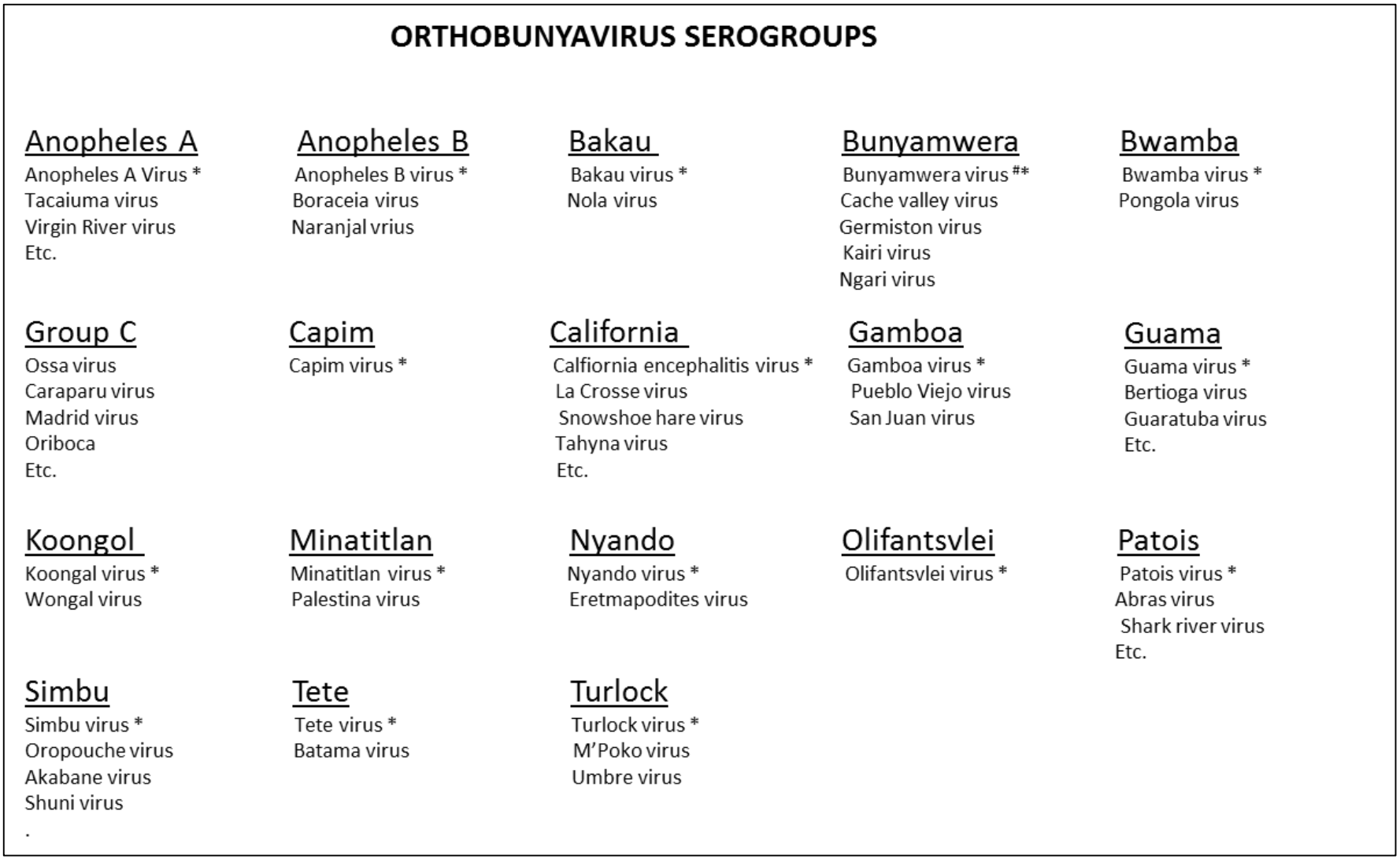


Figure 1.6 The Orthobunyavirus serogroups. #Indicates the genus prototype and *Serogroup prototype virus. Based on ICTV records (Fauquet and Fargette, 2005).

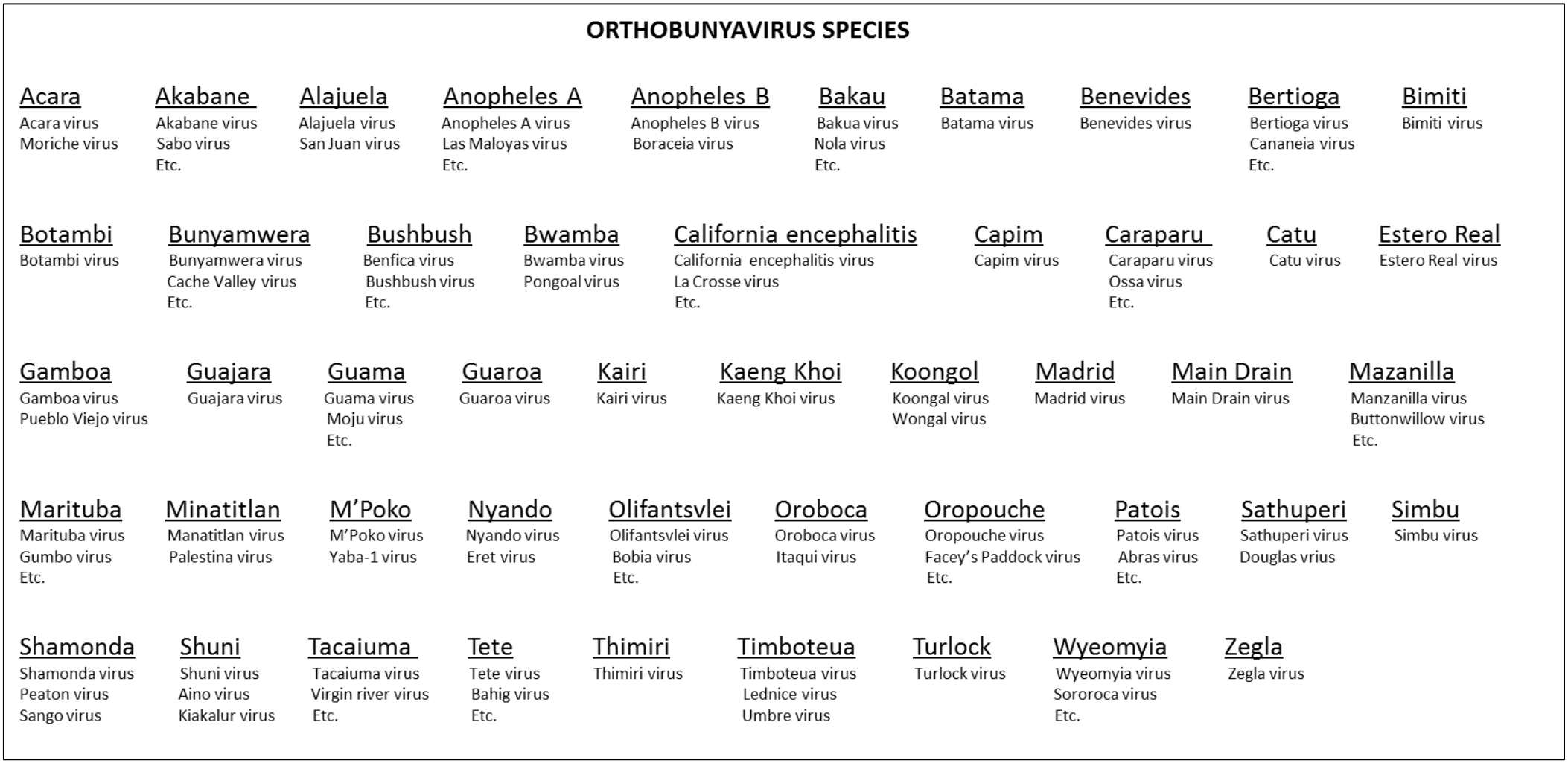


Figure 1.7 The Orthobunyavirus species. Based on ICTV records (Fauquet and Fargette, 2005)

1.5 SIMBU SEROGROUP

Shuni virus belongs to the Simbu serogroup in the *Orthobunyavirus* genus of the family *Bunyaviridae*. Of the 21 Simbu serogroup viruses listed on the DVBID (www.cdc.gov/ncidod/dvbid/misc/organiz.htm), only three are shown to have been isolated in South Africa. These are SHUV (Coetzer & Erasmus, 1994), Simbu virus (Weinbren *et al.*, 1957) and Ingwavuma virus (McIntosh *et al.*, 1965). The literature however suggests that there are at least 24 members in this serogroup (Calisher, 1996; Da Costa Mendes, 1984; Kinney & Calisher, 1981) and that at minimum, seven of these have been isolated in South Africa (Da Costa Mendes, 1984; Howell & Coetzer, 1998). These discrepancies are brought about by the large volume of comprehensive studies which do not reach the international circuit.

In the last 15 years at least three new Simbu serogroup members have been identified around the world (Aguilar *et al.*, 2011; Bryant *et al.*, 2005; Hoffmann *et al.*, 2012). Members of this group are widely distributed in Asia, Africa, Australia and North and South America (Calisher, 1996), the first isolation from Europe was in 2011 with the emergence of Schmallenberg virus (Hoffmann *et al.*, 2012). Additionally some members have been isolated in locations not previously defined (Matsumori *et al.*, 2002; Yanase *et al.*, 2004; Yanase *et al.*, 2005), further emphasising the wide distribution of these viruses. Members include important pathogens that may cause disease in humans and animals. Oropouche virus (OROV) causes human febrile illness, whilst Akabane (AKAV) and Aino (AINOV) viruses are associated with abortions, still births and congenital defects in cattle, sheep and goats (Gonzalez-Scarano *et al.*, 1996). A summary of the Simbu serogroup is presented in Table 1.3.

The most comprehensive analysis based on antigenic relationships of the Simbu serogroup viruses was performed by Kinney & Calisher (1981). They showed that although most Simbu serogroup viruses were readily distinguished in neutralization assays, they exhibited complex relationships by complement fixation tests. On the basis of cross reactivity in complement fixation tests, the authors divided the serogroup into five serocomplexes, Simbu, Manzanilla, Oropouche, Thimiri and Nola (Kinney & Calisher, 1981). The first study to analyse the phylogenetic relationship among members of the Simbu serogroup was conducted by Saeed *et al.*, (2001a).

Table 1.3 Summary of the Simbu serogroup members, including geographic location, hosts and disease association

Virus	Geographic distribution	Previously identified in South Africa	Hosts	Human infection	Vectors	Disease association	References
Aino	Asia, Australia	No	Cattle	Possibly	*Culex sp.* *Culicoides spp.*	Hydranencephaly, arthrogryposis, hypoplasia and abortion	(Doherty *et al.*, 1972; Ishibashi *et al.*, 1994; Noda *et al.*, 1998; Takahashi *et al.*, 1968)
Akabane	Asia, Australia, Africa	Yes	Cattle, horses, sheep	NN	*Anopheles sp.* *Culex sp.* *Culicoides spp.*	Abortion, arthrogryposis, hydranecephaly, ataxia, tremors	(Della-Porta *et al.*, 1976; Doherty *et al.*, 1972; Furuya *et al.*, 1980; Howell & Coetzer, 1998; Matsuyama *et al.*, 1960; Metselaar & Robin, 1976)
Buttonwillow	North America	No	Rabbit, hare	NN	*Culicoides spp.*	Unknown	(Reeves *et al.*, 1970)
Douglas	Australia	No	Cattle	NN	*Culicoides spp*	Unknown	(Cybinski, 1984)
Facey's Paddock	Australia	No	Unknown	No	*Aedes sp.* *Culex sp.* *Culicoides spp.*	Unknown	(Doherty *et al.*, 1979; Doherty *et al.*, 1972)
Ingwavuma	Africa, Asia, Cyprus	Yes	Weaver, birds, pigs	NN	*Culex sp.*	Unknown	(Causey *et al.*, 1972; McIntosh *et al.*, 1965; Pavri *et al.*, 1969; Top *et al.*, 1974)
Iquitos	Peru	No	Humans	Yes	Unknown	Fever	(Aguilar *et al.*, 2011)
Jatobal	Brazil	No	Raccoon	NN	Unknown	Unknown	(Saeed *et al.*, 2001b)
Kaikular	India	No	Unknown	NN	*Culex sp.*	Unknown	(Rodrigues *et al.*, 1977)
Manzanilla	Trinidad	No	Monkey	NN	Unknown	Unknown	(Anderson *et al.*, 1960)
Mermet	North America	No	Birds	NN	*Culex sp.*	Unknown	(Calisher *et al.*, 1969; Jakob *et al.*, 1979)
Oropouche	Brazil, Peru, Panama, Trinidad, Tobago	No	Humans	Yes	Unknown	Fever	(Anderson *et al.*, 1961; Dixon *et al.*, 1981; Pinheiro *et al.*, 1981a; Pinheiro *et al.*, 1981b; Pinheiro *et al.*, 2004)
Oya	Malaysia, Vietnam	No	Pigs	NN	Mosquito pools	Respiratory disease, encephalitis	(Bryant *et al.*, 2005; Kono *et al.*, 2002)

Table 1.3 continued

Virus	Geographic distribution	Previously identified in South Africa	Hosts	Human infection	Vectors	Disease association	References
Para	Brazil, Argentina	No	Mouse	NN	*Culex sp.*	Unknown	(Saeed *et al.*, 2001a)
Peaton	Japan, Australia	No	Cattle	NN	*Culicoides spp.*	Unknown	(Matsumori *et al.*, 2002; St George *et al.*, 1979; St George *et al.*, 1980)
Sabo	Africa	Yes	Cattle, goats	NN	*Culicoides spp.*	Unknown	(Causey *et al.*, 1972; Causey *et al.*, 1969; Da Costa Mendes, 1984; Kemp *et al.*, 1971; Lee, 1979)
Sango	Nigeria, Kenya	No	Cattle	NN	*Mansonia sp*. *Culicoides spp.*	Unknown	(Causey *et al.*, 1969; Kemp *et al.*, 1973; Kemp *et al.*, 1971; Lee, 1979; Metselaar *et al.*, 1974)
Shamonda	Japan, Africa	Yes	Cattle	NN	*Culicoides spp.*	Unknown	(Causey *et al.*, 1972; Causey *et al.*, 1969; Kemp *et al.*, 1973; Kemp *et al.*, 1971; McIntosh, 1980; Yanase *et al.*, 2005)
Sathuperi	Japan, Africa	Yes	Cattle	NN	*Culex sp.* *Culicoides spp.*	Unknown	(Causey *et al.*, 1972; Dandawate *et al.*, 1969; Howell & Coetzer, 1998; Lee, 1979; Yanase *et al.*, 2004)
Schmallenberg	Europe	No	Cattle	Possibly	Unknown	Fever, diarrhoea, porencephaly	(Garigliany *et al.*, 2012; Hoffmann *et al.*, 2012)
Shuni	Africa	Yes	Cattle, goats, horses	Possibly	*Culex sp.* *Culicoides spp.*	Fever, encephalitis	(Causey *et al.*, 1972; Coetzer & Erasmus, 1994; Kemp *et al.*, 1973; McIntosh, 1980; Moore *et al.*, 1975)
Simbu	Africa	Yes	Unknown	NN	*Aedes sp.*	Unknown	(Weinbren *et al.*, 1957)
Thimiri	India, Egypt, Australia	No	Birds	NN	*Culicoides spp.*	Unknown	(Carey *et al.*, 1971; Darwish & Hoogstraal, 1981; Standfast & Dyce, 1982)
Tinaroo	Australia	No	Cattle	NN	*Culicoides spp.*	Unknown	(Cybinski, 1984; St George *et al.*, 1979)
Yaba-7	Nigeria	No	Unknown	NN	*Mansonia sp.*	Unknown	(Da Costa Mendes, 1984)
Utinga	Brazil	No	Sloth	No	Unknown	Unknown	(Seymour, 1985)
Utive	Panama	No	Sloth	No	Unknown	Unknown	(Seymour *et al.*, 1983)

NN – not known

Based on a high nucleotide sequence identity (>85%) and/or high amino acid sequence identity (>90%), 16 of the 19 Simbu serogroup viruses studied, were divided into five distinct phylogenetic lineages, lineage I could be further dived into four clades (Figure 1.8). Viruses within a group exhibited very little (<8%) amino acid divergence, while variation between different groups exhibited significant (20-40%) variation. These results corresponded well with those obtained by Kinney and Calisher (1981) (Saeed *et al.*, 2001a). There is greater divergence between members of the Simbu serogroup than between other members of the *Orthobunyavirus* genus. The reason for this is unclear; however possible explanations include; that the earlier establishment of Simbu serogroup, the great geographic distribution and vector association of this serogroup (Saeed *et al.*, 2001a).

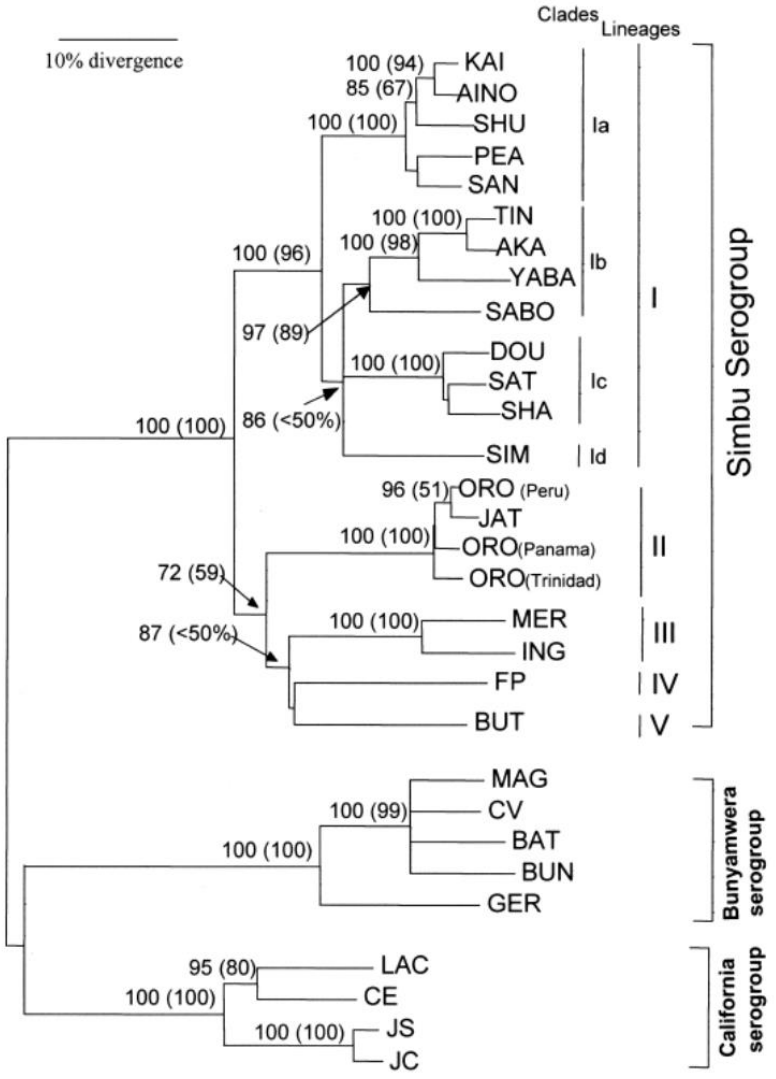


Figure 1.8 The phylogeny of the Simbu serogroup based on N ORF nucleotide sequence as determined by Saeed et al., 2001a

1.6 DIAGNOSIS OF ARBOVIRAL DISEASE

Roughly 125 arboviruses are known to cause illness in humans and animals. Diagnosis of arboviral infections can be simplified by the using the knowledge that geographical distribution, seasonality and vector presence are required for infection with these pathogens. Infection is confirmed by either the detection of the virus or antibodies against it. Virus isolation is often unsuccessful, due to low viraemia's and the probability that the virus may have been cleared by the time patients seek medical advice (Figure 1.9), for RVF however disease onset and viremia often coincide (Ikegami and Makino, 2011). Cases are thus more commonly confirmed by either a 4-fold change in serum antibody titre, the demonstration of viral antigen or viral RNA/DNA, or through the detection of specific IgM antibodies in blood, tissue or CSF (Alatoom & Payne, 2009). There are however several problems that exist in terms of interpreting IgM results. IgM antibodies can persist in a patients serum anywhere from a month to a few years (Sampathkumar, 2003), there is cross reactivity between different viruses within the same serocomplex (Martin *et al.*, 2002) and as such may lead to a misinterpretation of results. It is thus imperative that results are backed by both compatible clinical symptoms and plaque reduction neutralization tests (PRNT). The presence of IgM antibodies in the CSF is however confirmative of an acute infection.

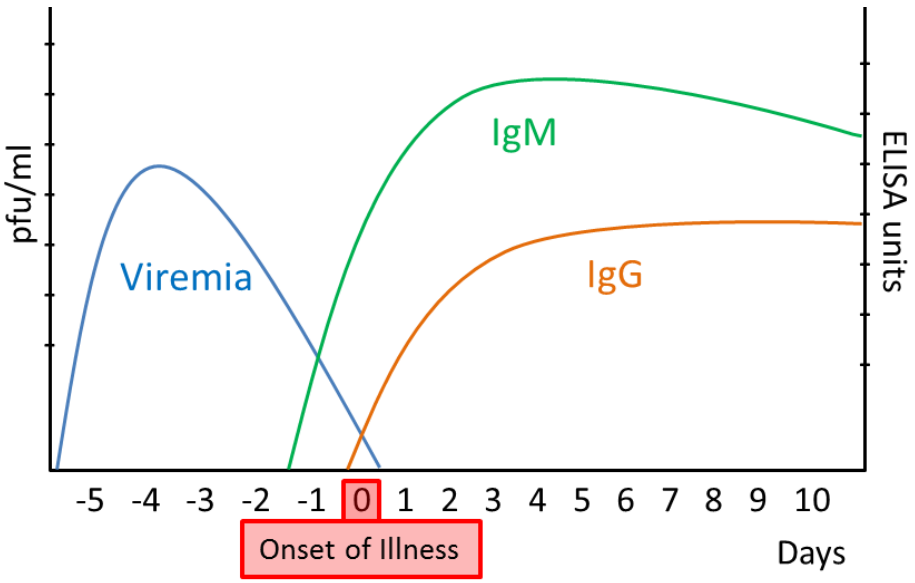


Figure 1.9 Diagram representing the viraemia phase, the onset of illness and the development of the antibody response following arbovirus infection (Authors own, based on graph by (Hazell, 2004).

Though real time PCR is the most sensitive method for the detection of the virus, it relies on the presence of viral particles and as viraemia is fleeting in arboviral disease, the detection of IgM antibodies remains the standard for diagnosing infection (Solomon *et al.*, 2003). Due to the non-specific symptoms of arboviral infections, an accurate clinical diagnosis may be problematic, which is why laboratory testing to confirm clinical suspicion is recommended. In this case, it would be imperative to consider other potential infectious agents that may present with similar clinical symptoms during a differential diagnosis.

1.7 SENTINEL ANIMAL SURVEILLANCE

A sentinel: one who watches or guards, to observe the approach of danger and give notice of it (Porter, 1913). Since the mid-twentieth century it has been recognised that animals can act as sentinels for a wide range of environmental health hazards (Halliday *et al.*, 2007). While working with *Culex tarsalis* mosquitoes and their transmission of western equine and St Louis encephalitis viruses, Dr Reeves, a medical entomologist, developed a method for tracking the insects, known as the "sentinel chicken" monitoring system. He found that infected chickens developed antibodies but did not become sick. Since chicken flocks are stationary, it became a useful way to detect the presence of infected mosquitoes in an area (Lenzer, 2004). If one looks at the importance of domestic and wild animal hosts in emerging human disease, it becomes clear that surveillance in animals is critical for the understanding and managing of emerging disease threats (Halliday *et al.*, 2007).

Animal sentinels may potentially be used to address a range of surveillance questions (Halliday *et al.*, 2007);

*Detection of a pathogen in a new area.
*Detection in the prevalence or incidence of a pathogen or disease over time.
*Determining the rates and direction of pathogen spread.
*Testing specific hypotheses about the ecology of a pathogen.
*Evaluating the efficacy of potential disease control interventions.

Recent examples of the emergence of zoonotic diseases in wildlife populations concurrent with novel outbreaks of human disease include; the identification of West Nile virus (WNV) in crows (*Corvus brachyrynchos*) during the 2002 epidemic in the United States (Watson *et al.*, 2004), palm civets (*Paguma larvata*) testing positive for Severe Acute

Respiratory Syndrome (SARS) in China, 2003 (Ng, 2003; Wang *et al.*, 2005), as well as the characterization of Avian Influenza H5N1 in poultry in Hong Kong, 1997-2002 (Shortridge *et al.*, 1998; Sims *et al.*, 2003). In 2002 the largest outbreak of WNV in horses was recorded in the USA, involving 15 257 cases (Dauphin *et al.*, 2004), this was followed by the largest outbreak in humans the following year (9 832 cases) (Hayes *et al.*, 2005).

Sentinel surveillance at the Zoonoses Research Unit (ZRU)

Several arboviral families; *Flavi*-, *Bunya*- and *Togaviridae* have emerged from Africa as new pathogens in previously unaffected regions and caused major epidemics and epizootics, including West Nile virus (WNV); Rift Valley fever (RVF) and Chikungunya virus (CHIKV) (Hollidge *et al.*, 2010). Following the emergence of West Nile virus lineage 1 (WNV1) as an important pathogen in Europe and North America (Ulbert, 2011), Venter et al. (2009), re-examined the pathogenicity and role of WNV lineage 2 (WNV2) in southern Africa. They proceeded to identify WNV2 as a cause of unexplained nervous disease of humans and horses in South Africa (Venter *et al.*, 2009; Venter & Swanepoel, 2010). Horses in particular are highly sensitive to some of these arboviruses and have thus been targeted as sentinel animals in the identification of zoonotic arboviruses associated with neurological disease in South Africa (Venter & Swanepoel, 2010). During the seasonal occurrence of more readily recognised vector-borne diseases such as African horse sickness and Equine encephalosis many horses exhibit febrile, neurological and fatal infections for which the aetiology remains unsolved.

1.8 VIRUS DISCOVERY TECHNIQUES

Viruses which have been discovered by molecular methods are diverse, with techniques such as simple cloning (Cotmore & Tattersall, 1984) and recombinant cDNA libraries (Choo *et al.*, 1989) being used before the development of the polymerase chain reaction (PCR) by the American biochemist Kary Mullis in 1983 (Mullis & Faloona, 1987). PCR is however based on primers that target specific genome regions which means it is only useful when searching for and amplifying specific virus genomes. Several adaptations to PCR were developed in the 1990's including, ligation of primer binding sites to DNA fragments, as well as sequence enrichment by amplification. The more well recognized techniques comprise representational difference analysis (RDA) (Lisitsyn & Wigler, 1993), sequence independent single primer amplification (SISPA) (Reyes & Kim, 1991) and arbitrarily primed PCR (McClelland *et al.*, 1993; Welsh & McClelland, 1991).

1.8.1 Representational difference analysis (RDA)

RDA combines the techniques of subtractive hybridization with gene amplification to detect differences between two similar clinical samples (Lisitsyn & Wigler, 1993), generally pre- and post-infection samples. The two DNA samples are hybridized together so as to reduce common sequences, leaving mainly viral sequences for downstream analysis. RDA reduces the genome complexity through restriction endonuclease digestion of the genomic DNA and subsequent amplification following the addition of specific linkers to the resulting DNA fragments. After obtaining the restriction enzyme representations of the two samples, a second set of specific links are then denatured and allowed to re-anneal. This is followed by PCR amplification of only those fragments where both strands are from the 'post-infection' sample are amplified in an exponential fashion. Thus sequences common to the pre- and post-infection samples are eliminated. The limitation of RDA is thus that two highly matched sources are required for efficient recovery of difference products. RDA was utilized in the discovery of a gamma herpes virus in AIDS associated Kaposi's sarcoma (Chang *et al.,* 1994) and in the identification of two flavivirus like genomes, GB viruses A and B a hepatitis patient (Simons *et al.,* 1995).

1.8.2 Sequence independent single primer amplification (SISPA)

SISPA was developed to amplify viral nucleic acids of unknown sequence at a low concentration (Reyes & Kim, 1991). The technique was based on the previously published methods of primer-directed enzymatic amplification (Akowitz & Manuelidis, 1989) and the digestion of chromosomal DNA (Johnson, 1990). SISPA involves endonuclease restriction of target DNA followed by the directional ligation of an asymmetric adapter onto both termini of a blunt ended cDNA. The common end sequences of the adapters allows the cDNA to be amplified in subsequent PCR using a single primer (Ambrose & Clewley, 2006). SISPA has the advantages of allowing for the identification of unknown viral nucleic acids in limited amounts, is culture independent and can detect ssRNA, dsRNA and DNA viruses. The major disadvantage of this technique is the requirement for virus particle purification to eliminate contaminating host and mitochondrial DNA which is highly time consuming. SISPA is associated with the discovery of GB virus C (Linnen *et al.*, 1996), bovine parvovirus (Allander *et al.*, 2001), Parvovirus 4 and TT-like viruses (Jones *et al.*, 2005).

1.8.3 Random PCR

Random PCR is based on the theoretical amplification of all nucleic acids present in a sample using PCR primers with a random nucleotide sequence at the 3' end and a defined sequence at the 5' end (Froussard, 1992). A subsequent PCR step is carried out with a second primer that is complementary to the 5' end of the first primer. The technique is similar to SISPA but removes the need for an adapter ligation step, which can render SISPA inefficient. Random PCR was used in the detection of HSV-1 (Stang *et al.*, 2005) and calicivirus RNA (Liu *et al.*, 1999).

1.8.4 Virus discovery cDNA AFLP (VIDISCA)

VIDISCA is based on the same principles as SISPA but uses two primers rather than one in the PCR amplification step and is done in the amplified fragment length polymorphism technique (AFLP). The DNA is digested with two frequently cutting restriction enzymes; only fragments with dissimilar overhangs are amplified in subsequent PCRs. The use of two adapters and two primers makes VIDISCA more sensitive and specific than SISPA (Ambrose & Clewley, 2006). VIDISCA also allows for a nested PCR step. This technique was linked to the discovery of a new human coronavirus HCoV-NL63 (van der Hoek *et al.*, 2004).

1.8.5 Arbitrarily Primed PCR

Arbitrary primed PCR uses single arbitrarily chosen primers and involves two cycles of low stringency amplification followed by PCR at a higher stringency. Amplification at low temperatures allows the primers to bind at many partially complementary sites, before more stringent and specific conditions are encountered (McClelland *et al.*, 1993; Welsh & McClelland, 1991). hMPV, a new human pneumovirus was discovered using this technique (van den Hoogen *et al.*, 2001).

1.8.6 Next Generation Sequencing (NGS)

There are many high throughput sequencing platforms available worldwide, but 454 Sequencing, Illumina and Ion Torrent are the most common platforms available will be described here.

1.8.6.1 Sample Preparation

The key to NGS techniques, is increasing the levels of viral nucleic acids while reducing background, prokaryotic and eukaryotic nucleic acids (Delwart, 2007), as these methods will amplify any nucleic acid present in a sample (Braham *et al.*, 2009). Enrichment of viral nucleic acids by particle purification of virus-like particles (Melcher *et al.*, 2008; Victoria *et al.*, 2008) partially achieves this. This is still not entirely generic since viruses with unstable particles or non-encapsidated agents, cannot be isolated (Kreuze *et al.*, 2009). Samples can be partially purified through a combination of filtration, density-dependent centrifugation in a caesium chloride gradient or ultracentrifugation (Braham *et al.*, 2009; Breitbart *et al.*, 2002; Ng *et al.*, 2009; Thurber *et al.*, 2009). Free nucleic acids are then removed through digestion with DNase, RNase or a combination of both, before DNA/RNA extraction is carried out (Allander *et al.*, 2001; Braham *et al.*, 2009; Jones *et al.*, 2005; Victoria *et al.*, 2008).

1.8.6.2 454 Sequencing

The 454 Life Sciences (454: Branford, CT, USA; now Roche Basel) sequencing platform was the first next generation technology to reach the market. 454 pioneered solutions to the three bottlenecks of high throughput sequencing; library preparation, template preparation and sequencing (Rothberg & Leamon, 2008). The first commercially available next-generation sequencer, the GS-20 was released in 2005. The technical specifications and sequencing chemistry of 454 are summarized in Table 1.4 and Figure 1.10.

454 Sequencing technology was the first technology other than Sanger's to sequence and assemble bacterial genomes *de nova* (Margulies *et al.*, 2005) and the first non-Sanger technology to sequence an individual human genome (Wheeler *et al.*, 2008). Other notable studies include revealing the complexity of rearrangements between individual human genomes (Korbel *et al.*, 2007), providing new approaches to understand infectious diseases (Palacios *et al.*, 2008) and sequencing the first million base pairs of a Neanderthal genome (Green *et al.*, 2006; Noonan *et al.*, 2006). 454 sequencing may also be advantages for resolving sequences with repetitive structures, palindromes or for metagenomic analysis, given the long read lengths obtained (Luo *et al.*, 2012). 454 sequencing was also used to determine the genome and phylogentically characterise the novel orthobunyavirus Leanyer virus (Savji *et al.*, 2011), as well as to identify Schmallenburg virus, as the novel orthobunyaviral cause of disease in cattle in 2011 (Hoffmann *et al.*, 2012).

1.8.6.3 Illumina

Illumina sequencing technology (Illumina, Inc, San Diego, CA, USA), leverages on a proprietary reversible terminator technology for rapid and large scale sequencing. Technical specifications are listed in Table 1.4 and the sequencing chemistry illustrated in Figure 1.11. Illumina sequencing has been employed in the characterization of quasispecies of pandemic 2009 influenza virus A virus (Kuroda *et al.*, 2010) and the complete sequencing of human cytomegalovirus genomes from cell cultures and clinical specimens (Cunningham *et al.*, 2010). This technology was also used to sequence the genome and transcriptome from metastatic tissues, from an individual with highly aggressive prostate cancer, proving personalized oncology may be viable (Wu *et al.*, 2012).

1.8.6.4 Ion Torrent

Ion Torrent or Ion Semiconductor Sequencing (Ion Torrent Systems Inc., San Francisco, USA) is a method of DNA sequencing based on the detection of hydrogen ions that are released during polymerization of DNA; this technology was made available in 2010. Ion Torrent technology differs from other sequencing technologies, in that no modified nucleotides or optics are used. In nature, the incorporation of a dNTP into a growing DNA strand, involves the formation of a covalent bond and the release of pyrophosphate and a positively charged hydrogen ion (www.iontorrent.com) and it is this chemistry that Ion torrent applies (Figure 1.12).

Due to the ability of alternative sequencing methods to achieve greater read lengths, Ion-Torrent technology may be best suited to small scale applications, such as microbial genome and transcriptome sequencing, amplicon and target sequencing (Perkel, 2011). It has been applied in the determination of the genetic diversity of the population structure of *Sarcophilus harrisii* through the sequencing of two full genomes (Miller *et al.*, 2011). This technology was also used to elucidate the unique genetic characteristics of a highly virulent Shigella toxin producing *E.coli* 0104:H4 (Mellmann *et al.*, 2011) and in 2012, was used for the genomic typing of meningococci, as a future tool in laboratory surveillance and outbreak investigation (Vogel *et al.*, 2012).

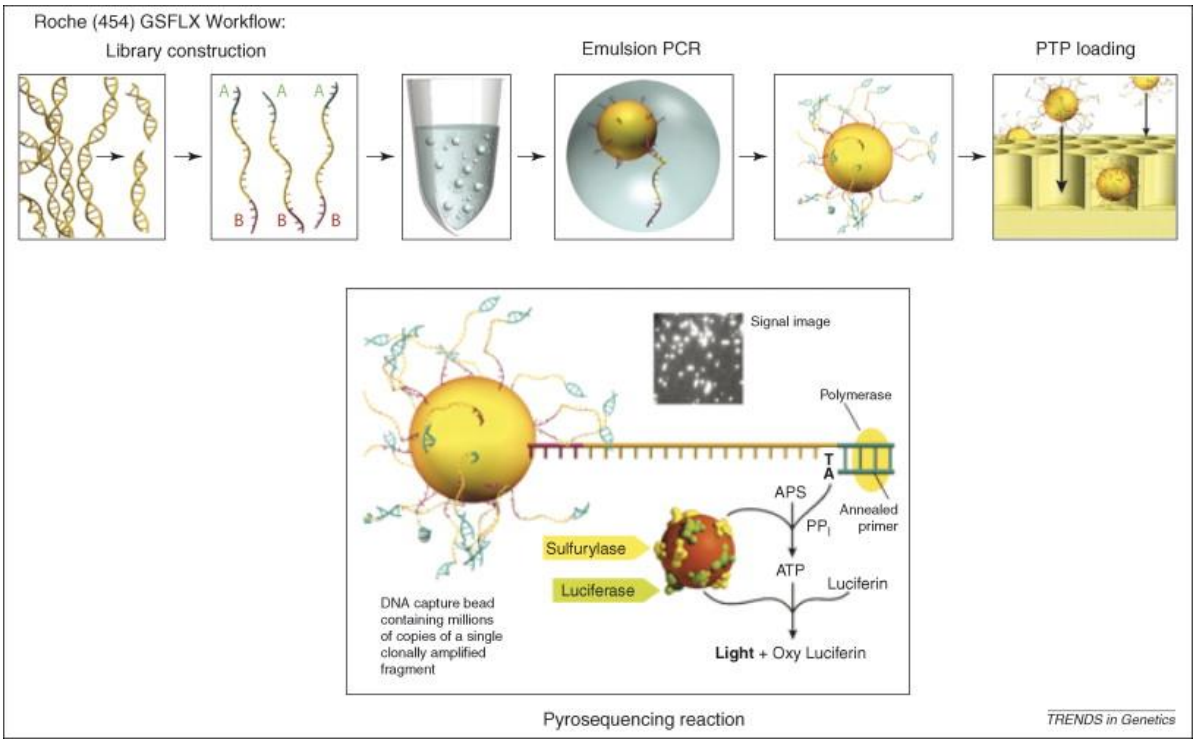


Figure 1.11 454 Workflow: library construction ligates 454-specific adapters to DNA fragments and couples amplification beads with DNA in an emulsion PCR to amplify fragments before sequencing. The beads are loaded into the picotiter plate (PTP). The bottom panel illustrates the pyrosequencing reaction that occurs on nucleotide incorporation to report sequencing by synthesis (Mardis, 2008).

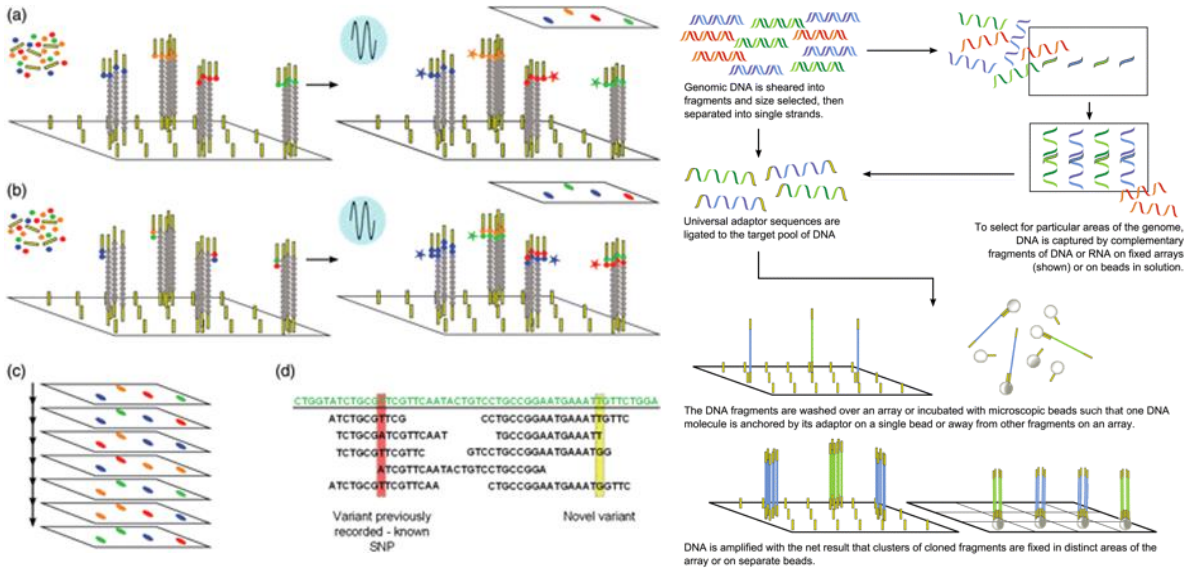


Figure 1.10 The Illumina sequencing process: (**A**) Arrays are washed with a solution containing non-specific primers, DNA polymerase enzyme and each of the four deoxyribonucleotides. Bases are incorporated into the sequence, complementary to the amplified template. After incorporation, laser excitation causes each base to fluoresce differently and the fluorescence is recorded. (**B**) The fluorescent tag is cleaved from the last base and a temperature cycle starts. During subsequent cycles, new bases are added and the fluorescence pattern is recorded. (**C**) The fluorescence pattern is used to construct the sequence at each position on the array. (**D**) Sequence fragments are aligned to the reference sequence (Raffan & Semple, 2011).

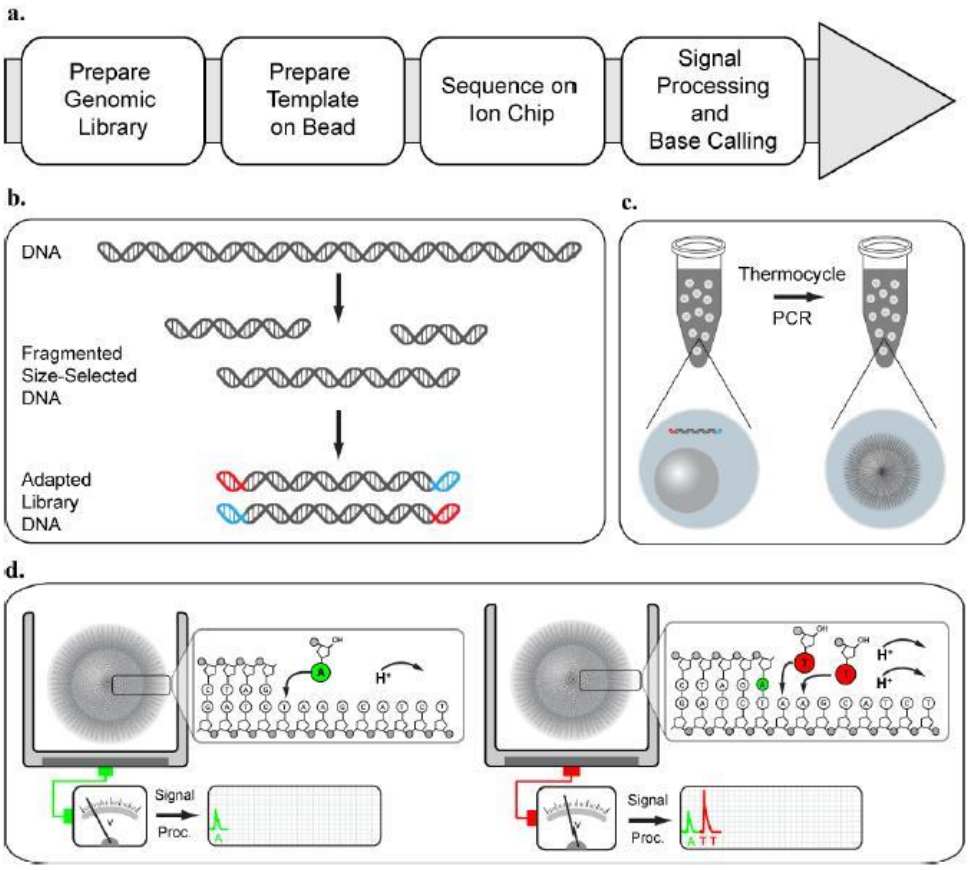


Figure 1.12 Ion Torrent workflow: (A) Overview of ion sequencing work flow, (B) Prepare genomic library, DNA is fragmented, sized, and forward and reverse adapters ligated, (C) Amplify template on bead, adapter-ligated libraries are clonally amplified onto beads, (D) Sequence on ion chip, sequencing primers and DNA polymerase are bound to the template-carrying beads are pipetted into the chip's loading port. The chip is installed in the sequencing instrument; all four nucleotides cyclically flowed in an automated 2- hour run. Signal processing, software converts the raw data into measurements of incorporation in each well for each successive nucleotide flow (Carr, 2012).

1.8.6.5 Bioinformatics for NGS

Although many laboratories have staff with the technical expertise to adapt to performing high throughput sequencing the overwhelming amount of sequence data generated, creates new challenges, requiring significant investment in bioinformatics infrastructure and personnel with programming expertise (Anderson & Schrijver, 2010). After the sequences have undergone quality assessment, the genomic sequences need to be reconstructed, either through alignment to a reference genome or *de novo* assembly. To perform efficient alignment of short read sequences data to a reference genome, a variety of methods have been developed. The two most common strategies are to either, convert the sequence data into a series of unique integer values, or to perform a Burrows-Wheeler transform to construct a matrix of all the possible rotations of a given sequence (Anderson & Schrijver, 2010).

Table 1.4 Summary of the technical specifications of three NGS platforms

Platform	Sequencing chemistry	Amplification approach	Read length	Total bases per run	Potential disadvantages	References
454 Sequencing	Pyrosequencing	Emulsion PCR	400bp	500Mb	Highest cost per base Long read length often unnecessary	(Margulies *et al.*, 2005; Rothberg & Leamon, 2008; Voelkerding *et al.*, 2009)
Illumina	Polymerase based sequence analysis	Bridge amplification	2x150bp	20Gb	Suppression of GC-rich reads Increased sequencing time	(Luo *et al.*, 2012; Quail *et al.*, 2008; Quail *et al.*, 2012)
Ion Torrent	Ion semiconductor sequencing	Emulsion PCR	150bp	1Gb	Reads may be too short for *de novo* assembly Not recommended for AT-rich genomes	(www.iontorrent.com) (Quail *et al.*, 2012)

In 2010, more than 20 short-read alignment softwares had been published a selection of these are listed in Table 1.5. BWA (Burrows-Wheeler aligner) works well for 454 reads, allowing gaps and clipping. Bowtie and MAQ (Mapping and assembling with qualities) allow base quality scores to be used, improving alignment accuracy (Magi *et al*., 2010). To perform *de novo* genomic assembly long stretches of DNA sequence must be created from shorter read length data. With Sanger technology the relatively long reads allows for sequence assembly based on the degree of overlap between sequencing reads. This application however is not computationally feasible for the short reads produced by NGS systems, thus new algorithms were developed which analyse the data as small fixed-length subsequence's (Anderson & Schrijver, 2010). Those algorithms have been incorporated into software programs, some of which are listed in Table 1.6.

Recently a company called CLC bio, developed a genome analysis tool that could carry out both read mapping and *de novo* assembly (Table 1.2), of sequences from data generated from any of the existing NGS platforms. CLC Genomics Workbench includes High Performance Computing accelerated assembly of High-Throughput Sequencing data as well as a large number of downstream analysis tools. CLC Genomics Workbench is the first comprehensive analysis package which can analyse and visualize data from all major NGS platforms, like SOLiD, 454, Sanger, Illumina and Ion Torrent (www.clcbio.com).

Once the sequences have been recreated, sequence similarity searches need to be carried out, studies however have shown that at least 50-60% and more typically 90% of the resulting DNA reads did not encode proteins that were significantly similar to others encoded in known genes of either viral or cellular origin (Angly *et al.*, 2006; Bench *et al.*, 2007). This low percentage of sequences with detectable similarity to known viral proteins might indicate that most of the sequences may represent novel virus genes that have no matches in the database because the diversity of viruses has not been adequately sampled (Breitbart & Rohwer, 2005). Alternatively despite all enrichment efforts many of the reads could be of cellular origin and have no matches in the database because these genes belong to the poorly conserved fraction of the pan-genome of cellular organisms (Koonin & Wolf, 2008; Lapierre & Gogarten, 2009).

Table 1.5 Programs available for short read alignment to reference sequences

Tool	Platform	Title	Author
SOAP	Illumina	SOAP: short oligonucleotide alignment program	(Li *et al.*, 2008b)
MAQ	Illumina, SOLiD	Mapping short DNA sequencing reads and calling variants using mapping quality scores	(Li *et al.*, 2008a)
SeqMap	Illumina	SeqMap: mapping massive amount of oligonucleotides to the genome	(Jiang & Wong, 2008)
BWA	Illumina, SOLiD,454	Fast and accurate short read alignment with Burrows-Wheeler Transform	(Li & Durbin, 2009)
Bowtie	Illumina	Ultrafast and memory-efficient alignment of short DNA sequences to the human genome	(Langmead *et al.*, 2009)
CLC genomics workbench	Illumina, SOLiD,454, Ion Torrent	CLC bio. A result of science	(www.clcbio.com)

Table 1.6 Programs available for de novo sequence assembly

Tool	Title	Author
ABySS	ABySS: a parallel assembler for short read sequence data.	(Simpson *et al.*, 2009)
VCAKE	Extending assembly of short DNA sequences to handle error	(Jeck *et al.*, 2007)
SSAKE	Assembling millions of short DNA sequences using SSAKE	(Warren *et al.*, 2007)
Edena	De novo bacterial genome sequencing: millions of very short reads assembled on a desktop computer	(Hernandez *et al.*, 2008)
CLC genomics workbench	CLC bio. A result of science	(www.clcbio.com)

PROJECT AIMS

- Investigation of unsolved cases of neurological disease in sentinel animals that tested negative for WNV and other common causes of neurological disease, using molecular virus discovery techniques to identify additional arboviruses which may pose a zoonotic risk to humans in South Africa.
- Characterization of the molecular biology of the newly identified agent (SHUV) through full genome analysis.
- Development of specific assays to detect this virus and to investigate the epidemiology of the identified agent (SHUV) within South Africa.
- Serological investigation into the prevalence of SHUV relative to WNV in humans with a high risk of exposure, in South Africa.

Specific Objectives

- To establish a random amplification technique for the identification of potentially novel agents that could be applied in a specialized viral diagnostic setting.
- To identify novel viruses in viral isolates and clinical specimens from animals with neurological disease, through electron microscopy, family specific PCRs and randomly primed amplification.
- To develop SHUV specific assays, including both a real-time nested PCR and a neutralization assay for use in both diagnostic and epidemiological investigations.
- To determine and annotate the full genome sequence of SHUV.
- To establish the epidemiology of SHUV within South Africa.
- To screen serum samples from veterinarians from across South Africa for the presence of WNV and SHUV antibodies, using IgG ELISAs (WNV) and neutralization assays (WNV and SHUV) to identify the risk of human infection.

CHAPTER 2
Establishment of a random amplification technique to solve undiagnosed neurological infections

2.1 INTRODUCTION

Arboviruses (arthropod-borne viruses) are maintained in nature by haematophagous arthropods such as mosquitoes, ticks, *Culicoides* midges and sand-flies, principally by biological transmission between susceptible vertebrate hosts. Many zoonotic arboviruses, are capable of causing major outbreaks, sometimes with severe morbidity and high mortality rates and are important emerging and re-emerging diseases (Weaver & Reisen, 2010). Several mosquito-borne zoonotic viruses in the families *Flavi-*, *Bunya-* and *Togaviridae* have emerged from Africa as new pathogens in previously unaffected regions and caused major epidemics and epizootics, including West Nile virus (WNV); Rift Valley fever (RVF) and Chikungunya virus (CHIKV) (Hollidge *et al.*, 2010).

Horses in particular are highly sensitive to some of these viruses and have thus been targeted as sentinel animals in the identification of zoonotic arboviruses associated with neurological disease in South Africa (Venter & Swanepoel, 2010). During the seasonal occurrence of more readily recognised vector-borne diseases such as African horse sickness and Equine encephalosis, many horses exhibit febrile, neurological and fatal infections for which the aetiology remains unsolved. Following the emergence of West Nile virus lineage 1 (WNV1) as an important pathogen in Europe and North America (Ulbert, 2011). Venter *et al*. (2009), re-examined the pathogenicity and role of WNV lineage 2 (WNV2) in southern Africa. They proceeded to identify WNV2 as a cause of unexplained nervous disease of humans and horses in South Africa (Venter *et al.*, 2009; Venter & Swanepoel, 2010). There were however numerous residual cases in which no diagnosis could be established.

Many advances in the development of virus discovery techniques have been made in recent years, each having their own advantages and disadvantages. In instances where the agent can be placed within a family or antigenic group, through electron microscopy or

serological analysis, sequence specific amplification still remains the preferred method of virus identification. For this route to be taken however, the pathogen would have to be significantly similar to an already known agent. In those instances where specific amplification is not possible, the sequence independent techniques of representational difference analysis (RDA) (Lisitsyn & Wigler, 1993), sequence independent single primer amplification, SISPA (Reyes & Kim, 1991), as well as random and arbitrarily primed (McClelland *et al.*, 1993; Welsh & McClelland, 1991) PCR become the methods of choice. Here, although any agent can theoretically be amplified, the processing of samples and the removal of contaminating DNAs presents a significant challenge. Recent developments in next generation sequencing (NGS) have enabled sequence independent pathogen discovery, however the low level of RNA/DNA found in clinical specimens necessitates sequence independent amplification of viral nucleic acids.

The objective of this chapter was to establish a random amplification strategy that could be used on a routine basis in a specific diagnostic or research setting, with the aim to identify potentially novel viruses associated with neurological disease in animals in South Africa.

2.2 METHODS

2.2.1 Clinical specimens and isolates

Kunjin virus (KUNV):

The MRM16 strain of KUNV was used as a known positive control in the establishment of this technique as it is a BSL2 pathogen, having been shown not to cause neurological disease or death in mice (Venter *et al.*, 2005). This allowed for numerous experiments to be performed and repeated without the need for special safety precautions.

SAE 18/09:

A cell culture isolate from a horse that had succumbed to neurological disease in 2009 was identified as Shuni virus (Chapter 3), very little sequence data was available for this virus at the time. The isolate was thus included to determine whether the random amplification technique could be used to acquire additional sequence information for uncharacterised viruses.

The following specimens were obtained through our surveillance program, where a causal agent of disease could not be identified.

SAE 66/09:

Near the end of May 2009, a 4 year old female Friesian, from Rust de Winter in the Limpopo province, collapsed and died during transport to an equestrian event in the Gauteng province. The owner had reported that the horse had appeared sick the previous day. Post mortem findings included severe anaemia, the abdomen filled with watery blood and no clotting of blood. Various tissue and fluid samples were sent for virological analysis.

SAE 112/11:

In May 2011, we received a cell culture isolate cultivated from the blood of a horse that had displayed symptoms of fever and congestion suggestive of an African horse sickness virus (AHSV) infection. The 6 year old mare was from the Bronkhorstspruit area of Gauteng, and survived the infection with no significant sequelae. At the time no causal agent could be identified.

2.2.2 Establishment of arbitrarily primed PCR technique

2.2.2.1 Particle purification and RNA extraction

This particle purification technique is a modified version of the method described by Victoria *et al*. (2008). In brief; cell cultures isolates were grown up in 75cm^3 flasks (3 per isolate) until 80% CPE was observed. The cultures were then freeze-thawed three times to disrupt the cells, hereafter they were spun down at 11 000g for 10 minutes to remove cellular debris. The 60ml (20ml x 3) was then put through a 0.2µm filter. For each 1 000µl filtered supernatant, 0.1g PEG-6000 (Merck, Darmstadt, Germany) and 0.02g NaCl (Merck), were added and the reactions incubated on ice with agitation for one hour and then on ice overnight. Samples were then spun at 11 000g for 20 minutes and the supernatant removed. The pellet was resuspended in 100µl 1x phosphate buffered saline (PBS) and incubated at room temperature for 10 minutes. 25 U Benzonase (Merck), 2.5 U DNase (Merck), 10µg/µl RNase (Merck), 42mM $MgCl_2$ and 40µl DNase buffer were then added and the reactions incubated at 37ºC for 90 minutes. 5mM EDTA was then added to stop the reaction. Samples were extracted with the RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.2.2.2 Reverse transcription

First strand cDNA synthesis was achieved by denaturing 10µl RNA and 20pmol of primer G52B, a Respiratory Syncytial virus (RSV) primer, selected due to its high melting temperature and non-specificity to arboviruses (Christensen *et al.*, 1999), at 65°C for 10 minutes. The reaction mixtures where then cooled on ice for 2 minutes, following this 10 mM dNTP mix, 4 µl 5x RT buffer, 100mM DTT solution, 50 U RNase inhibitor (Roche, Mannheim, Germany) and 50 U Expand reverse transcriptase (Roche) were added to each reaction. The reaction mix was then heated to 30°C for 10 minutes, 42°C for 45 minutes and then placed on ice.

2.2.2.3 PCR amplification

PCR reactions were performed using primer G52B and the Expand High Fidelity^{PLUS} PCR System (Roche, Mannheim, Germany) to produce multiple amplicons. The PCRs were conducted in two steps; firstly, an 11µl reaction using 6µl cDNA, 2µl 5x reaction buffer (no salt), 2mM of each dNTP, 100pmol primer, 40mM $MgCl_2$ and 1 U of Expand High Fidelity^{PLUS} enzyme mix. Reaction mixes were subjected to 94˚C for 2 minutes, 2 cycles: 94˚C, 1 min; 36˚C, 5 mins; 72˚C, 5 minutes, followed by 10 cycles: 94˚C, 1 min; 47˚C, 1 min; 72˚C, 2 minutes. To this reaction a further 18µl 5x reaction buffer, 10mM of each dNTP, 4 U of Expand High Fidelity^{PLUS} enzyme mix and 70 µl Nuclease free water were added to obtain a final reaction volume of 100µl. Reaction mixes were subjected to 30 cycles: 94˚C, 1 min; 60˚C, 1 min; 72˚C, 2 minutes, followed by a final extension of 72˚C for 7 minutes.

2.2.3 Differential diagnosis

All specimens were also screened for EHV (Kirisawa *et al.*, 1993), AHSV (Venter *et al.*, 2006a), EEV (van Niekerk *et al.*, 2003), WNV (Zaayman *et al.*, 2009), as well as alpha- and flaviviruses using family specific PCRs (Sanchez-Seco *et al.*, 2001; Zaayman *et al.*, 2009).

2.2.4 Cloning and sequencing

Amplicons were cloned using the CloneJET™ PCR Cloning Kit (Fermentas Life Sciences, Burlington, ON), using the manufacturer's instructions and screened by colony PCR according to the supplier's recommendations (Fermentas Life Sciences, Burlington, ON). After gel electrophoresis, amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions

and subjected to cycle sequencing using the Big Dye® Terminator V3.1 Kit as recommended by the supplier (Applied Biosystems, Foster city, CA). Reactions were precipitated using the EDTA/NaOAc/EtOH method according to the BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, 2002).

2.2.5 Phylogenetic analysis

Sequences were edited using Sequencher v4.6 and aligned using the ClustalW subroutine, which forms part of the Bioedit program (Hall, 1999). Sequences were then subject to BLAST analysis (Altschul *et al.*, 1990) and maximum likelihood trees were generated with Mega version 5 using 1000 bootstrap analysis and the Tamura-Nei model. P-distance analyses were carried out for nucleotide sequences using Mega v5 (Tamura *et al.*, 2011).

2.3 RESULTS

2.3.1 Random amplification optimization

The random amplification technique employed was based on the techniques of Welsh and McClelland, 1990 (Welsh & McClelland, 1991) and Williams et al. 1990 (Williams *et al.*, 1990). Different approaches were investigated (Refer to 2.3.2, Appendix A) and a final method selected (Refer to 2.2.2.1-2.2.2.3). Repeatability and specificity of the method was proven through the amplification and sequencing of Kunjin virus (KUNV) in multiple runs. In each instance a mock cell culture sample was also included, so as to determine a background contamination cut-off point, which was determined by gel electrophoresis.

2.3.2 Arbitrarily primed PCR on tissue samples (SAE 66/09)

Cell culture isolation was not attempted in this case and as such no family data was available, resulting in the need for a random amplification strategy. This case was investigated before the final optimization of the arbitrarily primed PCR technique, with the differences being as follows: during viral particle purification, the 10% tissue suspension supernatant was not put through a 0.2 µm filter, nor was the Benzonase (Merck) enzyme used. Additionally, the cDNA was made with the use of random hexamer primers (Appendix A). The results, although not as distinct as in the following case of SAE 112/11, showed unique bands in the samples that were distinct from the mock PCRs (Figure 2.1). These bands (lanes 11, 14 and 15) were individually cloned and sequenced.

Blast search analysis of the clones for SAE 66/09, revealed 50% to be contaminating host DNA and the other 50% to be *Clostridium perfringens* (Figure 2.2). Maximum likelihood phylogenetic analysis placed SAE 66/09 together with representative *C. perfringens* isolates. SAE 66/09 shared an average 98% identity with *C. perfringens* and 95% with *C. botulinum*. It is important to note that the finding of a bacterial agent is in this instance, directly associated with the inefficient particle purification system employed at this stage of the optimization process. This however does not diminish the significance of the finding, as although *C. perfringens* occurs as a colonizing agent in animals, it can cause disease, the symptoms of which are consistent with the clinical presentation in this case.

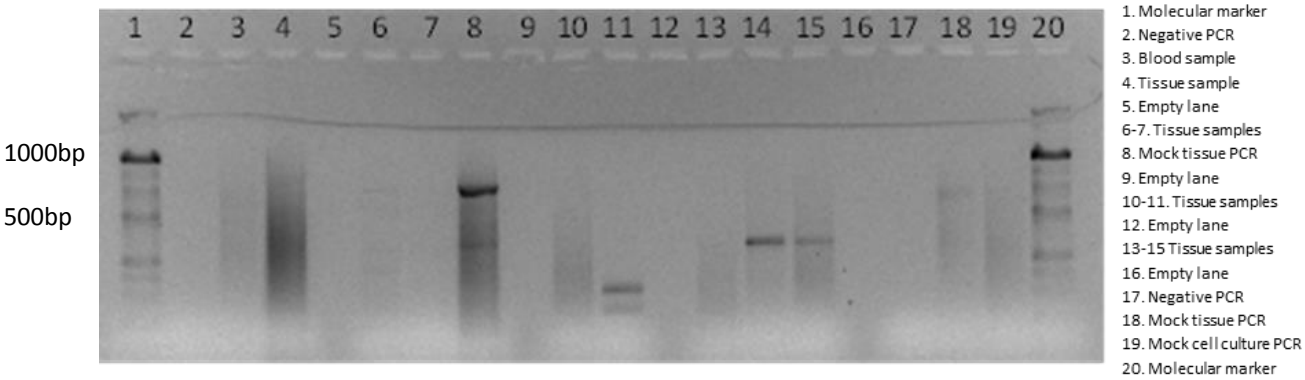


Figure 2.1 Randomly amplified PCR products from tissue samples, separated on a 1.5% agarose gel.

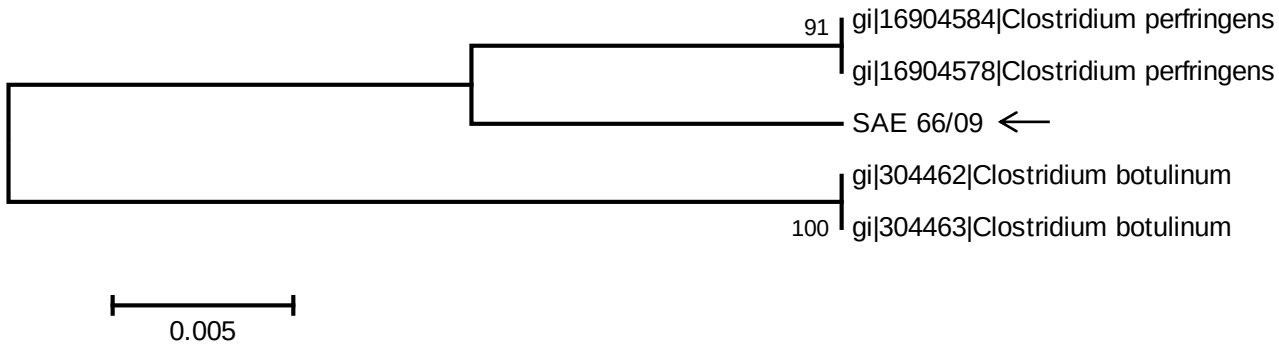


Figure 2.2 Maximum likelihood tree constructed using the Tamura-Nei model of MEGA v5, for a clone from the tissue sample of SAE 66/09. The bar indicates 0.005 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1000 replicates. Only values >70 are shown. Reference strains GenBank accession numbers are indicated.

2.3.3 SAE 18/09

Using the still unoptimized technique as discussed for SAE 66/09 (Refer to 2.3.2, Appendix A), randomly amplified fragments were generated for SAE 18/09 (Figure 2.3). The unique bands were cloned and produced 1000bp and 1300bp amplicons respectively. These amplicons were then sequenced, the larger amplicon contained non-specific sequences, but the 1000bp amplicon produced a 780bp fragment of the L segment of SHUV. These 780bp were the first sequenced for the L segment of SHUV and allowed for primer walking and further sequencing of this genomic segment (Chapter 4).

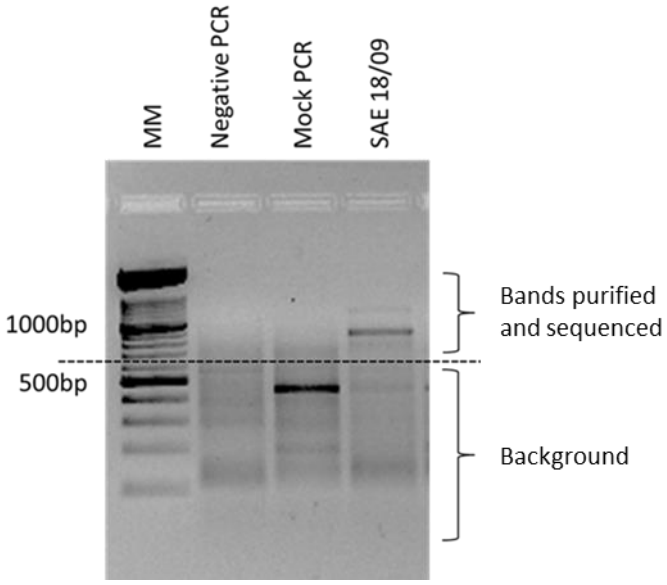


Figure 2.3 Randomly amplified PCR products of SAE 18/09, separated on a 1.5% agarose gel. Dashed line separation between products considered to be background and those to be purified and cloned.

2.3.4 SAE 112/11

The cytopathic agent isolated from the blood of case SAE 112/11 was not identified as one of the common horse pathogens tested for in our laboratory, and electron microscopic (EM) examination of negative stained preparations of culture fluid, revealed particles resembling bunyaviruses, the size of 47nm was however more representative of the togaviruses. Due to the inconclusive EM results, random amplification was carried out in this instance. The optimized random amplification technique as described in Section 2.2.2 was used in this instance. PCR amplification of the isolate with the random primer G52B produced amplicons between 200-1400bp. Amplicons larger than 500bp were cloned and sequenced as they were considered to be free of background through comparison with the mock PCR (Figure 2.4). Blast search analysis revealed that 85% of the clones were

Middelburg virus (MIDV), a member of the *Alphavirus* genus in the family *Togaviridae*, the other 15% were non-specific. Maximum likelihood phylogenetic analysis indicated high bootstrap support (99%) for the placement of SAE 112/11 with representative MIDV isolates. The isolate shared an average 97.5% identity with MIDV and 75% with Sindbis and Semliki forest viruses (Figure 2.5).

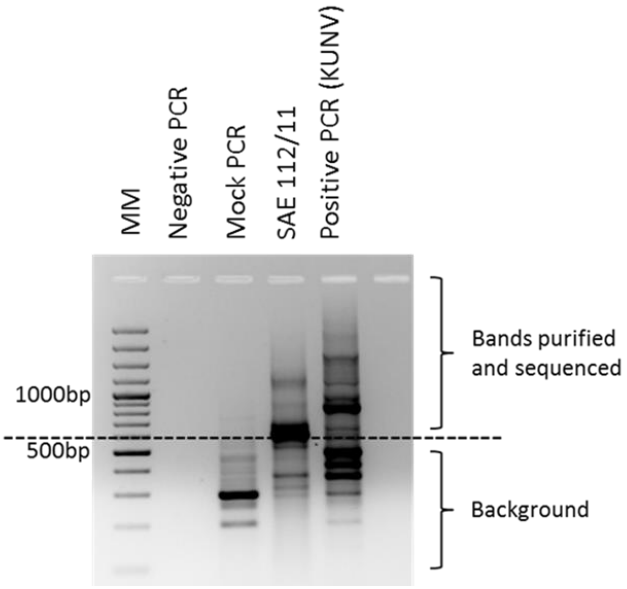


Figure 2.4 Randomly amplified PCR products of SAE 112/11, separated on a 1.5% agarose gel. Dashed line separation between products considered to be background and those to be purified and cloned.

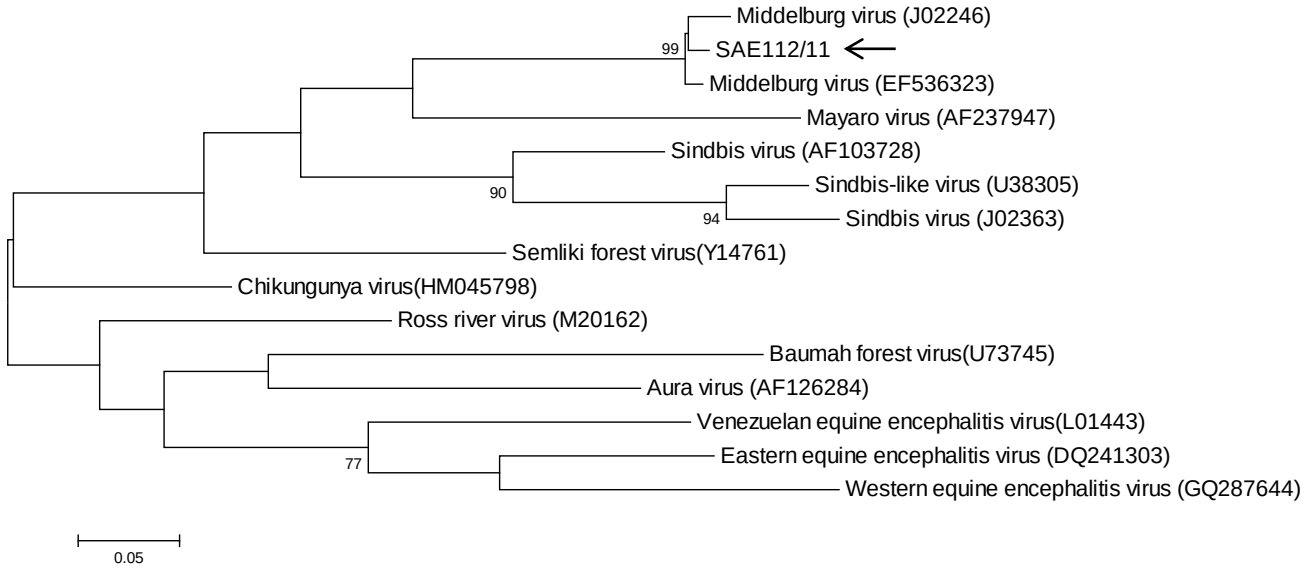


Figure 2.5 Maximum likelihood tree constructed using the Tamura-Nei model of MEGA v5, of a 196bp fragment of the nsp4 gene of representative alphaviruses. The unknown isolate SAE 112/11 is identified as Middelburg virus. The bar indicates 0.05 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1000 replicates. Only values >70 are shown. Reference strains GenBank accession numbers are indicated.

2.4 DISCUSSION

Annually an untold number of neurological and fatal infections in horses go unresolved. Symptoms of neurological disease are in most cases indistinguishable regardless of the causative agent involved. With most arbovirus laboratories testing only for the more common viruses such as AHSV, EEV and WNV, many cases remain unanswered. Our objective was to try and identify these unknown causal agents through the establishment and optimization of a virus discovery technique. Over the years, many sequence independent methods have been developed and used with great success (Ambrose & Clewley, 2006). We decided that for our application the technique of arbitrarily primed PCR would be best for use on a routine bases, as this method produces individual amplicons which can be individually cloned and sequenced.

Optimization of this technique involved multiple attempts at various strategies to purify the viral particles. This is indeed the most important step in sequence independent amplification as contaminating host DNA renders these techniques ineffective. In the case of SAE 66/09, the technique had not yet been fully optimized and was performed on various tissues and not cell culture. We were still however able to obtain a 50% success rate, where contaminating host DNA was found in only half the sequences obtained. In this instance *C. perfringens* was identified in the abdominal fluid of the horse. This same unoptimized technique was employed to try to amplify additional genomic fragments of SAE18/09, again of the sequences obtained only 50% were viral.

By the time we received SAE 112/11, we had optimized our technique and had found that it was imperative to filter the samples before nuclease treatment to remove contaminating bacteria. Additionally PEG precipitation had to be carried out to concentrate the viral particles, before nuclease digestion of free floating DNAs/RNAs. Another observation was that DNase and RNase digestion had to be carried out for extended periods of time and that the addition of Benzonase produced even cleaner products. Inefficiency at any of these steps would not allow for the differentiation of host associated bands in infected controls from those in infected specimens. When analysing SAE 112/11, this technique had brought the level of background down to 15% with 85% of the sequences being viral in nature, in both the sample and the control reactions. MIDV, a member of the *Togaviridae* was identified from cell culture supernatant in this case. MIDV was the focus of a fellow students project and as such was not further investigated in this study,

nevertheless this finding showed the potential of this technique to identify viruses in the major arboviral families including the *Flavi-*, *Toga-* and *Bunyaviridae*.

Our finding of *C. perfringens* in the abdominal fluid of SAE66/09 is not unexpected, as it is a natural bacterium of the gut. *C. perfringens* can nonetheless be associated with acute disease in horses (Diab *et al.*, 2011), although the enterotoxin toxin would need to have been detected to confirm the diagnosis. No toxicology was conducted in this case and as such *C. perfringens* could not be confirmed as the agent of disease. The fact that a bacterial agent was identified on this occasion is linked directly to the unoptimized technique used at the time, where filtering with a 0.2µm filter had not yet been employed, allowing the large bacterial DNAs to be detected. The identification of MIDV in a horse with symptoms similar to those of AHSV (SAE 112/11), reflects directly the initial isolation of MIDV, which was from a horse which had the same clinical presentation (Attoui *et al.*, 2007). This supports the possibility that MIDV was indeed the causative agent in this instance and highlights the significance of this technique in causal agent identification, when the more common methods fail to produce a result.

During this study we were able to successfully identify the possible causal agents in two cases of neurological and fatal disease in horses as the alphavirus MIDV and the orthobunyavirus SHUV, whilst consistently amplifying the flavivirus control. We employed our technique of arbitrarily primed PCR when the techniques of cell culture isolation, electron microscopy and generic family PCRs were unable to produce a result. Our strategy was used to great effect, with the reproducibility of our method further supporting its inclusion into our diagnostic setting. This technique can now be employed on a routine basis, to aid in the diagnosis of disease caused by unknown agents, thus allowing for a clearer and more thorough understanding of neurological disease in South Africa.

CHAPTER 3
Identification of Shuni virus as a cause of neurological disease in horses

3.1 INTRODUCTION

Arboviruses (arthropod-borne viruses) are maintained in nature by haematophagous arthropods such as mosquitoes, ticks, Culicoides midges and sand-flies, principally by biological transmission between susceptible vertebrate hosts. Many zoonotic arboviruses, are capable of causing major outbreaks, sometimes with severe morbidity and high mortality rates and are important emerging and re-emerging diseases(Weaver & Reisen, 2010). Several mosquito borne zoonotic viruses in the families' *Flavi-*, *Bunya-* and *Alphaviridae* have emerged from Africa as new pathogens in previously unaffected regions and caused major epidemics and epizootics, including West Nile virus; Rift Valley fever and Chikungunya virus (Hollidge *et al.*, 2010). Horses in particular are highly sensitive to some of these viruses and have thus been targeted as sentinel animals in the identification of zoonotic arboviruses associated with neurological disease in South Africa (Venter & Swanepoel, 2010). During the seasonal occurrence of more readily recognised vector-borne diseases such as African horse sickness (AHSV) and Equine encephalosis (EEV), many horses exhibit febrile, neurological and fatal infections for which the aetiology remains unsolved.

Following the emergence of West Nile virus lineage 1 (WNV1) as an important pathogen in Europe and North America (Ulbert, 2011) (Ulbert), the pathogenicity and role of WNV lineage 2 (WNV2) was re-examined in southern Africa, WNV2was identified as a cause of unexplained nervous disease of humans and horses in South Africa (Venter *et al.*, 2009; Venter & Swanepoel, 2010), there were however residual cases in which no diagnosis could be established. This report describes further studies, in which a virus isolated in cell culture from the brain of a horse euthanized with severe encephalitis was identified as Shuni virus (SHUV), a member of the Simbu serogroup of the *Orthobunyavirus* genus of the family *Bunyaviridae*. SHUV-specific primers were designed and used to perform RT-

PCRs on specimens from a further 111 horses with fever and nervous disease that had been screened for more common pathogens over the course of an 18 month study.

Index case (SAE 18/09):

In January 2009 a crossbreed yearling was found wandering aimlessly in her paddock in the Vaalwater district of the Limpopo Province, South Africa. She became progressively ataxic over the next 48 hours and when recumbent was referred to the veterinary hospital at the Faculty of Veterinary Science, University of Pretoria. On presentation she was unaware of her surroundings and was paddling constantly. Sedation including the use of ketamine (as a last resort) failed to calm her. She experienced several episodes of muscle spasm interspersed with tremors and was euthanized when terminal. An autopsy was performed and blood, brain and spinal cord samples submitted for histopathological and/or virological examination. An unidentified virus was isolated in cell culture.

Herein we describe the identification of this unknown agent as Shuni virus through the use of electron microscopy and family specific PCRs. Also discussed is the development of a nested SHUV specific PCR which we employed in the initial screening of further cases of acute disease between June 2009 and December 2010. This was done in the hope of further elucidating the role which SHUV may play in neurological disease in animals in South Africa.

3.2 METHODS

3.2.1 Immunohistochemistry on formalin tissues, index case SAE 18/09

Samples of kidney, liver, lung, spleen, heart, as well as lumbar, thoracic and cervical spinal cord regions, cerebellum, brainstem and cerebrum were preserved in 10% neutral buffered formalin. After >24 hours in formalin, these fixed tissues were cut into 2mm thick slices, dehydrated in alcohol, embedded in paraffin wax blocks, sectioned at 5µm and stained with haematoxylin and eosin (HE) for microscopic examination at the Faculty of Veterinary Science, Onderstepoort, by Dr. June Williams.

3.2.2 Virus culture

Specimens from both SAE 18/09 (brain) and MVA 35/10 (spinal cord) were grown up in cell culture as follows: clarified 10% tissue suspensions were inoculated onto Vero cell monolayers in 25cm^2 tissue culture flasks with Eagle Minimum Essential Medium

containing 2% foetal calf serum (Gibco BRL, Invitrogen). Cultures were incubated at 37ºC and monitored microscopically for cytopathic effects over a period of 10 days.

3.2.3 Electron microscopy

Vero cell cultures inoculated with specimens from SAE 18/09 which exhibited cytopathic effects were examined by electron microscopy. For negative stained preparations, cell culture fluid was centrifuged at 13 000 rpm for 45 minutes, the supernatant discarded and the pellet, mixed with a drop of water, added to 3% phosphotungstic acid (pH 6.5) for 30 seconds before placing a carbon coated formvar grid onto the mixture. Cell cultures were fixed in 2.5% glutaraldehyde in Millonig's buffer, pelleted at 3000 rpm for 3 minutes, rinsed in the same buffer and post-fixed in 1% osmium tetroxide. The pelleted cells were dehydrated through graded alcohols, embedded in an epoxy resin and ultra-thin sections cut and stained with uranyl acetate and lead citrate. The grids were examined by Dr. Gerdes of the Onderstepoort Veterinary Institute, in a Philips CM10 transmission electron microscope operated at 80kV.

3.2.4 Orthobunyavirus RT-PCR

RNA was extracted from infected cultures using the QIA-amp viral RNA mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. RT-PCRs were performed using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) with published primers; Bunya 1 (GTC ACA GTA GTG TAC TCC AC) and Bunya 2 (CTG ACA GTA GTG TGC TCC AC), which amplify a 550bp fragment of the N gene of the S RNA segment of orthobunyaviruses (Bowen *et al.*, 2001). Briefly, 10µl RNA was added to 10µl 5x reaction buffer, 5mM DTT solution, 40pmol of each primer, 10mM of each dNTP, 1µl Titan™ enzyme mix, 10 U of Protector RNase inhibitor (Roche, Mannheim, Germany) to a final volume of 50µl. The reaction mix was subject to initial incubations of 50˚C for 40 minutes, 94˚C for 2 minutes followed by 35 PCR cycles: 94˚C, 30 sec; 48˚C, 1 min; 68˚C, 45 sec, supplemented by a final extension for 7 min at 68˚C. Amplicons were separated on a 1.5% agarose gel following electrophoreses next to a Rift Valley Fever virus control.

3.2.5 Specimens

Post mortem samples from 111 horses were sent to the Department of Medical Virology, University of Pretoria (UP) between June 2009 and December 2010, by the Onderstepoort Veterinary institute and the UP Faculty of Veterinary Science, Onderstepoort, as well as by

veterinarians from around the country. The specimens were screened as appropriate for poisons and rabies virus.

3.2.6 SHUV specific nested RT-PCR

3.2.6.1 Primer design

Primers were designed based on the SHUV sequence (AF362405) available in the public domain: on GenBank (www.ncbi.nlm.nih.gov), through alignment using ClustalW multiple alignment program (Thompson, *et al.*, 1994) (Table 3.1). Genome position is based on this sequence.

Table 3.1 Shuni virus primers

Primer	Sequence	Genomic position	Reference
SHUVS111+	5' CGATACCGTTAGAGTCTTCTTCC 3'	111-134 (S)	(van Eeden *et al.*, 2012)
SHUVS688-	5' CGAATTGGGCAAGGAAAGT 3'	688-706 (S) rc	(van Eeden *et al.*, 2012)
SHUVS178+	5' CCGAGTGTTGATCTTACATTTGGT 3'	178-202 (S)	(van Eeden *et al.*, 2012)
SHUVS611-	5' GCTGCACGGACAGCATCTA 3'	611-630 (S) rc	(van Eeden *et al.*, 2012)

*rc, reverse compliment

3.2.6.2 Nucleic acid extraction

Nucleic acids were extracted from cell culture or EDTA blood using the QIA-amp viral RNA mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. For fresh tissue samples, approximately 30mg pieces were extracted with the RNeasy Plus mini kit (Qiagen, Valencia, CA).

3.2.6.3 First round amplification

RT-PCRs were performed using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) and SHUV specific primers designed for the present study, SHUVS111+ and SHUVS688- (Table 3.1). Briefly, 10µl RNA was added to 10µl 5x reaction buffer, 5mM DTT solution, 40pmol of each primer, 10mM of each dNTP, 1µl Titan™ enzyme mix, 10 U of Protector RNase inhibitor (Roche, Mannheim, Germany) to a final volume of 50µl. The reaction mix was subject to initial incubations of 50˚C for 40 minutes, 94˚C for 2 minutes followed by 35 PCR cycles: 94˚C, 30 sec; 52˚C, 1 min; 68˚C, 1 min, supplemented by a final extension for 7 min at 68˚C. Amplicons were separated on a 1.5% agarose gel following electrophoreses next to SAE 18/09 as a positive control.

3.2.6.4 Nested amplification

Nested PCRs were performed using primers SHUVS178+ and SHUVS611- and the Expand High Fidelity^{PLUS} PCR System (Roche, Mannheim, Germany) to produce approximately 430bp amplicons. The PCRs were conducted in a 50µl reaction volumes using 2µl of the RT-PCR product, 10µl 5x reaction buffer, 10mM of each dNTP, 20pmol of each primer and 2.5 U of Expand High Fidelity^{PLUS} enzyme mix. Reaction mixes were subjected to 95˚C for 2 minutes, 35 cycles: 95˚C, 30 sec; 50˚C, 1 min; 72˚C, 2 minutes, followed by a final extension of 72˚C for 7 minutes. A product of 430bp could be visualized on an agarose gel.

3.2.7 Differential diagnosis

Differential diagnosis was carried out as before (Refer to 2.2.3).

3.2.8 Cloning, sequencing and phylogenetic analysis

Purification, sequencing (Refer to 2.2.4) and phylogenetic analysis (Refer to 2.2.5) were carried out as before.

3.3 RESULTS

3.3.1 Macroscopic findings from index case SAE 18/09

Macroscopic analysis at the post mortem of SAE 18/09, carried out by Dr. June Williams at the Faculty of Veterinary Science, Onderstepoort, showed moderate visceral congestion and an atrophied spleen. Mild subcutaneous and intermuscular oedema of the neck and severe diffuse pulmonary oedema and congestion were also observed. Also observed was moderate serosanguinous hydropericardium.

3.3.2 Immunohistochemistry on index case SAE 18/09

Microscopic examination of tissues from the index case SAE 18/09, showed severe mononuclear encephalitis with scattered neutrophil presence, with lesions being most marked in cerebral white matter, midbrain and brain stem (Figure 3.1-A). In the cerebellum, Purkinje cells had degenerated, undergone necrosis or disappeared with attendant gliosis (Figure 3.1-B, D); there was also severe cerebellar mononuclear meningitis (Figure 3.1-C). These lesions were consistent with viral meningoencephalomyelitis.

3.3.3 Cell culture and electron microscopy from index case SAE 18/09

A cytopathic agent was isolated from the brain of case SAE 18/09 by Dr. Gerdes of the Onderstepoort Veterinary Institute and supplied to the Zoonoses Research unit (ZRU) for further examination. The isolate could not be identified as one of the more common horse pathogens tested for by the ZRU (Refer to 2.2.3). Electron microscopic examination of negative stained preparations of culture fluid and resin sections of infected cells, revealed 80–100nm particles resembling bunyaviruses (Figure 3.2).

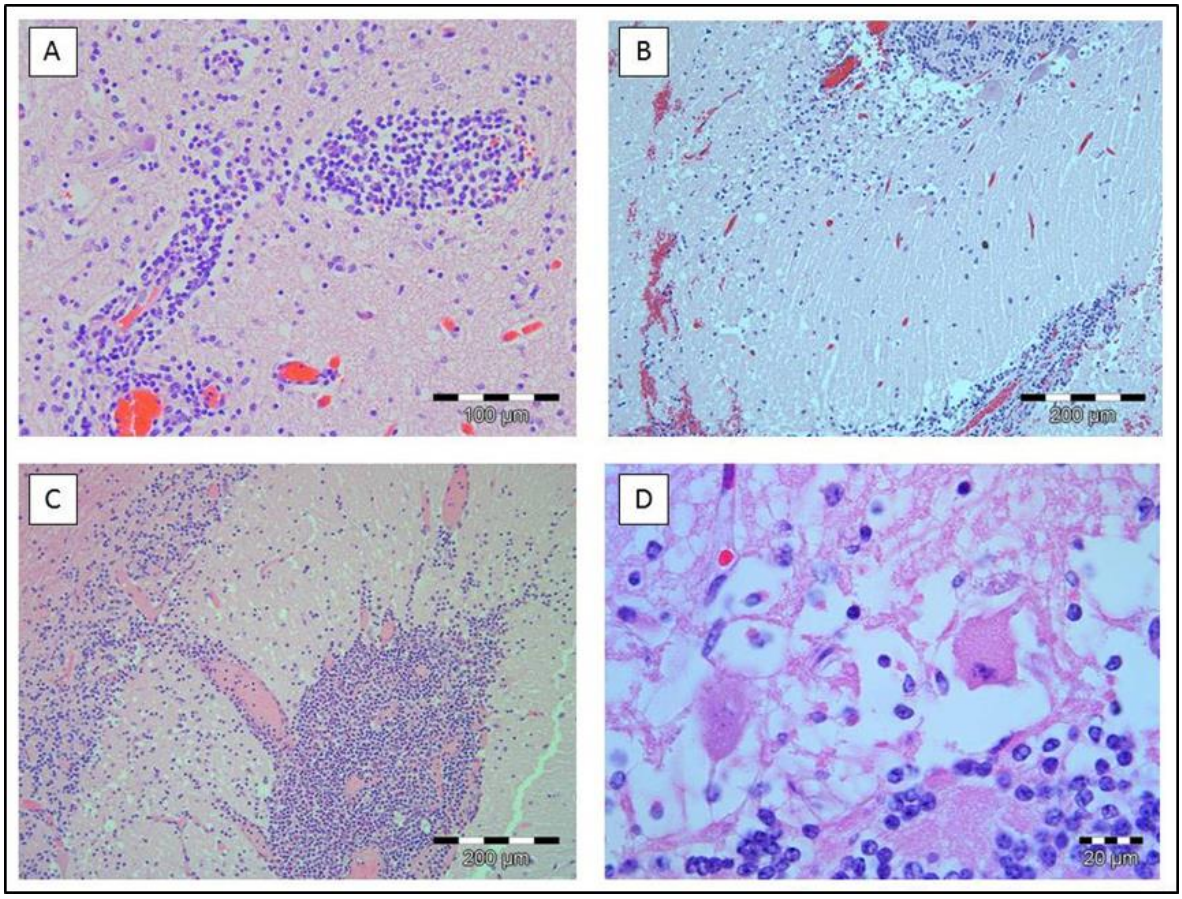


Figure 3.1 Micrographs of tissues from SAE 18/09. **A**, Severe mononuclear perivascular cuffing and white matter gliosis of the midbrain region. **B**, Cerebellum showing severe mononuclear meningitis, molecular layer haemorrhages, and loss of Purkinje cells. **C**, Severe mononuclear cerebellar meningitis, vascular congestion with cuffing. D, Two necrotic cerebellar Purkinje cells: nuclear pycnosis on the right and chromatolysis left, with surrounding gliosis, oedema and malacia. Images courtesy of Dr. June Williams, Faculty of Veterinary Science, Onderstepoort. (van Eeden et al., 2012).

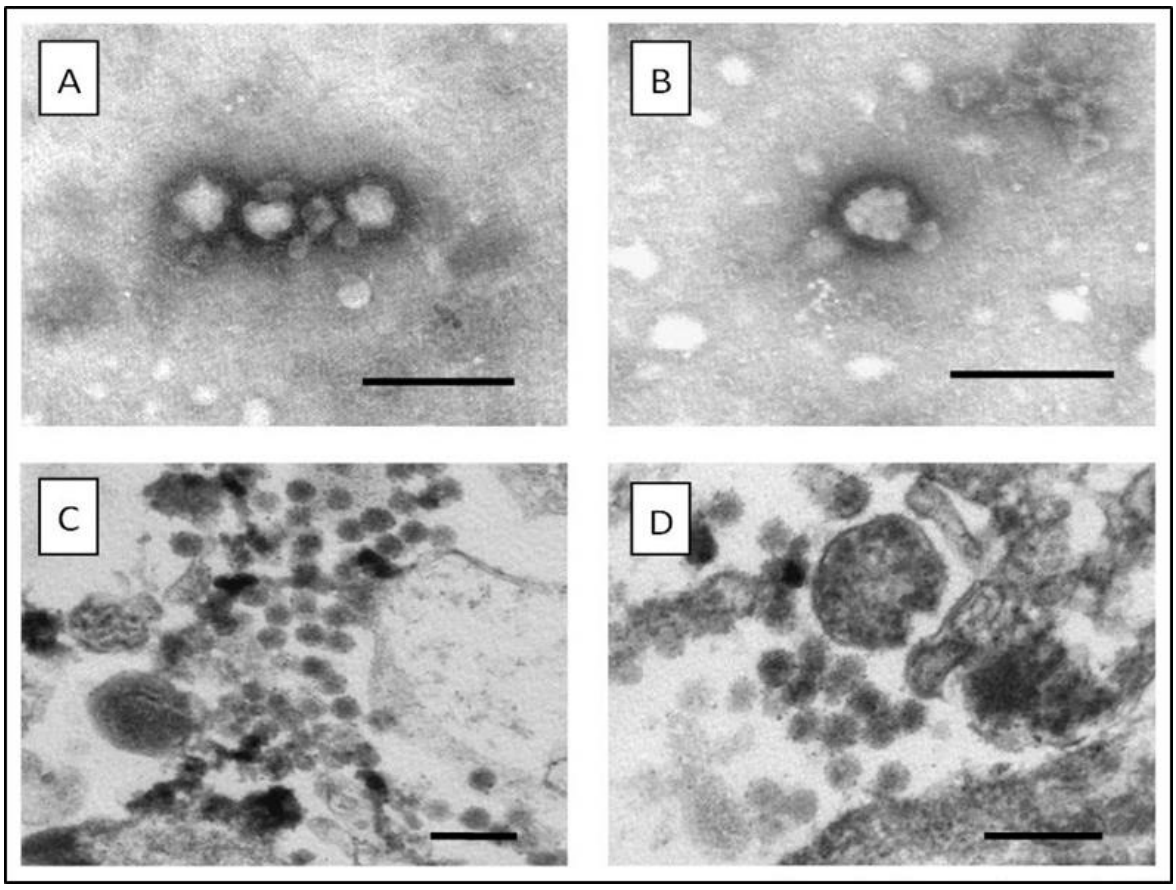


Figure 3.2 Electron micrographs of Vero cells infected with SAE 18/09. A, B; Negative stain EM showing fringed particles (bunyavirus size) with bleb formation. Bar = 250 nm. C, D; Resin section EM showing spherical and pleomorphic bunyavirus particles in the size range (80-100nm) Bar = 250 nm. Images courtesy of Dr. Gerdes of the Onderstepoort Veterinary Institute. (van Eeden et al., 2012).

3.3.4 Orthobunyavirus PCR

Following the identification of a probable bunyavirus, RT-PCR amplification of the isolate with orthobunyavirus generic primers produced an amplicon of 550bp that was cloned and sequenced. Blast search analysis of a 520nt fragment revealed that the amplicon was related to members of the Simbu serogroup of the *Orthobunyavirus* genus and maximum likelihood phylogenetic analysis indicated high bootstrap support (>91%) for the placement of SAE 18/09 within the Shuni, Aino and Kaikular branch of the serogroup. The isolate shared share 95.9% identity with SHUV and 91.1% with Aino virus (Figure 3.3).

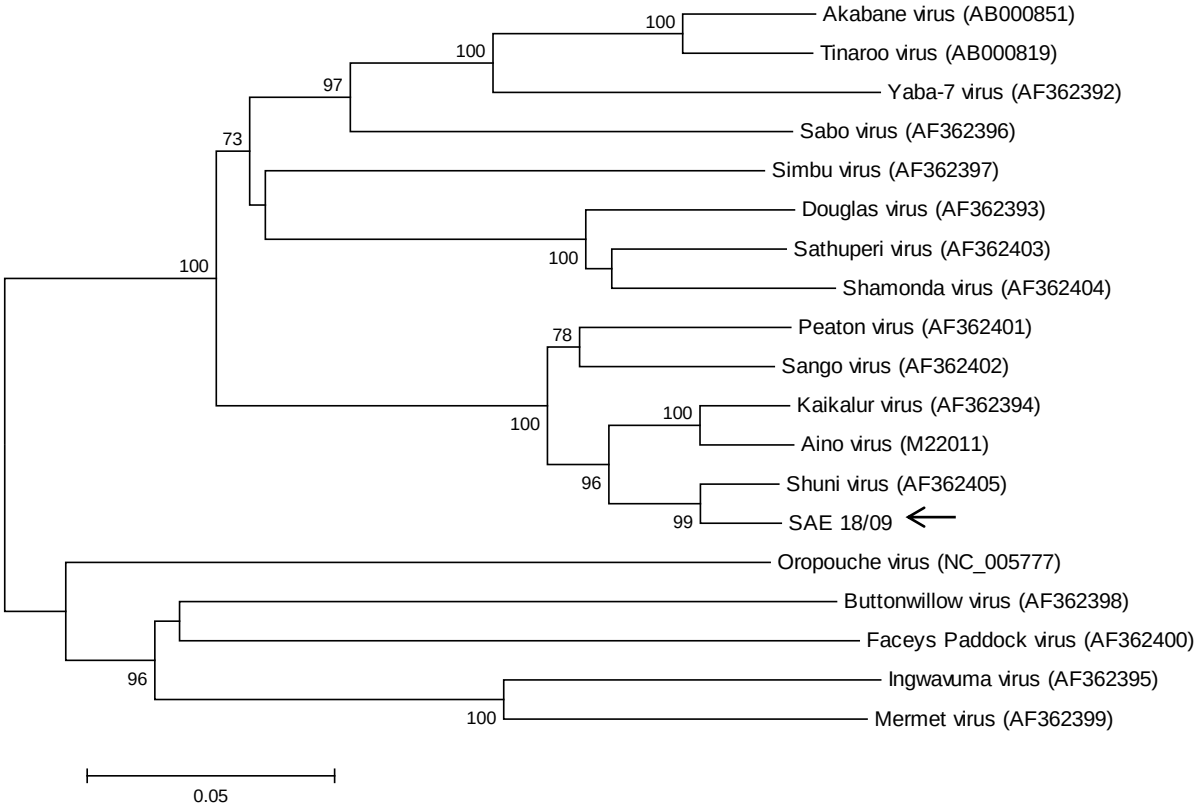


Figure 3.3 Maximum likelihood tree constructed using the Tamura-Nei model of MEGA v5, of a 330bp fragment of the S segment RNA of the orthobunyaviruses. The unknown isolate SAE 18/09 is identified as Shuni virus. The bar indicates 0.05 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1000 replicates. Only values >70 are shown. Reference strains GenBank accession numbers are indicated

3.3.5 Initial 18 month screen for SHUV

A total of 112 horses with unexplained fever and/or neurological disease submitted between June 2009 and December 2010 were screened with alphavirus, flavivirus and West Nile virus RT-PCRs as well as the nested SHUV-specific PCR to determine the prevalence of Shuni virus. SHUV was identified in an additional six horses, bringing the total to 7/112 (6.3%), 2/26 (8%) with unexplained fever and 5/86 (6%) with neurological disease. The epidemiology and clinical description of the disease are discussed in Chapter 6.

3.4 DISCUSSION

The use of electron microscopy and family specific PCRs allowed for the identification of Shuni virus as the causal agent of disease, in the horse from which isolate SAE 18/09 was obtained. In this case, bunyavirus-like particles could be clearly identified by EM and a genus specific PCR was able to amplify the agent, which was identified as SHUV, a member of the *Orthobunyavirus* genus, family *Bunyaviridae*. SHUV was first isolated in the 1960s from cattle and sheep in abattoirs; *Culicoides* midges tested in arbovirus surveys(Causey *et al.*, 1972; Kemp *et al.*, 1973; Lee, 1979) and in one instance from a febrile child in hospital, in Nigeria (Moore *et al.*, 1975). Subsequently, the virus was isolated from pools of *Culex theileri* mosquitoes caught near Johannesburg, and from cattle and a goat in KwaZulu-Natal Province, South Africa (McIntosh, 1972; McIntosh, 1980).

In 1977, the virus was isolated from the brains of two horses that succumbed to nervous disease, one in South Africa and one in Zimbabwe (Coetzer & Erasmus, 1994; Howell & Coetzer, 1998). Specific Shuni virus diagnostic tests were however never developed and no further investigations were undertaken to determine the importance of this virus as a cause of neurological disease in humans or animals. Identification of this virus in the brain of a horse with severe neurological symptoms, prompted us to design SHUV specific primers to screen further cases of acute disease.

Over a period of 18 months we identified seven SHUV cases, five of which were associated with significant neurological symptoms. For the index case SAE 18/09, the identification of the viral particles in the CNS and the amplification of the agent from brain tissue suggests the crossing of the blood brain barrier and provides evidence for this virus as the causative pathogen in this case. These findings suggest that the role of SHUV as a pathogen may be underestimated, and that it should be investigated routinely as a possible cause of unexplained nervous disease of humans and other animals, not only in South Africa but across the African continent. With this in mind we developed a nested real-time assay (Chapter 4) and investigated the epidemiology of SHUV in South Africa over a 5 year period (Chapters 6).

CHAPTER 4
Development of a SHUV real-time PCR assay

4.1 INTRODUCTION

Shuni virus was first isolated in 1966 from a cow bled in Sokoto, Nigeria during a program for the surveillance and study of viral infections that was instituted by the University of Ibadan between 1964 and 1969. During the course of the survey SHUV was also isolated twice again from cattle and once from a sheep with neutralizing antibodies being found in dairy and trade cattle as well as sheep (Causey *et al.*, 1972; Causey *et al.*, 1969; Kemp *et al.*, 1973). SHUV was also isolated twice from *Culicoides ssp.* during field surveillance and from a one and a half year old child at the General Outpatients Clinic at the University College Hospital (UCHGOP) in August 1966, as part of the arbovirus surveillance activity in Nigeria (Lee, 1979; Moore *et al.*, 1975).

In South Africa, SHUV was recovered twice from pools of *Culex theileri* mosquitoes caught near Johannesburg and from seven apparently healthy cattle and a goat in Natal between the years 1957 and 1979 (McIntosh, 1980; McIntosh *et al.*, 1972). In 1977, the virus was isolated from the brains of two horses with nervous disease submitted for rabies virus examination; one from South Africa (Coetzer & Howell, 1998) and one from Zimbabwe (Coetzer & Erasmus, 1994). Considering the public and veterinary health importance of other members of the *Orthobunyavirus* genus, surprisingly little attention was placed on uncovering the role SHUV may play in encephalitic disease in humans and animals. The identification of SHUV in seven horses (van Eeden *et al.*, 2012) with neurological symptoms between June 2009 and December 2010, led us to believe that this virus may play a noteworthy role in neurological disease in South Africa. To enable us to swiftly identify SHUV in acute cases submitted through our zoonotic arbovirus sentinel surveillance programme for neurological disease in animals, as well as from other laboratories to search for this virus in their region, we aimed to developed a rapid and specific test

We previously described a nested PCR for the detection of Shuni virus which, although effective is less practical in a diagnostic setting due to the need to confirm diagnosis by nucleic acid sequencing of amplicons (van Eeden *et al.*, 2012). To improve on the

turnaround time, sensitivity and specificity of this test we sought to design a real-time assay to incorporate into diagnostic setting. This assay can be implemented in both diagnostic and research laboratories, allowing for further investigations into the prevalence and association of SHUV with acute neurological disease in animals and humans in Africa as well as in other continents. In light of other recently described emerging orthobunyaviruses; Schmallenberg virus in Europe (Hoffmann *et al.*, 2012) and Iquitos virus in Peru (Aguilar *et al.*, 2011), the investigation of Shuni virus as a cause of unexplained outbreaks of neurological disease, especially in horses is warranted.

This report describes the development of a sensitive asymmetric nested real-time PCR assay, incorporating fluorescence resonance energy transfer (FRET) probes for the rapid detection of SHUV in diagnostic, surveillance and epidemiological studies. The assay was assessed for sensitivity and specificity against a dilution series of a cloned first round PCR product of known concentration. The assay was validated against known positive cases of Shuni virus and was applied in the screening of 386 unsolved cases of neurological disease in animals in South Africa.

4.2 METHODS

4.2.1 Primer and probe design

SHUV-specific primers were designed to amplify a portion of the Nucleoprotein gene (N) based on the sequence of the prototype Shuni virus isolate (Refer to 3.2.6.1). Sequence alignment of this strain with our own was carried out using the ClustalW subroutine, which forms part of the Bioedit program (Hall, 1999) to identify suitable areas for probe binding. A hybridization (FRET) probe set was designed with the aid of the LightCycler Probe design software package (Roche Applied Science, Mannheim, Germany), (Table 4.1).

4.2.2 Virus strains for real-time optimization and validation

Three SHUV strains were used during the real-time assay optimization, these included a cell culture isolate (SAE 18/09), a brain specimen (SAE 18/09b) and a blood specimen (SAE 87/11), all from horses which had displayed neurological symptoms and had proved to be positive for SHUV through conventional nested PCR. In order to validate the SHUV real-time PCR the assay was repeated from the RT-PCR step in triplicate. Four samples were

included, SAE18/09b, SAE87/11, the original cell culture isolate (Chapter 3) as the positive control, as well as a negative reagent control.

4.2.3 RNA Extraction

Nucleic acids were extracted as previously described (Refer to 3.2.6.2).

Table 4.1 Probe sequences for the SHUV real-time PCR

Primer	Orientation	Sequence	Genome position[a]	Reference
SHUVS111+	Sense	CGA TAC CGT TAG AGT CTT CTT CC	111-134 (S)	(van Eeden *et al.*, 2012)
SHUVS688-	Anti-sense	CGA ATT GGG CAA GGA AAG T	688-706 (S)	(van Eeden *et al.*, 2012)
SHUVS178+	Sense	CCG AGT GTT GAT CTT ACA TTT GGT	178-202 (S)	(van Eeden *et al.*, 2012)
SHUVS611-	Anti-sense	GCT GCA CGG ACA GCA TCT A	611-630 (S)	(van Eeden *et al.*, 2012)
Probe	**Orientation**	**Sequence**	**Genome position[a]**	**Reference**
SHUVProbe1	Sense	ATA TGC ACA GAG TGC TAA AAG ATG GGA TG –F[b]	488-516 (S)	This study
SHUVProbe2	Sense	LC Red 640 - GTC AAC TTC ATG CGA AAG GTT CTT CGC CAA CG –P[c]	520-551 (S)	This study

[a] SAE18/09 -KC510272 (in basepairs)
[b] F = Fluorescein
[c] P = Phosphate

4.2.4 SHUV-specific nested real-time PCR

RT-PCRs were performed using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) as previously described (refer to section 3.2.6.3). Nested real-time PCR was carried out in a LightCycler 2.0 using the FastStart DNA Master Plus Hybprobe kit (Roche Applied Science, Mannheim, Germany) according to the manufacturers' recommendations. Each reaction contained 2µl of the first round PCR product, 0.2µM of each probe (Probe 1 and Probe 2), 0.5µM of SHUVS178+, different reverse primer concentrations (0.5 to 5µM) SHUVS611- and 4µl of enzyme master mix in a 20µl reaction. Cycling started at 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 sec, 50˚C for 8 sec, 72˚C for 8 sec, followed by melting curve analysis between 30 and 80˚C, at a temperature ramp rate of 0.1˚C/s. A product of 430bp could be visualized on an agarose gel.

4.2.5 Real-time sensitivity

A first round PCR product from the cell culture isolate SAE 18/09 was cloned and the concentration of the amplicon determined through use of the NanoDrop 3300 (Thermo Scientific, Delaware, USA). A dilution series of this was then used to determine the detection limit of the assay through calculation of the copy number.

4.2.6 Sequencing

Purification and sequencing were carried out as before (Refer to 2.2.4).

4.3 RESULTS

4.3.1 Real-time PCR development and optimization

A nested real-time PCR with SHUV specific FRET probes was developed for direct detection of SHUV by melting curve analysis in clinical specimens. Two SHUV strains, a cell culture isolate and a brain specimen were used to test the efficiency of the assay. Both specimens could be detected with the SHUV nested real-time assay after melt curve analysis (Figure 4.1-A), however a double peak with melting temperatures of 59ºC and 65ºC was observed, although only a single band was visible by agarose gel electrophoresis (Figure 4.1-B). Neither sample concentration nor annealing temperature affected this outcome. We thus attempted asymmetric PCR, where the concentration ratios between the forward and reverse primers are adjusted. This modification is thought to circumvent competition from the non-target strand during probe analysis of double stranded DNA (Bernard *et al.*, 1999; Burggraf *et al.*, 2002).

We found that at a forward to reverse primer ratios of 1:5 to 1:6, the signal intensity increased and the additional peak disappeared (Figure 4.2). It was with these parameters that the assay was further evaluated. Computational analysis was carried out to assess the specificity of the assay, as no other Simbu serogroup viruses were available for experimental evaluation. Results showed that the mismatches between the different viruses are significant enough to type the viruses correctly (Figure 4.6), due to these mismatches the predicted Tm could only be predicted for SHUVs closest related virus (55.5°C). Based on the similarity of the predicted (64.1°C) and experimental (64.7-65.7°C) values obtained for SHUV the experimental values for the other Simbu serogroup members are likely to closely reflect the predicted values.

4.3.2 Evaluation against known positive specimens

Clinical specimens from a horse (brain SAE 18/09b) that succumbed to neurological disease and another horse (blood SAE 87/11) which survived were, used to test the diagnostic ability of the assay. SHUV was amplified directly from RNA extracted from the two clinical specimens (Figure 4.3), where detection signals became apparent after 10 cycles of PCR. These results could be confirmed with the conventional nested PCR.

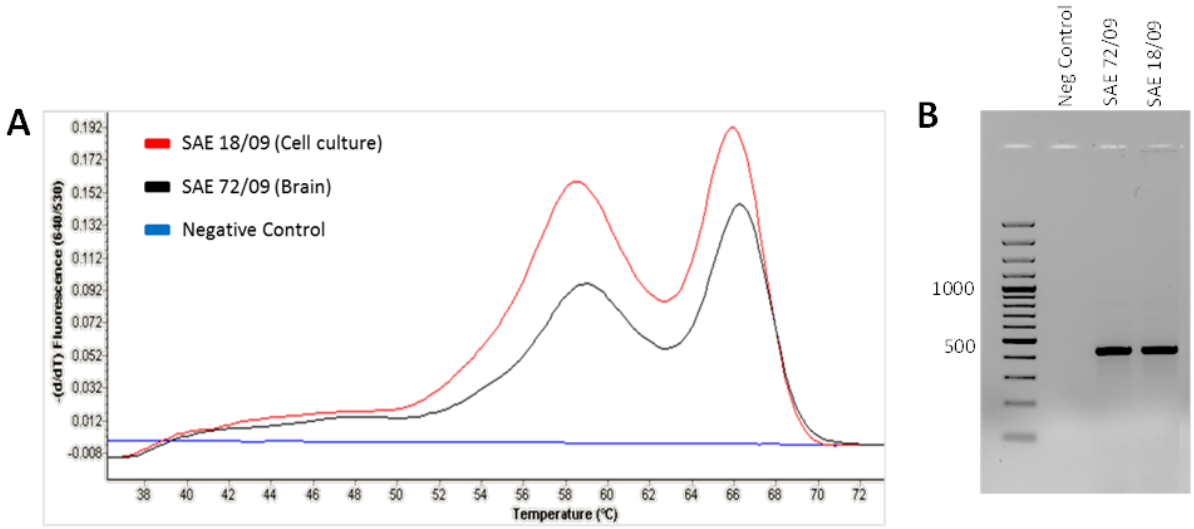


Figure 4.1 (A) Melting peaks showing both the cell culture isolate SAE 18/09 and the clinical specimen SAE 72/09 positive for SHUV following nested real-time PCR. (B) Agarose gel showing real-time PCR products.

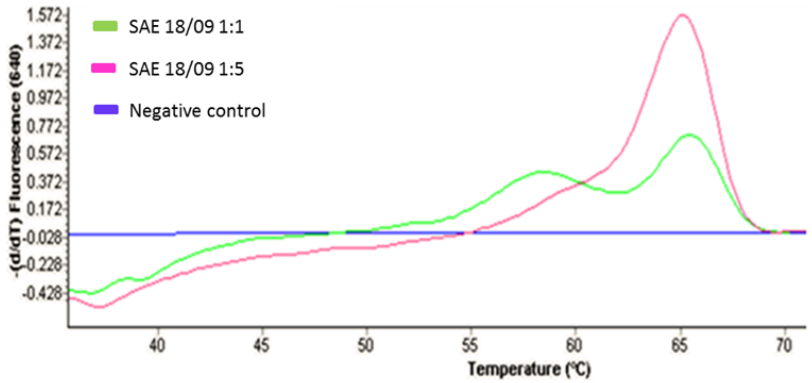


Figure 4.2 Melting peaks showing the detection of SAE 18/09 at both a 1:1 and 1:5 forward/reverse primer ratio. A ratio of 1:1 produced 2 peaks (59ºC and 65ºC) and a ratio of 1:5 only one (65ºC).

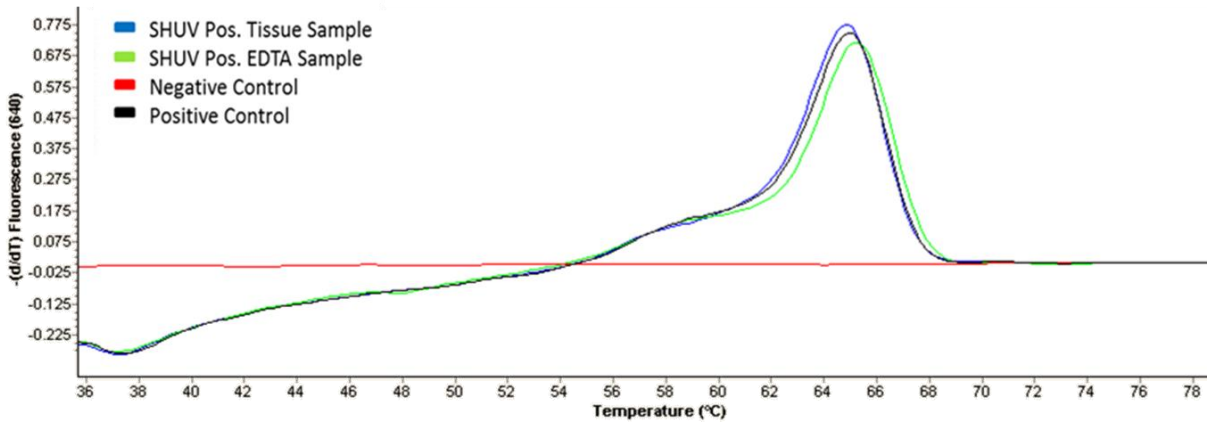


Figure 4.3 Melting peaks showing two clinical specimens positive for SHUV following nested real-time PCR. The specimens included a brain sample from a horse that had succumbed to neurological disease and blood from another horse which had survived the infection.

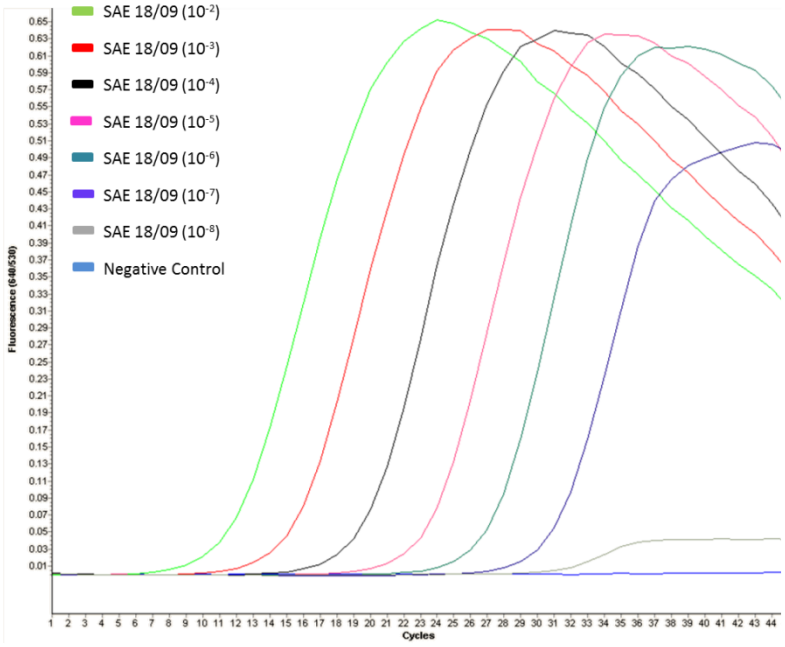


Figure 4.4 Amplification curve analysis of a dilution series of SAE 18/09 cloned RNA control, indicating the sensitivity of the nested real-time assay.

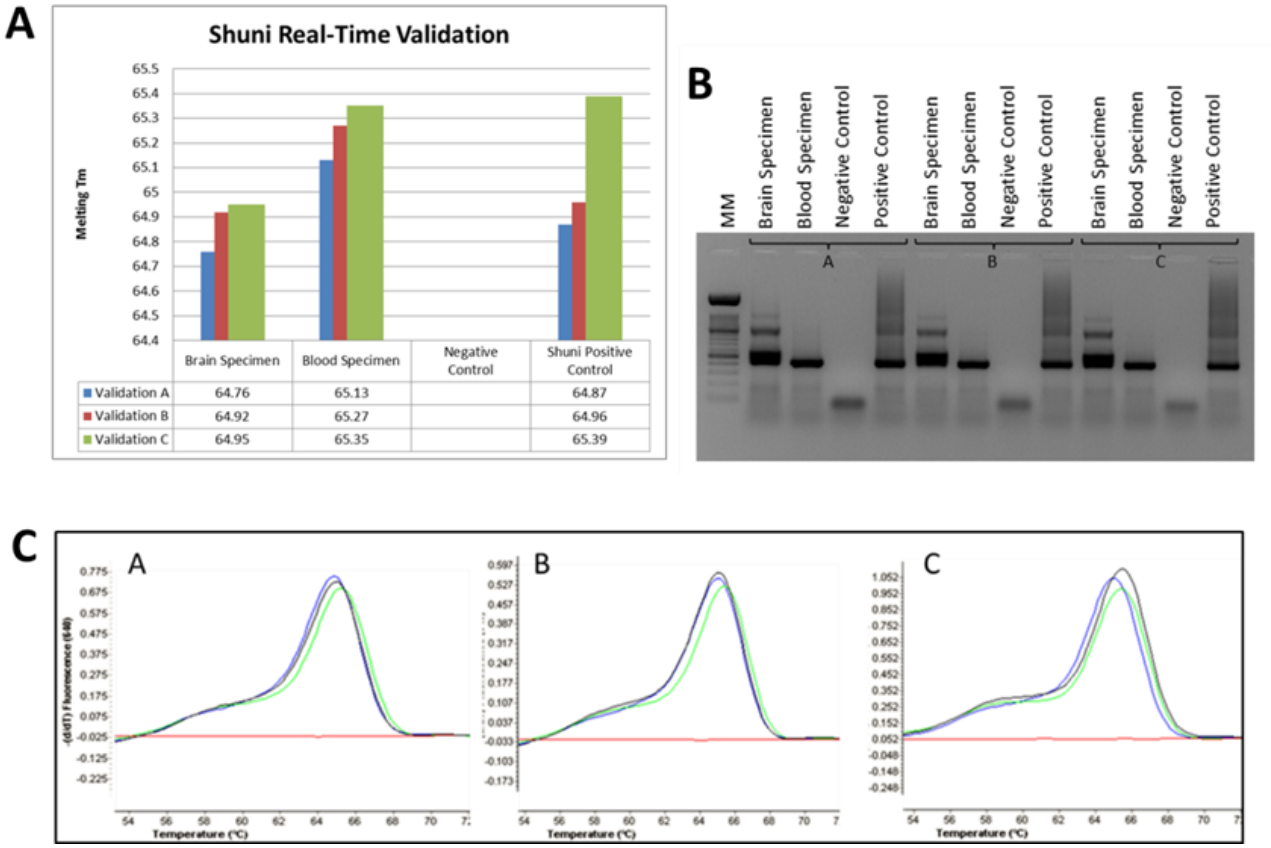


Figure 4.5 (A) Graph indicating the variation between melting temperatures on three separate real-time runs. (B) SHUV real-time validations, run on a 1.5% agarose gel. (C) SHUV real-time assay melt curves for validation runs, A, B and C

Probes	ATATGCACAGAGTGCTAAAAGATGGGATGG---TCAACTTCATGCGAAAGGTTCTTCGCCAACG	Predicted Tm	Experimental Tm
SHUV (KC510272)	.C.....ATG.....	64.1 °C	64.7/65.7°C
SHUV (HE800143)	.C.....ATG.....	64.1°C	NA
AINOV (HE795089)	.....G..G.....A....ATG.....A.G.....C.....G..	55.5°C	NA
PEAV (HE795095)	.C.....T.....A.....A....ATG.....T...A.....A..C.....	*	NB
KAIV (AF362394)	.C.....G..G..C.....ATG.....T...A.G.....C.....G..	*	NB
SIMV (HE795110)	.....T.....TT.....C....ATG.A.....T.....G..A.C.....GA.	*	NB
BUTV (AF362398)	.A.....AC.C.....A....ATG.....AAG..A..AA.AA.G..GA.	*	NB
SABOV (HE795098)	.....TT.G..G.....ATG.....G..A..A..C.....G..	*	NB
TINV (AB000819)	.C.....C.T...A.....ATG.....A.G.....C..A.....GA.	*	NB
AKAV (AB000851)	.C.....C.T...A....G.....A....ATG.....A.G.....CT.A..T..GA.	*	NB
DOUV (HE795092)	.G.....T.....C..C.....AAT....ATG.G.....AAG..A..C.....	*	NB
FPV (AF362400)	.A.....G.....CT.G..G.....A....ATG.A.....T.....C..A..AT.G.....A.	*	NB
INGV (AF362395)	.G.....C.T..TT.G.....C....AAG.A..T.....A.G.....G.....	*	NB
MERV (AF362399)	.G.....C.T.....C.....C.....ATG.A.....G.....C.....G..	*	NB
SANV (HE795101)	.C.....T.....G..C..A....ATG.....T...A....A..C..C..T..G..	*	NB
SATV (HE795104)	.G.....G..T..C.....AAT....ATG.A..T.....AA...A..C.....G..	*	NB
SHAV (HE795107)	.A.....T..G..C..T..G..CAAT....ATG.G..T..T...AA...A..C..C.....	*	NB
OROV (AF164549)	.G.....G..C..C..G..C..C....ATG.....T...AAG..A..C.....G..GA.	*	NB
SBV (HE649914)	.A.....T..G..C..C..G..CAAT....ATG.A..T..T...AA...A..C..C.....	*	NB
Y7V (AF362392)	.C.....TC.C..CT.....C..A....ATG.G.....A.....CT.A.....GA.	*	NB

* Contains too many mismatches for melting temperature prediction
NA Not available for testing
NB Probes do not bind

Figure 4.6 Comparison of FRET probe sequences to other members of the Simbu serogroup. Dots indicate conserved bases, whereas sequence variations are indicated by nucleotide bases. Probe melting temperatures that could be predicted or experimentally determined are also indicated.

The specimens exhibited melting peaks of between 64.76˚C and 65.73˚C, which correlated well with the 65.78˚C peak observed for the positive control. The sensitivity of the assay was determined by performing the PCR on a dilution series of cloned virus (SAE 18/09) for which the plasmid copy number had been calculated (Figure 4.4). The detection limit was determined to be $1.79x10^{-3}$ viral genome copies/µl.

The assay was then further validated by running known samples in triplicate, results were similar for all samples in each round with the average Tm (melting temperature), never deviating by more than 0.5ºC (Figure 4.5-A). Melting peaks were comparable in all runs, and were always unimodal (Figure 4.5-C), all products could be visualized by gel electrophoresis allowing for nucleotide sequencing (Figure 4.5-B).

4.4 DISCUSSION

In this study an asymmetric nested real-time PCR is described for the rapid identification of Shuni virus, an assay which may have applications in diagnosis as well as epidemiology and surveillance programs. This is particularly relevant following the identification of SHUV as a possible cause of neurological disease in horses in South Africa (van Eeden *et al.*, 2012) and the previous identification in the 1960s–1970s, of this virus in Zimbabwe and Nigeria which suggests it may occur across the African continent.

The S segment of the genome was selected as the target for this assay due to the high level of conservancy of this segment. The M segment of the orthobunyaviruses in particular is prone to reassortment and variants have been identified in members of the Simbu serogroup (Goller *et al.*, 2012; Kobayashi *et al.*, 2007; Saeed *et al.*, 2001b; Yanase *et al.*, 2010), thus making this segment an unsuitable target for diagnostic and surveillance programs. For evolutionary studies however the more variable M and L segments would be more appropriate. The nested real-time PCR approach greatly enhances the sensitivity of this assay, which may increase the time in which viral RNA can be detected in clinical specimens before the clearance of viremia. This increased sensitivity will also have a significant impact on surveillance studies, where low concentrations of virus need to be detected in pooled specimens. Although the risk of contamination is increased with the nested PCR approach, incorporation of Uracil-DNA-Glycosylase (UNG, Roche Applied Science, Mannheim, Germany), may prevent this. Alternatively the assay may be run as a single round PCR using primers SHUV178+ and SHUV611-, following cDNA synthesis

with random hexamer primers, this proved efficient for the amplification and detection of the control SAE 18/09 (Results not shown).

FRET probes combine the advantages of real-time with the ability to type samples, where sequence variation between the probe and template can be exploited by melting curve analysis thus eliminating the need for time consuming sequence and phylogenetic analysis. The specific melting temperature for a probe-template interaction will be determined by the number and location of mismatches between them, thereby generating distinct melting curves for different mismatch patterns (Yeh *et al.*, 2004). Analysis of the Simbu serogroup viruses showed sequence variations at the probe binding site should be suitable to distinguish between viruses based on the predicted melting temperatures, these viruses could however not be obtained for experimental analysis. The high sequence diversity observed between the probes and other orthobunyaviruses confirms that these cannot be detected by the SHUV probes.

Our initial experiments with symmetric primer concentrations resulted in a bi-modal peak, following melt curve analysis. Changes in virus concentration and annealing temperature did little to rectify this and we thus considered an asymmetric PCR (AS-PCR) strategy. The significance of AS-PCR in dual probe systems has been highlighted in many studies where it has been shown that competition between the complementary strand of DNA and the probes, as well as incorrect genotyping are reduced (Bernard *et al.*, 1999; Burggraf *et al.*, 2002) whilst fluorescent intensity and specificity in melt curve analysis is significantly increased (Szilvasi *et al.*, 2005).

We found that at a forward to reverse primer ratio of 1:5, the secondary peak we had seen earlier, disappeared. The sensitivity of the assay was demonstrated by amplification in both a tissue (brain) and a blood sample, both samples were from horses that had displayed neurological symptoms and had previously been confirmed as SHUV positive by conventional nested PCR (van Eeden *et al.*, 2012) and sequencing. The experimental melting temperatures of 64.7ºC and 65.7ºC correlated well with the predicted melting temperatures as calculated by the LightCycler Probe design software package (Roche Applied Science, Mannheim, Germany). The real-time PCR products could be visualized by agarose gel electrophoresis and be used for nucleotide sequencing.

This assay was applied in the screening of an additional 386 specimens collected between 2008-2012, this revealed two additional SHUV cases to those previously published (van

Eeden *et al.*, 2012), both from equines in 2011. Collectively 9 cases of SHUV were identified in horses with neurological disease. The phylogenetic and epidemiological aspects of this analysis are discussed in Chapter 5. Annually, a high percentage of neurological disease in animals remains undiagnosed due to the substantial number of potential pathogens, which are excluded from routine diagnostics. The described assay provides a sensitive diagnostic tool, which will also be of use in epidemiological and surveillance studies, which will aid in the rapid identification of SHUV in potentially undiagnosed cases of neurological disease leading to a broader understanding of neurological disease, especially in South Africa.

CHAPTER 5
Genomic and phylogenetic characterization of Shuni virus

5.1 INTRODUCTION

Shuni virus (SHUV) belongs to the Simbu serogroup of the Orthobunyavirus genus of the family Bunyaviridae. Recently SHUV has been identified as the cause of neurological disease in several horses in South Africa (van Eeden et al., 2012). Being that it was one of the less recognized orthobunyaviruses, with limited clinical descriptions of disease dating back to the 1960s and 1970s, genomic characterisation was never carried out. Recently the near complete genome of the prototype SHUV strain (An10107) was published on GenBank as part of a phylogenetic analysis of the Simbu serogroup (Goller *et al*., 2012). An10107 had been isolated from an apparently healthy cow, during arbovirus surveys at an abattoir in Nigeria in 1966 (Causey *et al*., 1972). The isolation of a highly pathogenic strain from the brain of a horse with severe neurological disease in South Africa raises several questions regarding difference in pathogenicity. Factors such as the geographic and time separation between the isolates and the possibility of recombination events could all influence the genetic makeup and thus the observed pathogenic differences between these two strains.

The orthobunyaviruses are enveloped and possess a genome composed of three segments of negative sense single-stranded RNA, designated as large (L), medium (M) and small (S) (Elliott, 1990). The L segment encodes the viral RNA polymerase which functions in replication and transcription of the genomic RNA segments. The M segment codes for a glycoprotein precursor which is co-translationally cleaved into the viral envelope glycoproteins, Gn and Gc, as well as a non-structural protein, named NSm (Elliott, 1990; Fazakerley *et al.*, 1988). The S segment encodes two proteins, the nucleocapsid (N) protein and a smaller non-structural protein (NSs), in overlapping reading frames (Elliott, 1990; Fuller *et al.*, 1983). A characteristic feature of the orthobunyavirus genome segments is the complementarity of the 3' and 5' termini, of which the base pairing results in so-called panhandle structures (Elliott, 1990).

Alignments of S segments of viruses from the Simbu serogroup have revealed nucleotide sequence identities range between 65% and 99.1% (Saeed *et al.*, 2001a). The nucleotide sequence identity of the M segment open reading frame (ORF) ranges from 47.9% to 56.0% between members of the Simbu serogroup. The amino acid (aa) sequences are highly variable (37.3- 49.7%), although several features are conserved. These features include 59 of the 71 cysteine residues and a conserved arginine (R) residue which is located proximal to the predicted cleavage site between Gn and NSm (Yanase *et al.*, 2003).

Analysis of the L segment has shown nucleotide identity between members of the Simbu serogroup to range between 60.4% and 92.9%. The conservation at the aa level varies from 57.5% to 98.4% (Goller *et al.*, 2012). Four conserved regions have been identified in the L protein. Region I flanks the strictly conserved dipeptide PD and region II flanks the conserved dipeptide RY. The corresponding Region I of the orthobunyavirus La Crosse virus (LACV) was shown to be active as a nuclease and its crystal structure revealed strong similarity with the influenza virus polymerase PA subunit (Reguera *et al.*, 2010). Region III contains a polymerase module, which consists of pre-motif A and motifs A, B, C, D and E are highly conserved (62.3–1.9%) (Elliott, 1990; Elliott *et al.*, 1991; Muller *et al.*, 1994). Region IV is located downstream of motif E and contains four conserved aa residues(Ogawa *et al.*, 2007).

As in the case of other viruses that have segmented genomes, the occurrence of genetic reassortments of orthobunyaviruses has been reported. Ngari virus (MRIV) for instance was generated as a result of reassortment between BUNV and Batai viruses (BATV) (Briese *et al.,* 2006; Yanase *et al.,* 2006). For the Simbu serogroup, the Jatobal (JATV) and Tinaroo (TINV) viruses are likely reassortments containing RNA segments from OROV and AKAV respectively (Kobayashi *et al.*, 2007; Saeed *et al.*, 2001b). Reassortment has also been observed between Australian field isolates of Aino (AINOV) and Peaton (PEAV) viruses (Yanase *et al.*, 2010), viruses which are closely related to SHUV both serologically and phylogenetically.

The objectives of this study were to; a) determine and describe the full genome sequence of the SAE 18/09 SHUV isolate, b) to investigate the phylogenetic relationships between the Simbu serogroup viruses at the full genome level and c) to investigate the antigenic, pathogenic and conserved domains on the SHUV genome.

5.2 METHODS

5.2.1 Specimen

Shuni virus isolate SAE 18/09 was grown up as previously described (Refer to 3.2.2) and used for the full genome amplification assays.

5.2.2 Full genome amplification, conventional PCR

5.2.2.1 Primer design

Primers were designed based on SHUV and other Simbu serogroup virus sequences available in the public domain: GenBank, through alignment using the ClustalW subroutine, which forms part of the Bioedit program (Hall, 1999) (Appendix B). Genome position is based on the full SHUV genome obtained in this study, S segment–KC510272, M segment–KF153117, L segment–KF153118.

5.2.2.2 RNA extraction

Particle purification and RNA extraction were carried out as before (Refer to 2.2.2.1).

5.2.2.3 RT PCR amplification

RT-PCRs were performed using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) and SHUV specific primers designed for the present study (Appendix C). Briefly, 10µl RNA was added to 10µl 5x reaction buffer, 5mM DTT solution, 40pmol of each primer, 10mM of each dNTP, 1µl Titan™ enzyme mix, 10 U of Protector RNase inhibitor (Roche, Mannheim, Germany) to a final volume of 50µl. The reaction mix was subject to initial incubations of 50˚C for 40 minutes, 94˚C for 2 minutes followed by 35 PCR cycles: 94˚C, 30 sec; 40˚C - 57ºC (Based on primer pair annealing temperatures), 1 min; 68˚C, 1 min, supplemented by a final extension for 7 min at 68˚C.

5.2.2.4 Cloning and sequencing

Purification and sequencing were carried out as before (Refer to 2.2.4).

5.2.3 Full genome amplification, Illumina

Although the full S segment and parts of the M and L segments could be determined by primer walking, the lack of conservancy in the latter, led to the need to employ a next generation sequencing technique to complete the viral genome.

5.2.3.1 Next generation sequencing - Sample preparation

45ml SAE 18/09 cell culture supernatant was put through Amicon Ultra 10K centrifugal filters (Merck, Darmstadt, Germany), resulting in 600ul of concentrate. The concentrate was then subject to Trizol LS (Sigma-Aldrich, Missouri, United States) RNA isolation. The extracted material was then purified with the RNA Clean and Concentrator-5 kit (Zymo research, California, United States), to remove all fragments smaller than 200bp, the in-column DNase step was included. The sample was then prepared for transport using GenTegra RNA tubes (IntegenX, California, United States) according to the manufacturer's instructions.

5.2.3.2 Next generation sequencing

The sample was processed at the Central Veterinary Institute of Wageningen University Research Centre in the Netherlands for full-genome sequencing. Briefly, after reconstitution, the sample was purified to remove additional small fragments (RNA Clean and Concentrator-5 kit) and the libraries were prepared using the ScriptSeqv2 RNA-Seq Library Preparation kit (Epicentre Biotechnologies, Wisconsin, United States). Both the protocol for FFPE samples and the protocol for alternative fragmentation (2 min 85ºC, instead of 5 min) were utilized according to manufacturer's instructions unless otherwise stated. Quality of the libraries was determined using the Bioanalyzer with the High Sensitivity DNA kit (Agilent Technologies, California, United States) and quantity, using the Qubit dsDNA HS Assay kit (Life Technologies, New York, United States). Cluster generation and paired-end 250bp sequencing of the libraries was done on an Illumina MiSeq V2 instrument. Raw sequencing data was first trimmed and QC filtered using in-house scripts. Possible contaminants were identified (host cell fragments) by short-read mapping using Bowtie2 (Langmead & Salzberg, 2012) and subsequent filtering.

5.2.3.3 Next generation sequencing - Bioinformatics

The full viral genome sequences of all three segments (S, M and L) were identified using two different approaches being either a full *de novo* reconstruction or a mapping based approach starting from earlier determined sequences of the three segments. Full *de novo* genome reconstruction was achieved by sequence assembly using ABySS (version 1.3.3; Canada's Michael Smith Genome Science Centre). Repeated short-read mapping using Bowtie2 and manually inspecting any strange mapping densities were done to reduce any errors in the assembly process. Finally the full genome was reconstructed using closely related reference sequences where we determined the synteny of the initial contigs from

ABySS. The mapping-based approach where sequence reads were mapped using Bowtie2 on the earlier determined sequence fragments of the three segments confirmed/refined earlier derived sequences. In addition it allowed the reconstruction of the full 5-prime and 3-prime sequence ends of all three segments. Further manual curation by both complementary approaches into a single high-quality sequence was performed using SeqMan (DNASTAR Lasergene version 11).

5.2.4 Phylogenetic analysis

Nucleotide phylogenetic analysis was carried out as before (Refer to 2.2.5). Amino acid analysis was carried out using the Jones-Taylor-Thornton (JTT) model (Jones *et al.*, 1992) which forms part of Mega version 5 using 1000 bootstrap analysis (Tamura *et al.*, 2011).

5.2.5 Analysis of sequences

Obtained sequences were edited using the Sequencher^{TM} Version 4.6 software package (Gene Codes Corporation, Ann Arbor, MI). Alignments carried out using the ClustalW subroutine (Thompson *et al.*, 1994) which forms part of the Bioedit program. Sequence similarity was determined using the distance estimation program of Mega v5 (Tamura *et al.*, 2011). Genetic distances were calculated for both the nucleotide and deduced amino acid sequences using the p-distance model (Nei & Gojobori, 1986). Analysis of cleavage sites, glycosylation sites and trans-membrane domains was accomplished with the programs listed in Table 5.1.

Table 5.1 Functional domain prediction programs

Program	Website	Reference
SignalP	http://www.cbs.dtu.dk/services/SignalP	(Petersen *et al.*, 2011)
NetNgly	http://www.cbs.dtu.dk/services/NetNGlyc	(Gupta *et al.*, 2004)
TopPred2	http://www.sbc.su.se/~erikw/toppred2	(von Heijne, 1992)
TMHMM	http://www.cbs.dtu.dk/services/TMHMM	(Krogh *et al.*, 2001)
HMMTOP	http://www.enzim.hu/hmmtop	(Tusnady & Simon, 1998)
TMpred	http://www.ch.embnet.org/software/TMPRED_form.html	(Hofmann & Stoffel, 1993)
DAS	http://www.sbc.su.se/~miklos/DAS/maindas.html	(Cserzo *et al.*, 1997)
MEMSAT	http://bioinf.cs.ucl.ac.uk/psipred	(Jones *et al.*, 1994)
SOSUI	http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html	(Hirokawa *et al.*, 1998)

5.3 RESULTS

5.3.1 S segment

5.3.1.1 The full S segment sequence

The S segment of SAE 18/09 was found to be 850 nucleotides (nt.) in length, with a 3' untranslated region (UTR) of 33 and a 5' UTR of 115 nucleotides (Figure 5.1). The N gene was found to contain 699 nt. coding for 233 amino acids (aa), whilst the NSs gene comprised 273 nt. and encoded a 91 aa protein, reflecting the sizes found in other members of the Simbu serogroup (Table 5.2), sequence identity is also indicated.

5.3.1.2 Distance and phylogenetic analysis

Full S segment

A set of 11 complete S segment sequences of members of the Simbu serogroup were analysed using the maximum likelihood (ML) method. Phylogenetically the S segment of SAE 18/09 groups with Aino and Kaikular viruses, although bootstrap support was low (38%), the separation of SAE 18/09 as an outgroup in this clade is however supported by a 100% bootstrap value (Figure 5.2a). Nucleotide identity was determined for the full S segment of SAE 18/09 and other Simbu serogroup viruses, percentage identity values obtained showed SAE 18/09 to be most similar to Aino (AINOV) (93.9%) and Kaikular (KAIV) (93.7%) viruses, and most divergent from Ingwavuma virus (66.6%) (Table 5.2).

Nucleoprotein (N)

The N protein was analysed at both the nucleotide and amino acids levels. At the nt. level SAE 18/09 grouped with the prototype Shuni virus strain (An 10107) (97.9%). In the Aino/Kaikular clade, the grouping was similar to what was obtained from analysis with the whole S segment. At the aa level however, SAE18/09 groups with Shuni, Peaton and Sango viruses, the bootstrap values for this branching is however low (55%) (Figure 5.3a). Distance analysis revealed identity between the two SHUV isolates to be 100%, with interspecies values of 99.6% (Peaton, PEAV), 99.6% (AINOV), 99.6% (Sango, SANV) and 98.3% (KAIV) (Table 5.2).

Non-Structural protein S segment (NSs)

Phylogenetic analysis of NSs revealed similar groupings at both the nucleotide and amino acid levels (Appendix B, Figure 1), which corresponded with the N protein analysis.

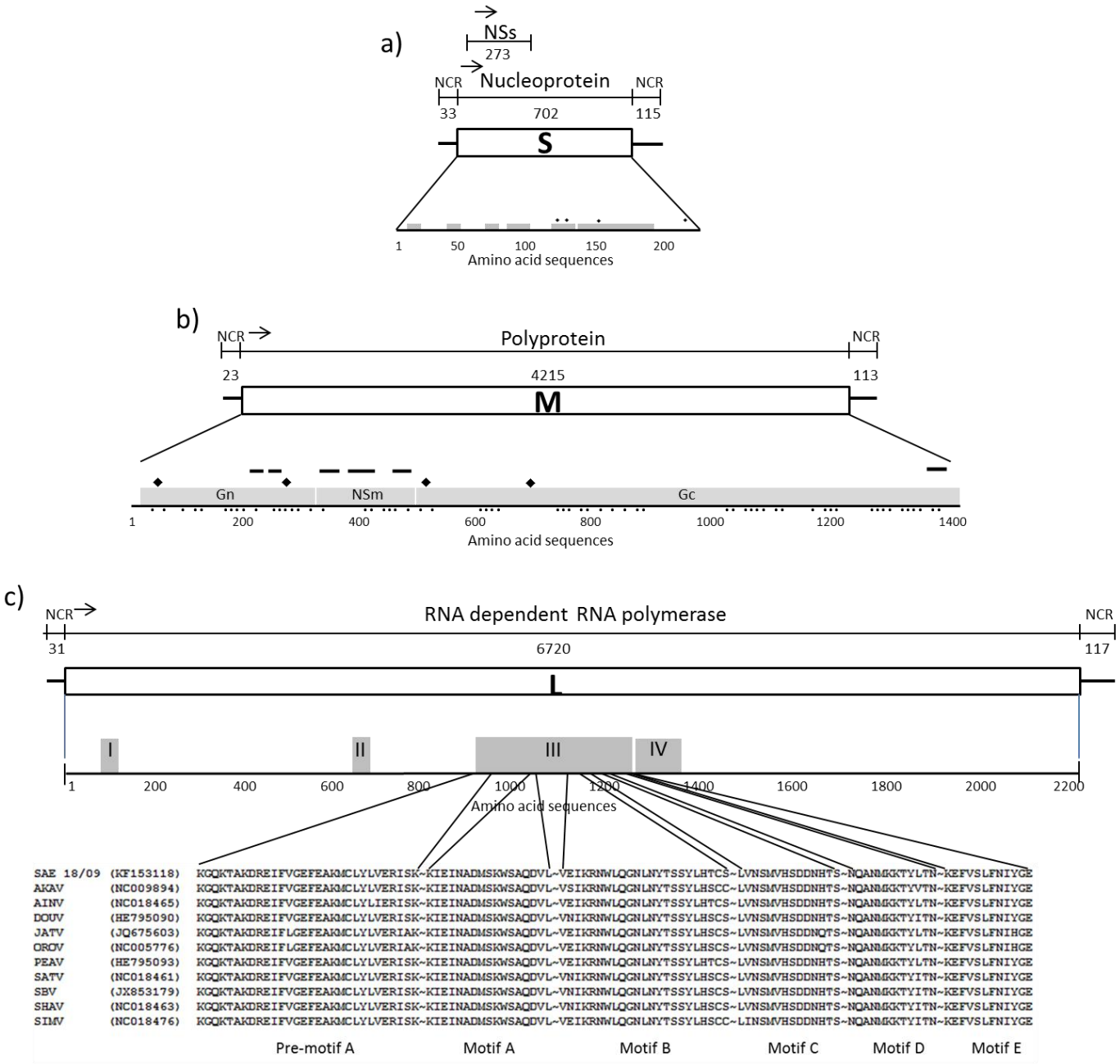


Figure 5.1 Genome characteristics of Shuni virus (SAE 18/09). (a) The S segment codes the N and NSs proteins in overlapping reading frames, the grey boxes indicate the conserved regions identified by Saeed et al. (2001) and the black diamonds the four residues involved in the formation of the ribonucleoprotein complexes. (b) The M segment codes the GPC which is co-translationaly cleaved into the Gn, NSm and Gc, the black diamond indicate the glycosylation sites and the black boxes the transmembrane regions. (c) The L segment codes the RdRp, regions I-IV are indicated by the grey boxes and the pre-A, A, B, C, D and E motifs from region III are indicated. Genome segments are depicted in the antigenomic sense orientation.

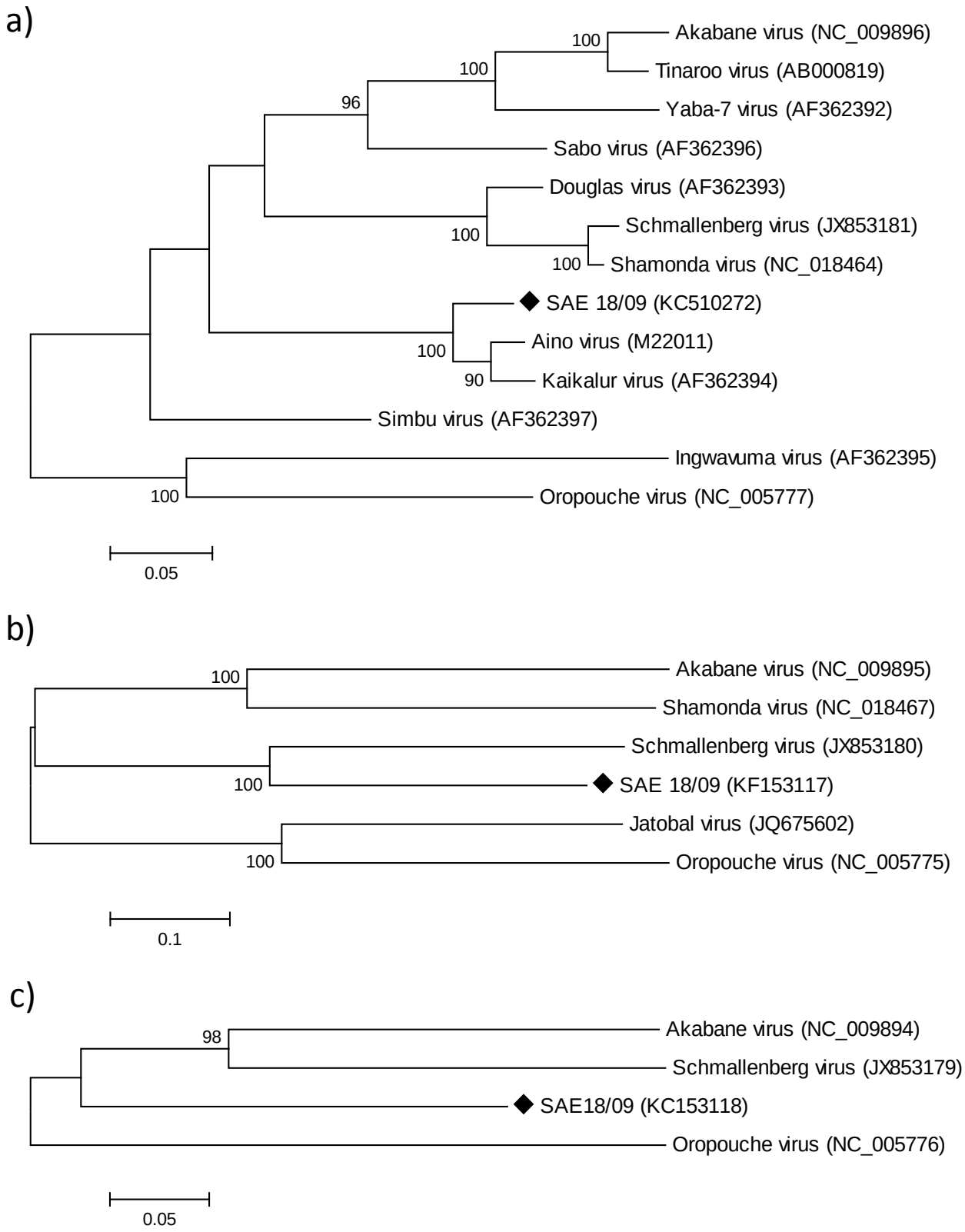


Figure 5.2 Phylogenetic relationship between the S (a), M (b) and L (c) segments of SHUV (SAE 18/09). Maximum likelihood trees constructed under the Tamura-Nei substitution model using the Mega 5 program, with representative full genome sequences of other Simbu serogroup viruses. Estimates were based on bootstrap resampling carried out with 1000 replicates. Reference strains GenBank accession numbers are indicated.

Phylogenetic analysis of the NSs revealed similar groupings at both the nt. and aa levels. SAE 18/09 grouped with SHUV (An 10107), AINOV, KAIV, and SANV although bootstrap support was low. Analysis of NSs at the nucleotide level showed an identity of 99.3% between the SHUV isolates, and interspecies values of 99.3% (KAIV) and 98.6% (AINOV). Amino acid analysis revealed a similar result, with a value of 98.9% between the SHUV isolates and interspecies values of 98.9%. (KAIV) and 96.7% (AINOV).

Conserved domains

Certain regions of the N protein are conserved amongst members of the *Orthobunyavirus* genus, and are presumed to be of functional importance (Elliott, 1990). These six domains (Figure 5.1; Appendix C, Figure 1) are well conserved between SAE 18/09 and other members of the Simbu serogroup, as was observed by Saeed *et al*. (2001a), however several amino acids identified as being globally conserved between four of the major serogroups, were not all conserved even between members of the Simbu serogroup in this analysis, a finding previously highlighted by Savji *et al.,* (2011). Of significance is the observation that within the first conserved domain, the original Shuni isolate from Nigeria (AF362405) has an Asparagine (N) residue at position 9 when all other members of the serogroup have an Aspartic acid (D) residue. The four residues involved in the formation of the ribonucleoprotein complexes (P_{125}, G_{131}, Y_{158} and I_{231}) (Eifan & Elliott, 2009) are all conserved in SAE 18/09 (Figure 5.1; Appendix C, Figure 1).

5.3.2 M segment

5.3.2.1 The full M segment sequence

The M segment of SAE 18/09 was found to be 4351 nucleotides in length, with a 3' UTR of 23 and a 5' UTR of 112 nucleotides (Figure 5.1). The glycoprotein precursor coding for 1404 amino acids (aa) (Table 5.2). The predicted signal peptide for SAE 18/09 determined by SignalP 4.1, lies between aa 17 and 18 (GVP-IP) (Appendix C, Figure 2), corresponding with what was previously found for AKAV and AINOV (Yanase *et al.*, 2003) and confirmed with our analysis (results not shown). The Gn/NSm cleavage site KSLRAAR found to be conserved amongst members of the California and Bunyamwera serogroups (Briese *et al.*, 2007; Briese *et al.*, 2004; Fazakerley *et al.*, 1988; Pollitt *et al.*, 2006) is less well conserved within the Simbu serogroup though OROV and PEAV maintain these residues.

Table 5.2 Characteristics of the S, M and L segments of 5 Simbu serogroup viruses compared with SAE 18/09

S Segment							
	Nucleotide Sequence					**Amino Acid Sequence**	
Virus	**Total***	**3' UTR**	**5' UTR**	**N***	**NSs***	**N‡**	**NSs‡**
SAE18/09 (KC510272)	850	33	115	702	276	233	91
SHUV (HE800143)	_	_	_	702(97.9)	276(99.3)	233 (100)	91 (98.9)
KAIV (AF362394)	850 (93.7)	34	114	702(93.6)	276(99.3)	233 (98.3)	91 (98.9)
AINOV (HE795089)	850 (93.9)	34	114	702(94)	276 (98.6)	233 (99.6)	91 (96.7)
PEAV (HE795095)	_	_	_	702(91.7)	276 (97.5)	233 (99.6)	91 (94.5)
SIMV (HE795110)	867 (77.5)	33	132	702(72.2)	276 (83)	233 (79.8)	91 (62.7)
M Segment								
	Nucleotide Sequences						**Amino Acid Sequences**		
Virus	**Total***	**3' UTR**	**5' UTR**	**Gn***	**NSm***	**Gc***	**Gn‡**	**NSm‡**	**Gc‡**
SAE18/09 (KF153117)	4351	23	113	873	468	2820	291	156	940
SHUV (HE800142)		_	_	873 (99.2)	471 (97.9)	2817 (98.8)	291 (98.7)	157 (96.2)	939 (97.9)
AINOV (HE795088)	_	_	_	873 (92.5)	468 (70.3)	2820 (83.7)	291 (89.3)	156 (59)	940 (74.4)
SATV (HE795103)	_	_	_	873 (82.9)	468 (56.7)	2817 (68.4)	291 (73.4)	156 (39.3)	939 (53.8)
SBV (HE649913)	_	_	_	873 (82.7)	468 (57.6)	2817 (68.3)	291 (72.7)	156 (42.7)	939 (53.1)
SIMV (HE795109)	_	_	_	873 (78.5)	486 (57.4)	2814 (64.9)	291 (66.6)	162 (40.4)	938 (48.2)
L Segment					
	Nucleotide Sequences				**Amino Acid Sequence**
Virus	**Total***	**3' UTR**	**5' UTR**	**RdRp***	**RdRp‡**
SAE18/09 (KF153118)	6910	31	117	6762	2253
SHUV (HE800141)	_	_	_	6762 (93.5)	2253 (98.9)
AINOV (HE795087)	_	_	_	6762 (85.1)	2253 (95.2)
PEAV (HE795093)	_	_	_	6759 (78.1)	2252 (88.9)
SANV (HE795099)	_	_	_	6762 (78.7)	2253 (90.5)
SIMV (NC018476)	_	_	_	6762 (67.9)	2253 (70.9)

* Values are lengths in nucleotides (percentage identity with SAE 18/09)
‡ Values are lengths in amino acids (percentage identity with SAE 18/09)
_ Not available

Based on alignment with these viruses and comparison to the data obtained by Yanase *et al.*, (2003); the suggested Gn/NSm cleavage site for SAE18/09 is 308-309 following the conserved arginine (R) residue (Figure 5.1; Appendix C, Figure 2). The cleavage site between NSm and Gc is poorly conserved amongst the Simbu serogroup (Yanase *et al.*, 2003), though between other members of the *Orthobunyavirus* genus cleavage usually

follows a conserved alanine residue (Briese *et al.*, 2004; Pollitt *et al.*, 2006; Savji *et al.*, 2011). SignalP predicts cleavage of SAE18/09 at VDA_{464}-ND, which results in -3=V and -1=A one of the most frequent combination in signallase sites. Based on these analyses, the Gn protein comprises 291aa, NSm 156aa and Gc 940aa (Table 5.2), sizes which correlate well with what was found previously for other Simbu viruses (Savji *et al.*, 2011). SHUV isolate An 10107 however differs at three positions with SAE18/09 at the NSm/Gc cleavage site, resulting in a predicted cleavage at AAS_{465}-DK, changing the size of the NSm to 157aa and Gc to 939aa.

The number and positions of N-linked glycosylation sites (N-X-S/T) are not well conserved amongst the Simbu serogroup (Yanase *et al.*, 2003) and in this analysis using NetNGlyc 1.0, AKAV and SBV viruses had 10 potential sites each whereas, AINOV and PEAV had six and five respectively (Appendix C, Table 1). Four potential glycosylation sites were identified in the glycoprotein precursor of both SAE 18/09 and An10107, two in Gn and two in Gc (Figure 5.1; Appendix C, Figure 2). The site at the amino terminus of Gn (N_{40}) is well conserved amongst the Simbu serogroup, as is N_{492}, at the amino terminus of Gc. Analysis showed 60 of 71 cysteine residues to be conserved between all Simbu serogroup viruses analysed, which is one more than originally found by Yanase et al., 2003 (Figure 5.1).

Based on the comparison of seven transmembrane prediction software packages (Appendix C, Table 2), the results produced by TMHMM best reflect the true membrane domains. Two regions are identified in Gn (212-229) and (234-253) which together form a very long hydrophobic sequence (212-253) that is followed by charged amino acids (254-267) similar to stop-transfer sequences seen in the transmembrane domains of other viral envelope proteins (Elliott, 1990; Fazakerley *et al.*, 1988). In NSm three regions were identified (315-337, 371-393, 445-462) and in Gc one (1357-1379), the latter likely functions as a membrane anchor (Fazakerley *et al.*, 1988; Pekosz *et al.*, 1995).

5.3.2.2 Distances and phylogenetic analysis

Full M segment

Only five complete Simbu group M segments were available for this analysis and as such SAE 18/09 grouped with Schmallenberg virus (SBV) as it was the most closely related member present. Separation of the six strains into three distinct groups (SAE 18/09–SBV, AKAV–SHAV, JATV–OROV) was supported by 100% bootstrap values. Nucleotide

identity for the full M segment revealed SAE 18/09 to be most similar to SBV (61.4%) followed by JATV (51.8%) and SHAV (49.4%) (Figure 5.2b).

Glycoprotein precursor (GPC)

Phylogenetic analysis revealed that SAE 18/09 groups with SHUV and AINOV at both the aa and nt. levels with 100% bootstrap support for the complete GPC (Appendix B, Figure 2a). Distance analysis at the nucleotide level showed identity values of 98.8% between the SHUV isolates and interspecies values of 84.9% (AINOV) and 70.7% (SATV). At the amino acid level the SHUV isolates share 97.9% identity. The interspecies values were much lower with 75.9% (AINOV) and 56.4% (SATV), reflecting the low level of conservation in the orthobunyavirus GPC.

Gn – NSm - Gc

Amino acid (Figure 5.3c) and nucleotide analysis of the Gn protein of SAE 18/09 showed the same topology as the full GPC analyses, where SAE 18/09 groups with SHUV and AINOV within a clade that comprises DOUV, SATV and SBV. Distance estimation showed the Gn protein to be more conserved than the precursor with nt. identity values of 99.2% between the SHUV isolates, 92.5% to AINOV and 82.9% to SATV. Amino acid identity values between the SHUV isolates was 98.7%, followed by interspecies values of 89.3% (AINOV) and 73.4 (SATV). Analysis of the NSm sequence revealed a topology similar to that of Gn. Genetic distance between the Simbu group viruses is low for NSm (Fazakerley *et al.*, 1988; Yanase *et al.*, 2003), in the nt. analysis SAE 18/09 shared 97.9% identity to SHUV (An 10107) and only 70.3% similarity to AINOV. At the aa level the two SHUV isolates shared 96.2% identity followed by a 59% identity to AINOV.

Phylogenetic analyses of Gc showed the same topology as was observed for Gn at both the nt. and aa levels (Figure 5.3d). Nucleotide identity between the two SHUV isolates was 98.8%, with an interspecies identity of 83.7 to AINOV. Amino acid analysis showed 97.9% identity between the SHUV isolates and 74.4% identity to AINOV. The lower level of homology in the Gc protein is well documented for the *Orthobunyaviruses* (Elliott, 1990; Fazakerley *et al.*, 1988; Yanase *et al.*, 2003) (Fig. 3d).

5.3.3 L segment

5.3.3.1 The full L segment sequence

The L segment of SAE 18/09 is 6910 nt. in length with a 31 nucleotide 3' UTR and a 117 nt. 5'UTR. The RNA-dependent RNA polymerase (RdRp) codes for 2253 amino acids. These nt. and aa lengths are comparable with those seen in other members of the Simbu serogroup (Table 5.2), although SAE 18/09 has a significantly longer 5' UTR.

5.3.3.2 Distance and phylogenetic analysis

Full L segment

Only 3 complete Simbu group L segments were available for this analysis. SHUV strain SAE 18/09 branched separately from the other Simbu serogroup members (Figure 5.2c), including SBV with which it grouped in the full M segment analysis (Figure 5.2b). Nucleotide identity showed the full L segment of SAE 18/09 to be most similar to SBV (66.2%), followed by AKAV (65.8%) and OROV (61.8%).

RNA-dependent RNA polymerase

Phylogenetic analysis of the RdRp protein of SAE 18/09 grouped together with SHUV (An 10107) in a clade with AINOV, with high bootstrap support at both the nucleotide and amino acid levels (Figure 5.3b). Sequence identity at the nucleotide level demonstrated SAE 18/09 to be 93.5% similar to SHUV (An 10107), 85.1% to AINOV and 78.7% to SANV. At the amino acid level it was found to be 98.9% similar to SHUV (An 10107), 95.2% to AINOV and 90.5% to SANV.

Conserved domains

Comparing the L protein aa sequences of SAE 18/09 with other Simbu serogroup members shows homology at the endonuclease active site H…D…PD….DxK…..T at positions 34, 53, 76, 77, 90, 92 and 103 respectively (Reguera et al., 2010). Conserved regions I and II containing conserved residues (PD, aa76-77) and (RY, aa 650-651) respectively were also identified in all members investigated. Pre-motif A as well as motifs A, B, C, D and E which make up region III (aa 947-1238), are highly conserved with the only significant difference being that AINOV has a V→I_{971} substitution which is not shared by any other members including SAE 18/09 (Fig. 1c). The four conserved residues of region IV; G_{1274}, Y_{1294}, G_{1323} and G_{1336} (Ogawa *et al.*, 2007) showed 100% homology. (Figure 5.1c; Appendix C, Figure 3).

5.3.4 Terminal sequences

The 3' untranslated region (UTR) sequences of all three SAE 18/09 genomic segments are identical for the first 10 nt, as are the corresponding 5' UTR sequences (Figure 5.4). In addition, these 3' and 5' sequences exhibit complementarity for all but one of the first 11 nt; the mismatch at position 9 is conserved amongst members of the *Bunyaviridae* (Elliott, 1990; Elliott *et al.*, 1991; Fauquet & Fargette, 2005). Complementarity extends to 15 nt. in the S segment, 17 nt. in the M segment and 19 nt. in the L segment (Figure 5.5), this is similar to what is seen in Bunyamwera virus where complementarity extends to 15, 18 and 19 nt. respectively (Barr *et al.*, 2003; Barr & Wertz, 2004). The AACA motif which is associated with the minireplicon activity (Kohl *et al.*, 2006) was found to be conserved for all three segments of SAE 18/09 ($AACA_{24}$).

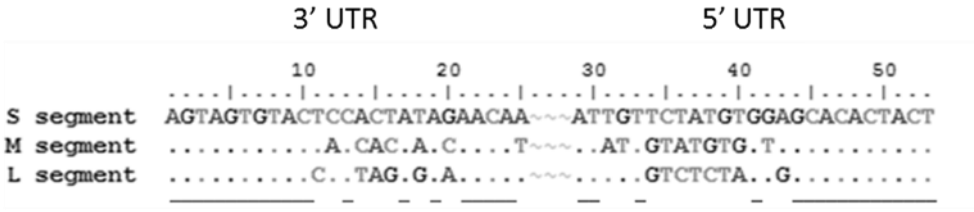


Figure 5.3 Sequence comparisons to identify the highly conserved nucleotides in the untranslated regions of the S, M and L segments of SAE 18/09. Conserved nucleotides are underlined.

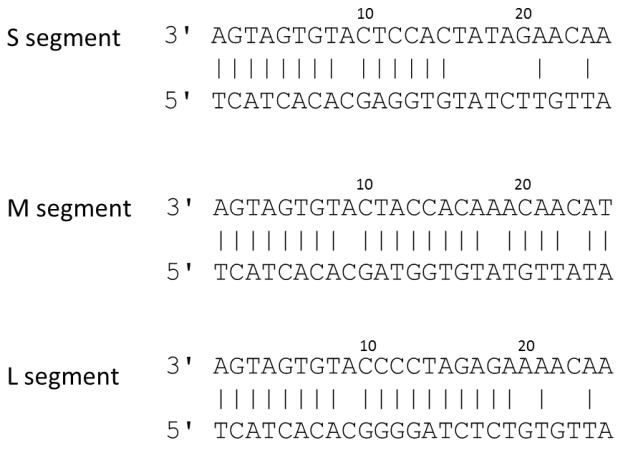


Figure 5.4 Analysis of the complementarity between the 3' and 5' untranslated regions of the S, M and L segments of SAE 18/09.

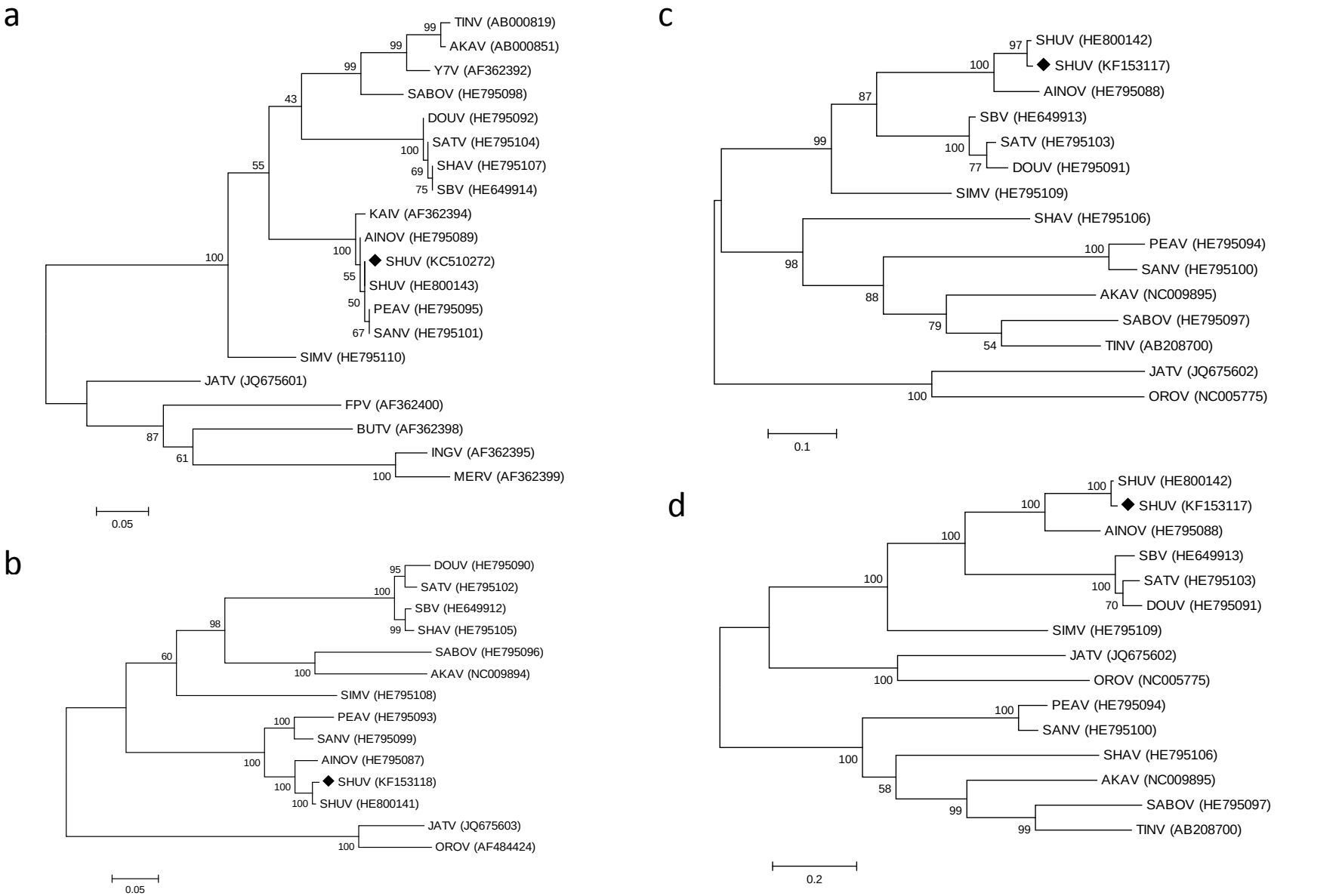


Figure 5.5 Relationship of Shuni virus (SAE 18/09) to other members of the Simbu serogroup. Maximum likelihood trees of the amino acid sequences of the N (a), L (b), Gn (c) and Gc (d) proteins, constructed under the Jones-Taylor-Thornton (JTT) substitution model using the Mega 5 program. Estimates were based on bootstrap resampling carried out with 1000 replicates. Reference strains GenBank accession numbers are indicated.

5.4 DISCUSSION

Given the importance of the orthobunyaviruses assigned to the Simbu serogroup in both public and veterinary health, surprisingly little genetic information is available. Following the identification of SHUV in multiple cases of neurological disease in equines (van Eeden *et al.*, 2012) (Chapter 3), the need to investigate its molecular biology arose in preparation for reverse genetics studies. In a recent study the near complete genome for the prototype SHUV isolate (An10107), isolated from an asymptomatic bovine in Nigeria in 1966, was determined (Goller *et al.*, 2012), this gave us the opportunity to compare SAE18/09; an isolate which had been associated with neurological disease in an equine (van Eeden *et al.*, 2012), to this isolate from an asymptomatic bovine (Causey *et al.*, 1972).

Although only a limited number of viral sequences were available for the full segment analyses, the overall topology for the S, M and L segments was uniform and reflected the results seen in other partial genome studies (Goller *et al.*, 2012; Saeed *et al.*, 2001a), nevertheless more full genomes are required to fully elucidate the relationship between the Simbu serogroup viruses.

Analysis of the viral proteins was more comprehensive and for all the proteins SHUV SAE18/09 grouped with SHUV An10107 and AINOV, its closest serological relative, the topology reflecting the original molecular analysis (Saeed *et al.*, 2001a). Sequence identity between the two SHUV isolates was high and ranged between 93.5–99.3% at the nt. level and 96.2-100% at the aa level, of all viral proteins. For the N ORF in particular the values of 97.9% (nt.) and 100% (aa) are very high for isolates from different geographic locations, for Aino and Akabane viruses for instance the identity between Australian and Japanese strains can result in up to 7-8.9% difference (Akashi *et al.*, 1997b; Matsumori *et al.*, 2002).

The only noteworthy variation being that the prototype SHUV isolate's (An10107) predicted cleavage site for NSm/Gc, produces an NSm and Gc protein of one aa longer and shorter, respectively due to three amino acid changes at the cleavage site. The significance of these changes will need to be investigated in studies of expression and pathogenicity, as such mutations can lead to changes in the efficiency of signal peptide cleavage, which in turn can affect the production of infectious virions (Lee *et al.*, 2000). The high level of conservancy between these two isolates; one of neurologic origin and the other

asymptomatic origin further highlights the need to investigate the factors which influence SHUV pathogenicity.

In summary the genome of SHUV shared all the features and conserved domains previously identified for the Simbu serogroup. Phylogeny of the viral proteins was similar throughout, with no indication of genomic reassortment. Mutations between the two SHUV strains at the NSm/Gc cleavage site will need to be investigated further. This genome sequence and phylogenetic analysis marks an important step towards the establishment of a reverse genetics system to analyse the biology and pathogenicity of SHUV, as well as further elucidating the molecular relationship between members of the Simbu serogroup.

CHAPTER 6
The epidemiology of Shuni virus in horses over a period of five years

6.1 INTRODUCTION

Considering the public and veterinary health importance of other members of the *Orthobunyavirus* genus, surprisingly little attention has been placed on uncovering the role SHUV may play in encephalitic disease. Recently described emerging orthobunyaviruses; Schmallenberg virus in Europe (Hoffmann *et al.*, 2012) and Iquitos virus in Peru (Aguilar *et al.*, 2011), further highlighting the potential of orthobunyaviruses as emerging pathogens.

Following the identification of WNV as a cause of neurological disease in horses, a surveillance network for neurological disease in horses was established with veterinarians from across the country. Retrospective and prospective investigation of cases sent to the Zoonoses Research unit (ZRU) between the years 2008 and 2012, enabled us to investigate other causes of neurological disease, to assess geographic distribution, seasonality and epidemiology in horses.

The identification of SHUV in seven horses with unexplained fever and/or neurological symptoms between June 2009 and December 2010 (van Eeden *et al.*, 2012) (Chapter 3), led us to believe that this virus may play a noteworthy role in neurological disease in South Africa. We aimed to investigate the epidemiology of SHUV in South Africa over a five year period, by screening our diagnostic population of acute cases submitted through our zoonotic arbovirus sentinel surveillance programme for neurological disease. To aid in the identification of acute disease, we developed a SHUV real-time PCR assay (Chapter 4) to improve on turnaround time, sensitivity and specificity.

This report describes the application of the previously described SHUV nested real-time PCR to the screening of an additional 386 cases of neurological disease in horses in South Africa, with the aim of further elucidating the epidemiology of Shuni virus.

6.2 METHODS

6.2.1 Specimens

In addition to the 112 horses screened in our original study (van Eeden *et al.*, 2012), we screened an additional 378 neurological horse cases submitted to the ZRU for diagnostic testing. Samples were sent by the Onderstepoort Veterinary institute, the UP Faculty of Veterinary Science, Onderstepoort, as well as by veterinarians from around the country. The specimens were screened as appropriate for poisons and rabies virus.

6.2.2 SHUV specific nested real-time PCR

Nucleic acid extraction (Refer to 3.2.6.2) and nested real-time PCRs (Refer to 4.2.4) (van Eeden *et al.*, 2013) were carried out as previously described.

6.2.3 Differential diagnosis

Differential diagnosis was carried out as before (Refer to 2.2.3).

6.2.4 Sequencing and phylogenetic analysis

Purification, sequencing (Refer to 2.2.4) and nucleotide phylogenetic analysis were carried out as before (Refer to 2.2.5).

6.3 RESULTS

6.3.1 SHUV positive cases

In our initial investigation (June 2009 through December 2010) (Chapter 3), we screened 112 horses and identified seven SHUV positive animals, including the index case (SAE 18/09). In screening the additional 378 horses, two new SHUV cases were identified. These bring the total number of SHUV infections identified in horses, in our study, to 9/490 (1.8%). The total number of cases per year ranged between 0% and 3.7%. In three of the nine cases SHUV was amplified from brain tissue, indicating the crossing of the blood-brain barrier, the rest were detected in the serum of acute horses.

Table 6.1 Clinical Signs and Symptoms of Shuni Virus Cases Identified in Horses in South Africa

Case	Specimen	Age (Years)	District, Province	Outcome	Date Received
SAE 18/09	Brain, Cell culture	1	Vaalwater, Limpopo	Euthanized	16/01/2009
SAE 72/09	Brain	5	Bapsfontein, Gauteng	Euthanized	02/07/2009
SAE 27/10	Blood	18	Unrecorded, Gauteng	Euthanized	13/04/2010
SAE 38/10*	Blood	4	Kimberly, Northern Cape	Survived	14/04/2010
SAE 39/10	Blood	4	Kimberly, Northern Cape	Survived	14/04/2010
SAE 48/10	Blood	4	Norvalspont, Northern Cape	Survived	23/04/2010
SAE 109/10	Blood	13	Bronkhorstspruit, Gauteng	Survived	27/07/2010
SAE 15/11	Blood	13	Nelspruit, Mpumalanga	Died	09/02/2011
SAE 87/11	Blood	N/A	Villiersdorp, Western Cape	Survived	13/04/2011

*Indicates Middelburg co-infection

6.3.2 SHUV demographics

Geographically, acute SHUV cases were identified in the Gauteng, Limpopo, Northern Cape and Mpumalanga provinces over a five year period (Figure 6.1, Table 6.2). As we rely on veterinarians from around the country to send us cases, the apparent lack of cases in the central and eastern regions of the country may only be a reflection of the limited neurological cases submitted from these regions.

Table 6.2 Distribution of SHUV in South African Provinces

Province	2008*	2009*	2010*	2011*	2012*	Total Positive
Gauteng	(38)	1 (21)	2 (37)	(49)	(21)	3/166 (1.8%)
Limpopo	(2)	1 (1)	(0)	(1)	(1)	1/5 (20%)
Mpumalanga	(0)	(1)	(3)	1 (4)	(2)	1/10 (10%)
Northern Cape	(4)	(4)	3 (15)	1 (37)	(3)	4/63 (6.3%)
Western Cape	(1)	(4)	(25)	(41)	(24)	0/100
Eastern Cape	(0)	(0)	(5)	(1)	(3)	0/9
Kwa-Zulu Natal	(1)	(24)	(36)	(11)	(14)	0/86
Free State	(0)	(2)	(8)	(4)	(5)	0/19
North West	(1)	(0)	(2)	(1)	(1)	0/5
Not specified	(8)	(1)	(4)	(12)	(7)	0/32
Total	(55)	2 (58)	5 (135)	2 (161)	(81)	9/490 (1.8%)

*Positive specimens (Screened specimens)

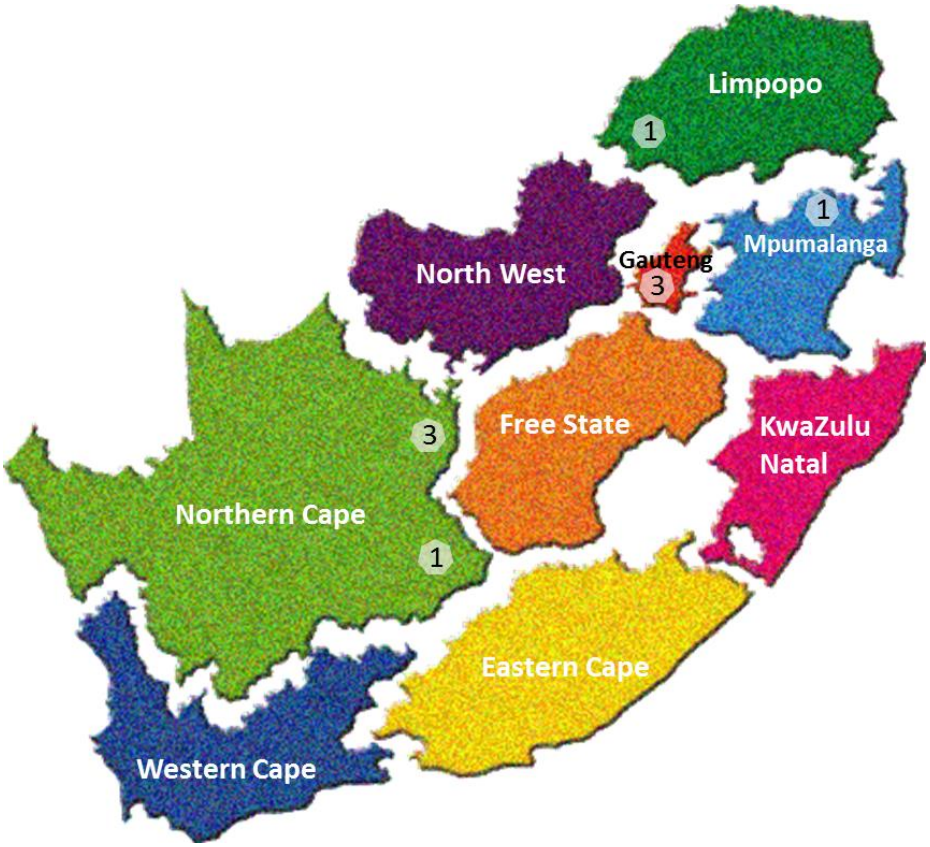


Figure 6.1 Map of South Africa indicating the geographic location of the Shuni virus equine cases.

6.3.3 SHUV seasonality

SHUV infections were identified between the months of January and July, from late summer through winter, with the majority of cases occurring towards the end or after the rainy season in autumn (April) (Figure 6.3). Our five year analysis showed no SHUV cases in the years 2008 and 2012 but a definite peak in 2010 (Figure 6.2).

6.3.4 SHUV clinical disease description

Clinical SHUV cases were identified in horses displaying both fever and severe neurological disease (n=9). Horses displaying only febrile disease (n=2) survived the illness, while 4/6 (67%) of those horses displaying neurological signs died or were euthanized (Table 6.2). In one case the horse was found dead and as such no clinical signs were submitted. The majority of cases presented with anorexia and ataxia, with other prevalent signs including depression, tremors, fever, leucopaenia, paddling of legs and paralysis. Additional symptoms are listed in Table 6.3.

A SHUV isolate was obtained from the brain of index case SAE 18/09 (Chapter 3) (van Eeden *et al.*, 2012). This horse became ataxic and recumbent, after two days constant

paddling, muscle spasms and tremors led to humane euthanasia. An autopsy was performed and blood, brain and spinal cord samples submitted for histopathological and/or virological examination.

Macroscopic analysis at the post mortem showed moderate visceral congestion and an atrophied spleen. Mild subcutaneous and intermuscular oedema of the neck and severe diffuse pulmonary oedema and congestion were also observed. Microscopic examination of tissues showed severe mononuclear encephalitis with scattered neutrophil presence, with lesions being most marked in cerebral white matter, midbrain and brain stem. In the cerebellum, Purkinje cells had degenerated, undergone necrosis or disappeared with attendant gliosis; there was also severe cerebellar mononuclear meningitis. These lesions were consistent with viral meningoencephalomyelitis.

In case SAE 72/09, there were multifocal widespread perivascular cerebral and cerebellar petechiae, some neuronal and Purkinje cell degeneration, diffuse gliosis and minimal leucocyte inflammatory response.

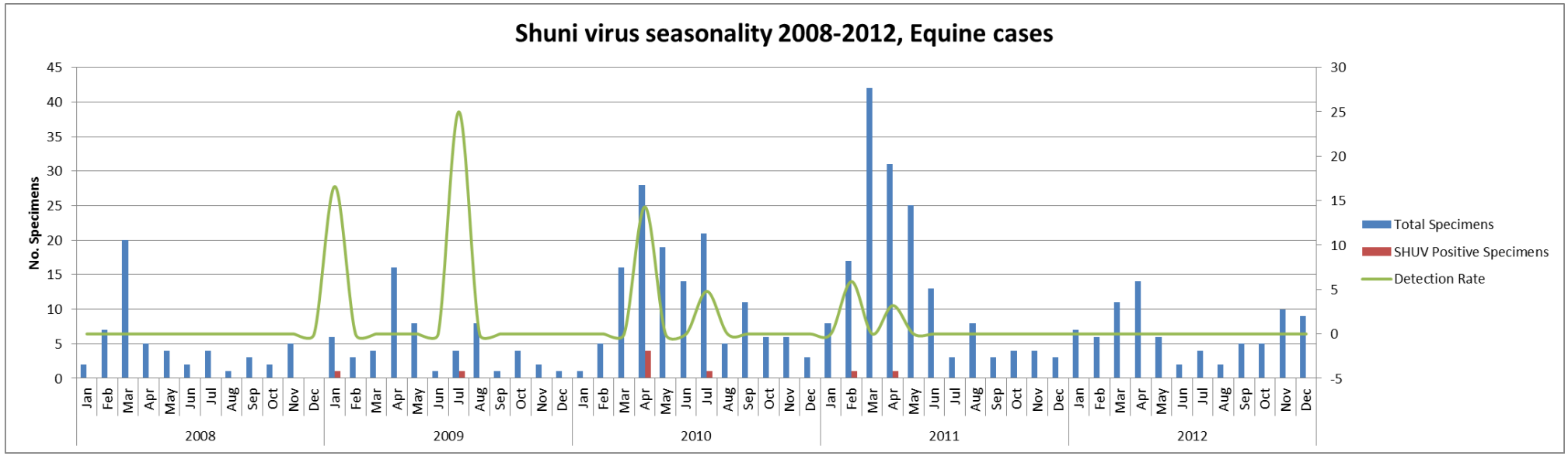


Figure 6.2 Graph indicating both the specimens received, as well as the SHUV positive equine cases for the years 2008-2012.

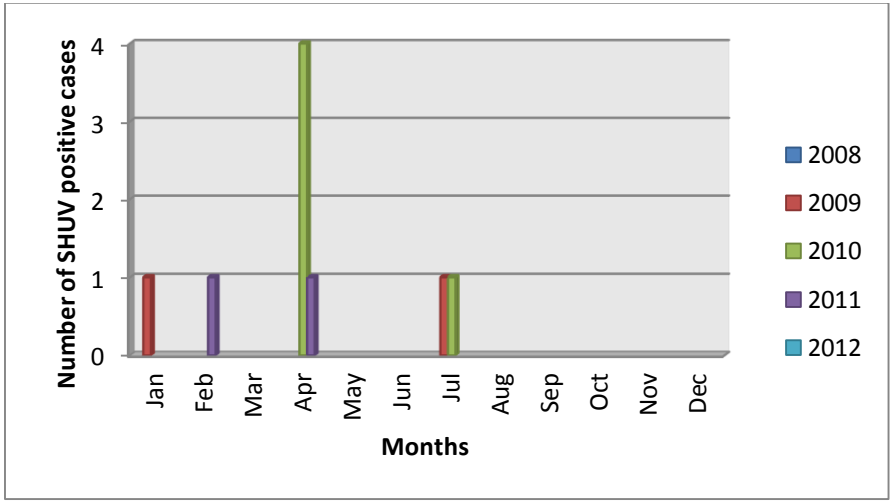


Figure 6.3 SHUV positive equine cases for the years 2008-2012.

Table 6.3 Summary of SHUV cases 2008-2012, indicating the most common symptoms associated with SHUV infection in horses

	2008	**2009**	**2010**	**2011**	**2012**	**Total**	**% (n/9)**
Total no. specimens	55	58	135	161	81	490	-
SHUV positive cases	0	2 (3.4%)	5 (3.7%)	2 (1.2%)	0	9 (1.8%)	-
Neurological	-	2 (3.4%)	3 (2.2%)	1 (0.6%)	-	6 (1.2%)	66.6
Fever	-	-	2 (1.5%)	0	-	2 (0.4%)	22.2
Deaths/Euthanasia	-	2 (3.4%)	1 (0.7%)	2 (1.2%)	-	5 (1%)	55.5
Co-Infections	-	-	1[#] (0.7%)	-	-	1 (0.2%)	11.1
Clinical sign	**2008**	**2009**	**2010**	**2011**	**2012**	**Total**	**% (n/8)***
Anaemia	-	-	1	-	-	1	12.5
Anorexia	-	1	4	-	-	5	62.5
Ataxia	-	2	2	-	-	4	50
Convulsions	-	1	-	-	-	1	12.5
Depression	-	1	2	-	-	3	37.5
Fever	-	-	2	-	-	2	25
Hepatitis/Icterus	-	-	1	-	-	1	12.5
Hyperesthesia	-	-	1	-	-	1	12.5
Leucopaenia	-	-	2	-	-	2	25
Paddling of legs	-	2	-	-	-	2	25
Paralysis	-	-	2	-	-	2	25
Petechiae	-	-	1	-	-	1	12.5
Recumbent	-	2	-	-	-	2	25
Tremors	-	1	2	-	-	3	37.5

6.3.5 Phylogenetic analysis

All SHUV amplicons were sequenced and the data deposited in GenBank (accession numbers: SAE 18/09–HQ610137, SAE 72/09–HQ610138, SAE 27/10–HQ610139, SAE 38/10–HQ610140, SAE 39/10–HQ610141, SAE 48/10–HQ610142, SAE 109/10–HQ610143, SAE 15/11–KC525996 and SAE 87/11–KC525997). To determine the relationships of the nine SHUV cases to the SHUV reference strain as well as other Simbu serogroup viruses, a partial 330nt fragment of the N gene was aligned with those sequences available in the public domain, GenBank.

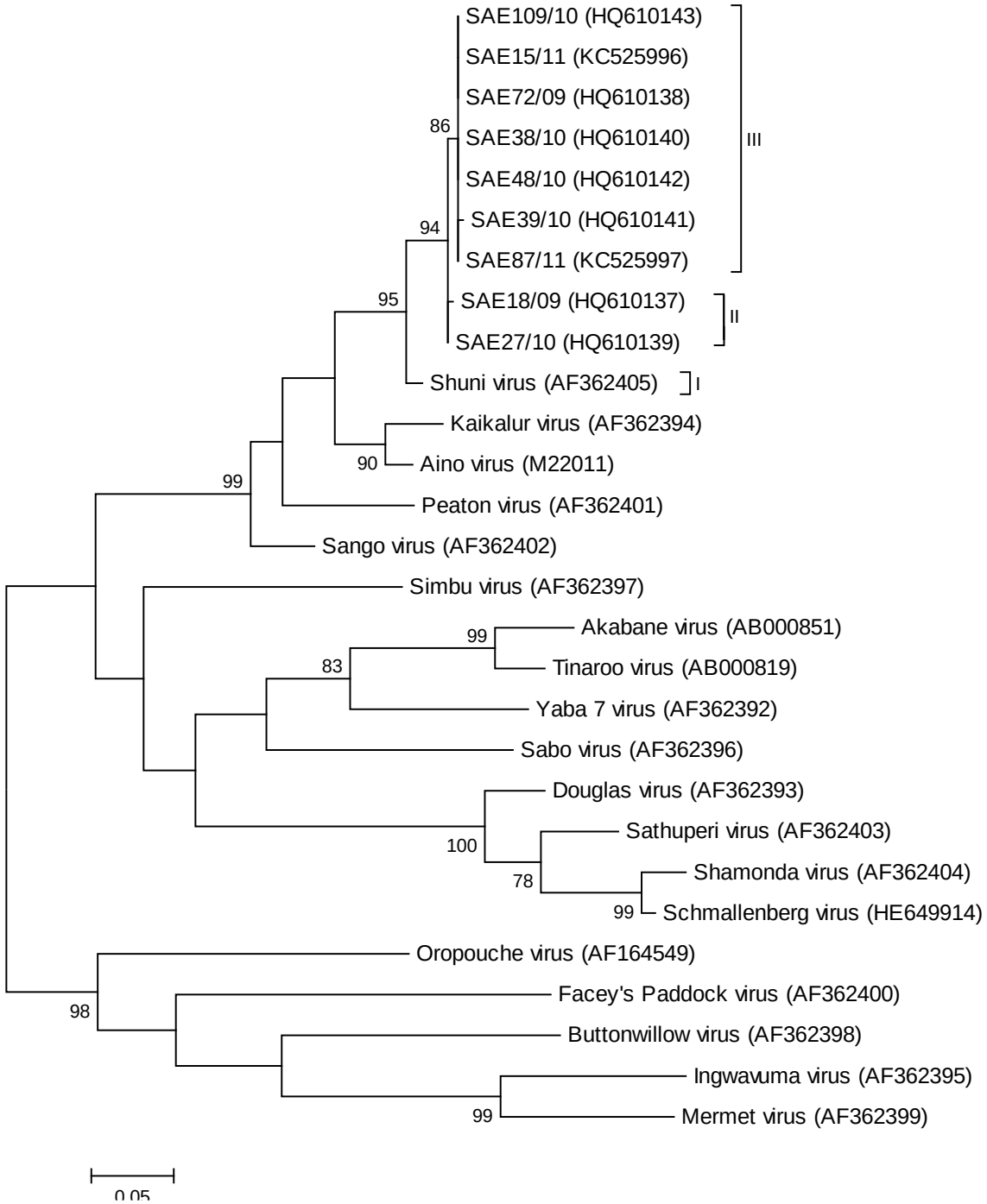


Figure 6.4 Maximum likelihood analysis of SHUV virus strains identified in horses in South Africa 2008-2012, constructed using the Tamura-Nei model of MEGA v5, of a 330bp fragment of the S segment RNA of Shuni virus identified in horses in South Africa, with representative sequences of selected other Simbu serogroup viruses. The bar indicates 0.05 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 100 replicates. Only values >70 are shown. Reference strains GenBank accession numbers are indicated.

Maximum likelihood analysis resulted in a tree with high bootstrap support for the clustering of all the SHUV strains with the prototype SHUV isolate (Figure 6.4). A very high degree of similarity was observed, further illustrating the high level of conservation in the N gene, between members of this serogroup. The SHUV strains were separated into three clusters; the nine South African strains could be divided into two clusters (II, III), separate from the prototype SHUV strain (I). Analysis of the genetic distances between the SHUV strains, showed the intrinsic variation between them to be low (0.3-0.9%). For this short sequence analysis it was found that the 9 SHUV strains averaged a 4.2% (3.6-4.8%) distance to the prototype SHUV strain isolated in Nigeria in 1966 (Causey *et al.*, 1972) and 0.7% from each other (0-1.8%).

6.4 DISCUSSION

We investigated the epidemiology of SHUV in South Africa over a period of five years (January 2008–December 2012). In total 9/490 (1.8%) positive cases were identified, 2/9 (22%) had unexplained fever, 6/9 (67%) showed neurological signs and 1/9 (11%) died before any symptoms were observed. The disease presentation was mostly severe, 44% of horses either succumbed due to their symptoms or were euthanized on humane grounds. In three cases SHUV was amplified from CNS tissues, the crossing of the blood-brain barrier suggests SHUV is likely the cause of the observed nervous disease. Differential diagnosis was carried for alpha- and flaviviruses, one co-infection with Middelburg virus was identified, implying that broad screening for arbovirus infections in unexplained illness is warranted.

SHUV was present in 0-3.7% of cases per year based on RT-PCR diagnosis; the short period of viraemia in most arbovirus infections suggests that the addition of serological testing would likely see an increase in the number of cases detected. The observation of disease peaks in certain years is similar to the seasonality observed for Rift valley fever virus, a well-studied member of the *Bunyaviridae*, where outbreaks of disease follow climatic events and higher than average rainfalls, resulting in the proliferation of vector species (Gerdes, 2002). Indeed statistics for the central region of South Africa indicate that rainfall was significantly higher in 2010 and 2011, than in 2008 and 2009 (www.cra.org.za), this correlates well with the observed peaks of SHUV infection and is likely as a result of an increase in the number of vectors species.

Additionally it was observed that most cases annually, occurred during the later stages of the rainy season (January – April), again highlighting the role of rainfall and increased vector species on arbovirus infection. This observed seasonality has some overlap with AHSV, EEV and WNV outbreaks in South Africa (Quan *et al.*, 2008; Venter *et al.*, 2006; Venter *et al.*, 2009), which may contribute to the under recognition of viruses such as SHUV, due to the routine diagnostic investigation being limited to the above mentioned viruses.

Phylogenetically there was very little intrinsic variation between the SHUV strains, which is largely due to the high level of conservancy of the N gene in orthobunyaviruses (Saeed *et al.*, 2001a; Yanase *et al.*, 2005). Additional studies focusing on the less conserved regions of the genome may better define the molecular epidemiology of SHUV, as would investigations into disease occurrence in other parts of Africa, as has been witnessed for other members of the Simbu serogroup (Akashi *et al.*, 1997a; Saeed *et al.*, 2000). These findings suggest that the role of SHUV as a pathogen may be underestimated, and that it should be investigated routinely as a possible cause of unexplained nervous disease in animals. Investigation of SHUVs role in human disease is also warranted.

CHAPTER 7
Shuni virus as a cause of disease in wildlife

7.1 INTRODUCTION

Though the main focus of the Zoonoses Research unit (ZRU) is to survey cases of neurological disease in sentinel horses, every year a number of other animal cases are submitted with similar symptoms. Following the identification of SHUV as a cause for neurological disease in horses and its previous isolation from apparently healthy domestic animal species such as cattle and goats (Causey *et al.*, 1972; Causey *et al.*, 1969; Kemp *et al.*, 1971; McIntosh, 1980b), it was decided to investigate the other animal cases also, to determine whether SHUV may be a cause of similar severe disease in wildlife and domestic animals.

This report describes the screening of 136 livestock and wildlife species for SHUV through the use of the previously described nested real-time PCR. The study covered a period of three years (January 2010 – December 2012).

7.2 METHODS

7.2.1 Specimens

Post mortem specimens, plasma and serum from 136 animals, other than equines were submitted to the ZRU with signs of neurological disease, between January 2010 and December 2012. Many of these cases formed part of disease outbreaks with only one or two reference specimens being sent for testing. All samples were sent to the Department of Medical Virology, University of Pretoria (UP), by the Onderstepoort Veterinary institute, the UP Faculty of Veterinary Science, Onderstepoort, as well as by veterinarians from around the country. The specimens were screened as appropriate for poisons and rabies virus.

7.2.2 SHUV specific nested real-time PCR

RNA extraction, SHUV specific real-time, differential diagnosis and phylogenetic analyses were all carried out as described in Chapter 6, Section 6.2.

7.3 RESULTS

7.3.1 Animal species investigated

Of the 136 animals screened for SHUV, 44 were livestock species (32%) and 92 (68%) wildlife. The most common domestic species tested were bovine (59%), followed by ovine (16%), alpaca (11%) and caprine (10%). The wildlife species were dominated by buffalo (30%) and rhinoceros (29%), followed by warthog (18%) and small birds (13%). Additional species are listed in Table 7.1.

Table 7.1 Summary of the domestic and wild animal species tested for SHUV

		Total	2010	2011	2012
Livestock	Caprine	4	2		2
	Ovine	7		7	
	Bovine	26	9	9	8
	Porcine	0			
	Ostrich	1	1		
	Alpaca	5	5		
	Canine	1	1		
Wild animals	White rhinoceros	23	8	10	5
	Vulture	1	1		
	Buffalo	25	15	2	8
	Crocodile	6	2		4
	Sable antelope	1	1		
	Avian	11	4	6	1
	Warthog	15	11	4	
	Eland	1	1		
	Jackal	2	2		
	Roan antelope	1	1		
	Bushbuck	1		1	
	Zebra	1			1
	Blue wildebeest	1			1
	Lion	3			3
	Total	136	64	39	33

7.3.2 SHUV positive animals and the disease presentation

Four SHUV positive animals were identified (Table 7.2), all were wildlife species; rhinoceros, buffalo, crocodile and warthog. The rhinoceros (MVA 11/10) proved to be co-infected with an alphavirus that was identified as Middelburg virus. All four animals suffered paralysis, two died and two were euthanized on humane grounds. For the two animals which died, the white rhinoceros and the buffalo, it is likely that they succumbed before euthanasia due to the weight of their limp bodies inhibiting their respiratory function (Personal communication with referring veterinarian, Dr. Johan Steyl). They weigh on average between 1 400-2 300kgs and 680-820kgs respectively (Cillie, 2011). All positive specimens were from the CNS of the affected animals.

7.3.3 Seasonality and demographics

All cases were identified in 2010 in the months April, May, July and August (Figure 7.2); this correlates well with what was observed for the equine cases both with regards to season and to the observation that the virus is not identified on a yearly basis (Refer to 6.3.3). All four cases were also identified in the Limpopo Province (Figure 7.1), this province is home to numerous wildlife reserves and the warmer climate of this region may both have an influence on the occurrence of disease.

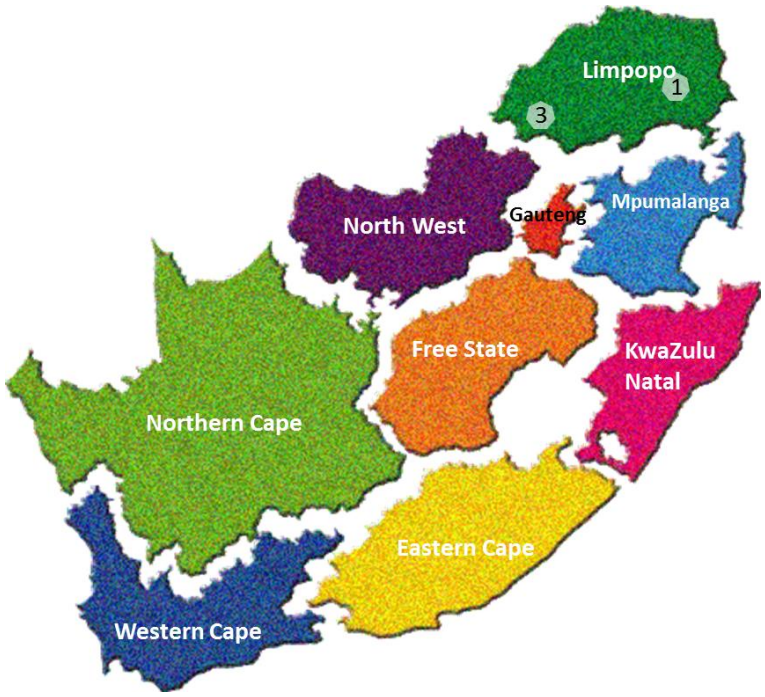


Figure 7.1 Map of South Africa indicating the geographic location of the Shuni virus wildlife cases.

Table 7.2 Clinical signs and symptoms of Shuni virus cases identified wildlife in South Africa

Case	Specimen	Species	Age (Years)	District, Province	Clinical Signs	Outcome	Date Received
MVA 08/10	Spinal Cord	Crocodile	3	Tzaneen, Limpopo	Discharge from eyes, lethargic, partial paralysis	Euthanized	29/04/2010
MVA 11/10*	Brain	Rhinoceros	6	Thabazimbi, Limpopo	Paralysis	Died	05/05/2010
MVA 35/10	Spinal Cord	Warthog	2	Thabazimbi, Limpopo	Recumbent, tremors, paralysis	Euthanized	27/07/2010
MVA 43/10	Spinal Cord	Buffalo	7	Thabazimbi, Limpopo	Paralysis	Died	04/08/2010

*Indicates Middelburg co-infection

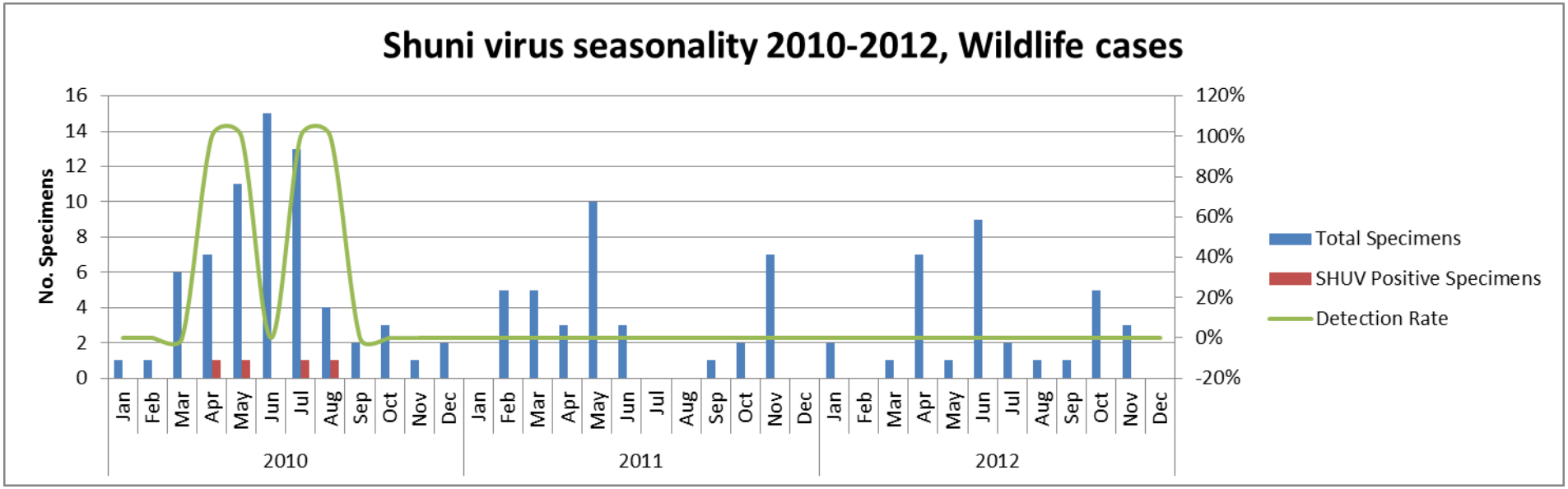


Figure 7.2 Graph indicating both the specimens received, as well as the SHUV positive wildlife cases for the years 2008-2012.

7.3.4 Phylogenetic analysis

All amplicons were sequenced and the data deposited on GenBank; MVA 08/10–JQ726396, MVA 11/10–JQ726395, MVA 35/10–JQ726398 and MVA 43/10–JQ726397.

To determine the relationships of the 13 SHUV cases to the SHUV reference strain as well as other Simbu serogroup viruses, a partial 330nt fragment of the N gene was aligned with those sequences available in the public domain, GenBank. Maximum likelihood analysis resulted in a tree with high bootstrap support for the clustering of all the SHUV strains with the prototype SHUV isolate (Figure 7.4). A very high degree of similarity was observed, further illustrating the high level of conservation in the N gene, between members of this serogroup.

Analysis revealed the 13 SHUV strains could be divided into two clusters (I, II), separate from the prototype SHUV strain (III), the first cluster comprised both equine and wildlife strains, whilst the second cluster contained only our original SHUV strain (SAE 18/09) and a later equine case (SAE 27/10). Analysis of the genetic distances between the SHUV strains, showed the intrinsic variation between them to be low (0.9-2.2%). For this short sequence analysis it was found that the 13 SHUV strains averaged a 4.3% relation to the prototype SHUV isolate and a 12% relation to its closest relative Aino virus. Investigation into the various factors which may impact on phylogenetic relationships identified no obvious influence of either host or geographic location on the separation of strains (Figure 7.3).

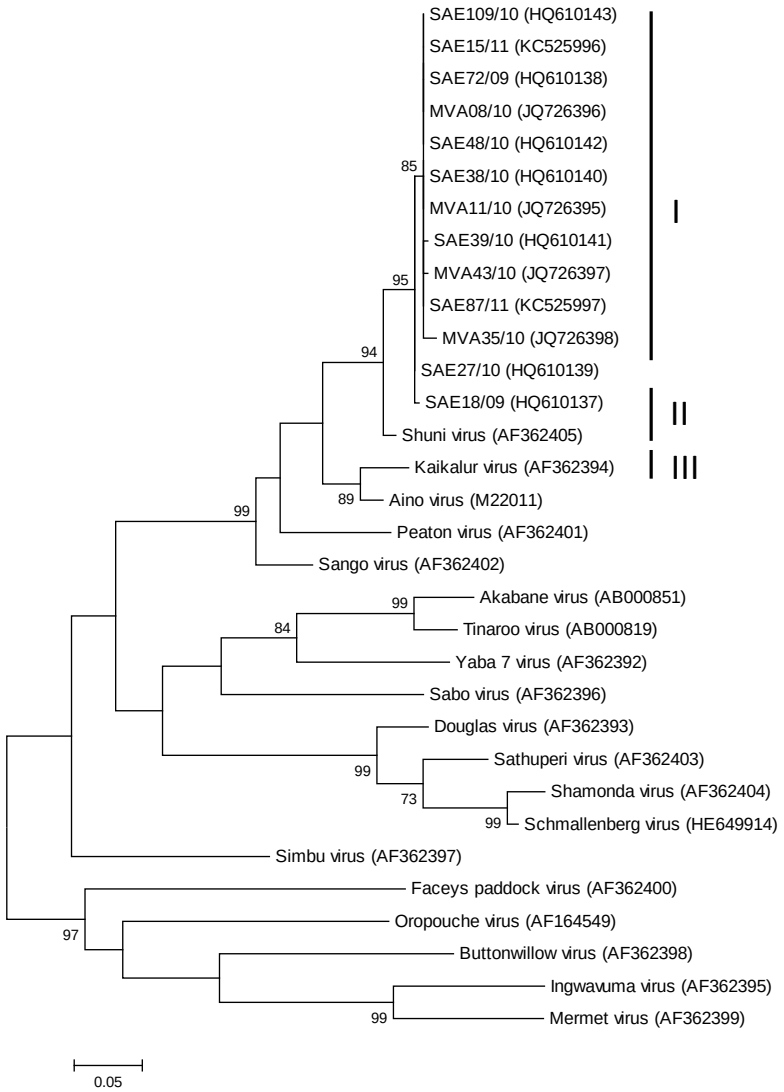


Figure 7.4 A maximum likelihood tree constructed under the Tamura-Nei substitution model using the Mega 5 program, of a 330bp fragment of the S segment RNA of Shuni virus identified in horses and wildlife in South Africa, with representative sequences of selected other Simbu serogroup viruses. The bar indicates 0.05 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 100 replicates. Only values >70 are shown. Reference strains GenBank accession numbers are indicated.

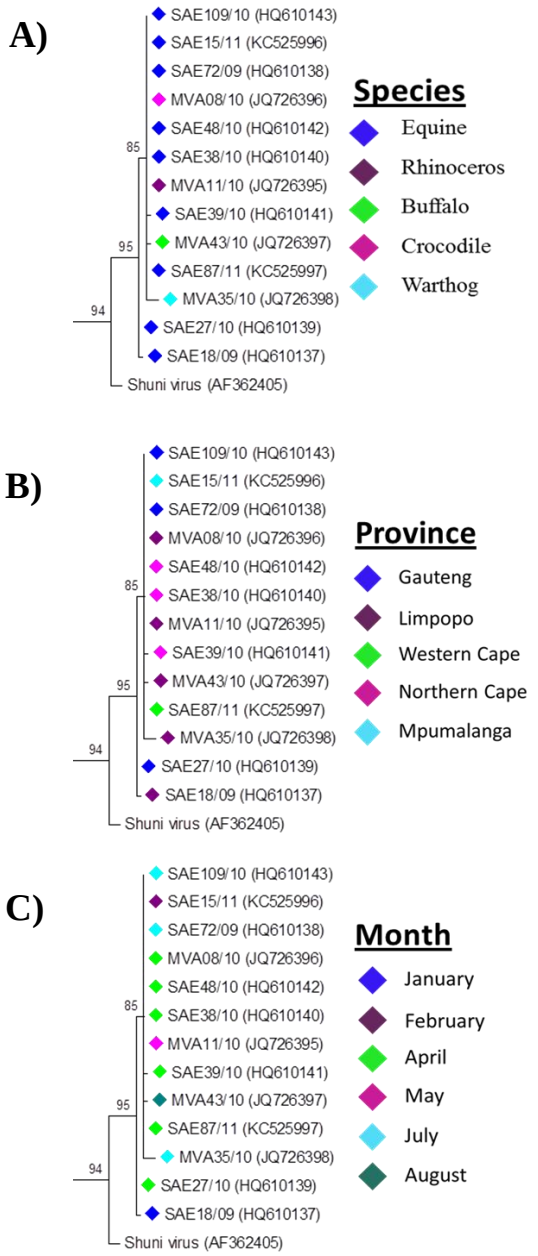


Figure 7.3 Analysis of the SHUV strains with reference to factors which may influence phylogenetic relationships. (A) Host. (B) Geographic location. (C) Date of sample collection.

7.4 DISCUSSION

Over a period of three years (2010-2012) four cases of SHUV were identified in wildlife species in South Africa, all four cases presented with paralysis and resulted in death. SHUV was amplified from the CNS of the animals, demonstrating crossing of the blood-brain barrier and indicating SHUV to be the causal agent of the observed neurological signs. Differential screening revealed one co-infection with MIDV.

An important feature of the wildlife analysis was that in most instances the specimens submitted to us were from individual animals sent as reference cases for disease outbreaks affecting multiple animals. Thus the positive cases do not only indicate single infections, but also identify SHUV as the possible cause of disease outbreaks.

The observation that all cases were from the Limpopo Province is likely as a direct result of the species from which they were identified, as this province is largely dominated by wildlife reserves. Additionally the climate of this province is much warmer than other regions of the country, which likely benefits the proliferation of the vector species responsible for the spread of disease.

Geographically and seasonally the wildlife cases followed the patterns observed for the equine cases. Phylogenetically the wildlife cases grouped with the equine cases, with no obvious influence of geographic distribution, seasonality or host species on topology.

Future studies into the epidemiology of SHUV in wildlife would greatly benefit from the analysis of increased samples sizes, combined with serological analysis to confirm infections in animals where either the viraemic phase has passed or the disease association is less severe, especially in outbreak investigations. This report confirms that such studies are warranted and that lesser known viruses such as SHUV, may be playing a significant role in neurological disease in animals other than horses, further increasing the risk for zoonotic transmission.

CHAPTER 8

Seroprevalance of Shuni virus relative to West Nile virus in veterinarians South Africa

8.1 INTRODUCTION

Arboviruses in the *Bunya-* and *Flaviviridae* families have a wide host range and are often associated with zoonotic disease and are spread with relative ease by their arthropod vectors. Humans can also become infected through exposure to the tissues and bodily fluids of infected animals. Due to the fact that both West Nile virus (WNV) and Shuni virus (SHUV) are associated with disease in horses, and that the health of these valuable animals is closely monitored by veterinarians both in life and death, we hypothesized that veterinarians may be at greater risk to exposure and may thus have a higher seroprevalence of protective antibodies.

WNV is a well-studied zoonosis which is largely endemic in South Africa, especially where the principal vector *C. univittatus* and avian hosts are present. Serosurveys conducted between 1950 and 1970 suggested that 17-20% of people in Karoo (Jupp, 2001; Kokernot *et al.*, 1956); 4-8% in the Highveld (Jupp, 2001; Kokernot *et al.*, 1956; McIntosh *et al.*, 1962a) and 1-3% in the Natal and the Eastern Cape had antibodies against WNV (Kokernot *et al.*, 1956; McIntosh *et al.*, 1962b). Most cases of human infection are sporadic and are characterised by mild febrile illness with myalgia, anthralgia and macupapular rash (Kokernot & McIntosh, 1959), however severe disease has been documented, including; renal failure, necrotic hepatitis and non-fatal encephalitis (Burt *et al.*, 2002). WNV is a causal agent of neurological disease in horses in South Africa (Venter *et al.*, 2009; Venter & Swanepoel, 2010), an association which in 2008 led to the zoonotic transmission of WNV to a veterinary student whom had conducted an autopsy on a WNV infected horse (Venter *et al.*, 2010).

Investigation of animals with neurological symptoms led to the description of SHUV as a neurological pathogen in both horses (van Eeden *et al.*, 2012) and wildlife species (Chapter 7) in South Africa. SHUV has only once been documented in human infection, from a febrile child during an arbovirus survey conducted in Nigeria in 1966 (Moore *et al.*, 1975). Due to the fact that both WNV and SHUV are associated with disease in horses,

and that the health of these valuable animals is closely monitored by veterinarians, we hypothesized that veterinarians may be at a greater risk to infection either through either contact with vector species or with infected animal tissues, allowing for the opportunity to detect human exposure in South Africa.

The objectives of this study were; a) to develop neutralization assays for the detection both WNV and SHUV neutralizing antibodies, and b) to screen the sera of veterinarians to determine the level of neutralizing antibodies present for both WNV and SHUV.

8.2 METHODS

8.2.1 Sample collection

A total of 125 veterinarians from around South Africa volunteered blood samples for this study. They were approached during four veterinary conferences in the years 2011 and 2012 and gave informed consent to be screened for zoonotic pathogens. Only those veterinarians whom had regular contact with animals which may have been exposed to WNV and/or SHUV (horses, domestic and wildlife species) were asked to donate serum samples.

8.2.2 Virus strains

The viruses (Kunjin virus MRM61C, lineage 1b strain of WNV (Zaayman *et al.*, 2009) and Shuni virus SAE 18/09) were cultured on Vero cells in EMEM (Lonza, Basel, Switzerland), supplemented with 5% foetal calf serum (FCS) (Invitrogen, California, USA), 100 U/ml Penicillin and 100µg/ml Streptomycin (Lonza, Basel, Switzerland) under BSL3 conditions. Viral infections were allowed to proceed until a cytopathic effect (CPE) of 80% became apparent, after which virus was harvested and stored until further use.

8.2.3 Virus titrations

For viral titration, 100µl diluted virus (10^{-1} to 10^{-9}) prepared in Leibowitz culture medium (Invitrogen) supplemented with 5% FCS (Invitrogen, California, USA), was co-incubated with 100µl Leibowitz (Invitrogen) supplemented with 2% FCS and 25µl Vero cells at a concentration of $8x10^5$ cells/ml, prepared in 2% FCS at 37ºC for 96 hours in a flat-bottomed 96 well culture plate (Corning, Lowell, MA). Cells were inspected daily for the presence of CPE. Viral titre was calculated as previously described (Reed & Muench, 1938) and expressed in 50% tissue culture infectious dose/ml, TCID50 U/ml. Briefly, this

calculation uses the formula I = (The % of wells that display more than 50% CPE for the first dilution closest to 50%-50%)/ (The % of wells that display more than 50% CPE for the first dilution closest to 50%-50% of wells infected at the first dilution below 50%). The calculated value of I is substituted into the formula 10 log (The dilution factor for the first dilution in which wells display more than 50% CPE) – (I x log h), where h is the dilution factor, giving us the 50% endpoint titre.

8.2.4 Virus neutralization assays

In order to detect the presence of WNV and SHUV specific neutralizing antibodies in serum samples, a modified version of a previously published method for a micro-neutralisation assay was used (Swanepoel *et al.*, 1976). Before experimentation, patient serum samples were heat inactivated at 56°C for 30min. Briefly, 100µl of 100TCID50 U/ml of either SHUV or WNV virus, prepared in 2% FCS (Invitrogen, California, USA), was incubated together with 100µl of each 2-fold duplicate dilutions of patient sera (1:10-1:640 for WNV and 1:8-1:32 for SHUV), in a flat-bottomed 96 well culture plate (Corning, Lowell, MA) at 37°C and 45 minutes. Following this, 25µl of Vero cells prepared in 2% FCS (Invitrogen, California, USA), 100 U/ml Penicillin and 100µg/ml Streptomycin (Lonza, Basel, Switzerland) was added to the virus/serum combination at a concentration of $8x10^5$ cells/ml and incubated at 37°C 96 hours. Each well was then examined for the presence of CPE. Titres were expressed as the reciprocal of the serum dilution that inhibited ≥75% of CPE. A serum sample was considered positive when it had a titre of $\geq \log_{10}$ 1.0, equivalent to a serum dilution ≥1:10 for WNV and ≥1:8 for SHUV.

8.2.5 Statistical analysis

95% confidence intervals were determined using STATA 12 (Stata Corporation, Texas, USA), by Dr. Stefano Tempia of the CDC, South Africa. A binomial distribution for the veterinary serosurvey data was used. Intervals that did not cross the value one were considered to be statistically significant.

8.3 RESULTS

8.3.1 Veterinarian serosurvey, West Nile virus

Of the 125 veterinarian serums screened for WNV, 10 (12.5%) were found to be positive (titres ≥1:20). We further analysed these findings with regards to age, gender (Figure 1)

and geographic locality (Figure 2). Five of the positive veterinarians were male, three in the 25-50 and two in the 50-75 age groups. Of the five positive female veterinarians, three were in the 25-50 age group and 2 in the 50-75 group. Three positive cases were identified for both the Gauteng (6%) and Kwa Zulu Natal (23%) provinces, with one each in the Free State (25%), Eastern Cape (12.5%), Northern Cape (20%) and Western Cape (6%) provinces. An additional six veterinarians had titres of 1:10; these weak positives were not included due to the potential for cross reactivity at this level.

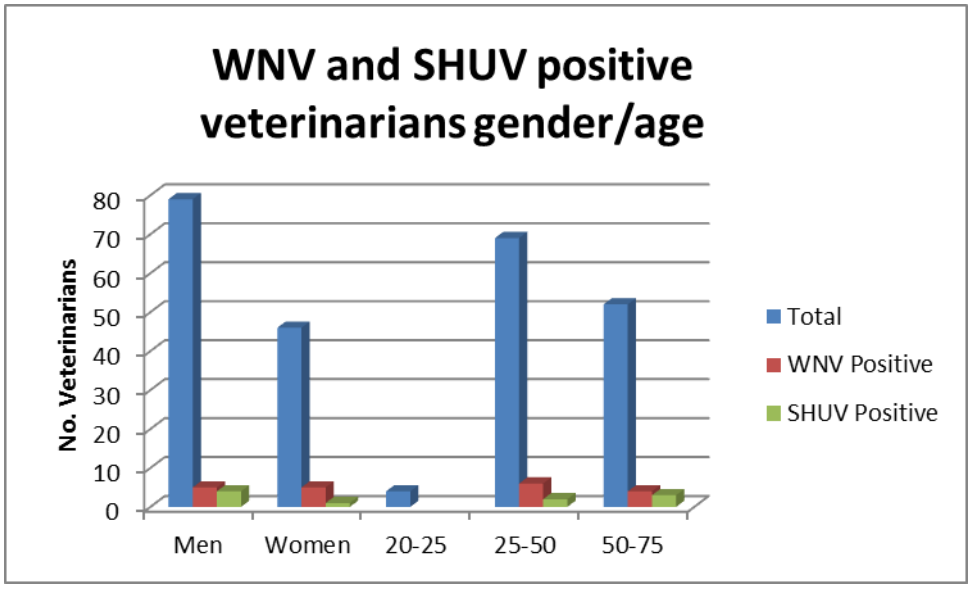


Figure 8.1 Graph indicating the total number of veterinarians screened as well as those that were found to be positive for either WNV or SHUV, with regards to both gender and age.

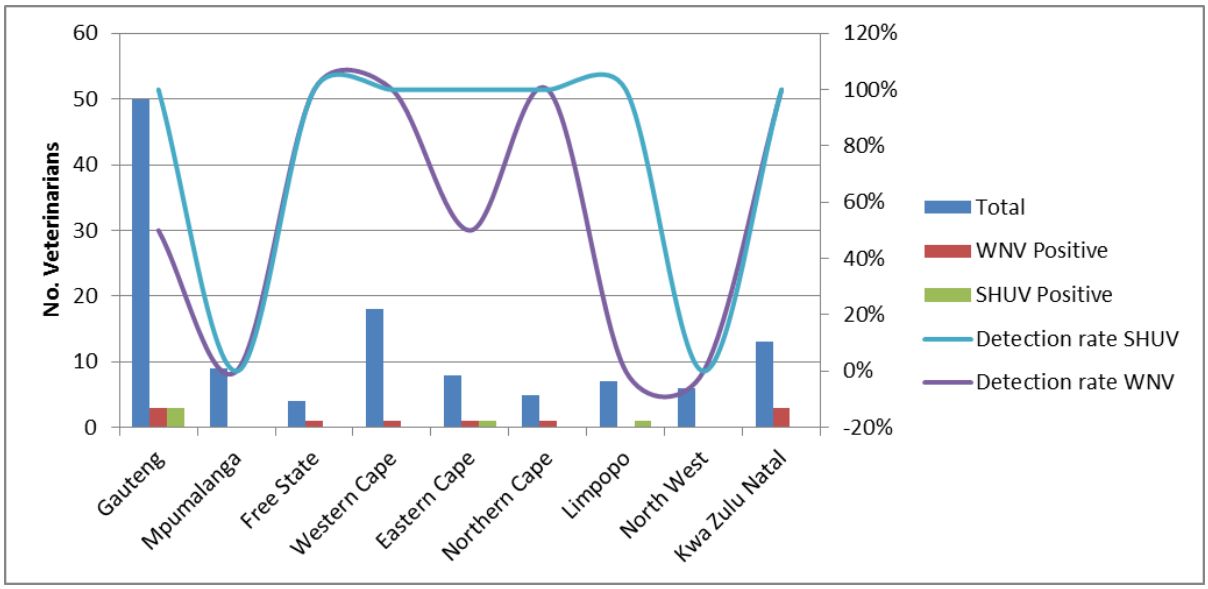


Figure 8.2 Graph indicating the total number of veterinarians screened as well as those that were found to be positive for either WNV or SHUV, with regards to province of residence.

8.3.2 Veterinarian serosurvey, Shuni virus

Of the 125 veterinarian serums screened for SHUV, five (4%) were found to be positive (titres ≥1:16). We further analysed these findings with regards to age, gender (Figure 8.1) and geographic locality (Figure 8.2). Four of the five positive veterinarians were male, two in the 25-50 and two in the 50-75 age groups. The female veterinarian fell into the 50-75 age group. Three of the positive cases were identified in Gauteng (6%) and one each in the Eastern Cape (12.5%) and Limpopo (14%) provinces. An additional 11 veterinarians had titres of 1:8; these weak positives were not considered due to the potential for cross reaction to other orthobunyaviruses at this level.

95% confidence intervals indicate significant associations for both men (95% CI 2.1-14.1) and women (95% CI 3.6-23.6) with regards to WNV exposure and men (95% CI 1.3-12.4) for SHUV. The 26-50 and 51-75 age groups were significant for WNV exposure and the 51-75 age group for SHUV (Appendix D). Confidence intervals for the total number of positive veterinarians were significant for both WNV (95% CI 3.9-14.2) and SHUV (95% CI 1.3-9.1), though geographic location was in most instances not significant (Appendix D).

DISCUSSION

Investigation into the levels of neutralizing antibodies to WNV in veterinarians from around South Africa, revealed much higher levels than had been observed in the general population in the 1960s-1980s. In the Highveld region (Gauteng and Free State provinces) we observed values of 6% and 25% respectively, which is much higher than the 4-8% previously observed (Jupp, 2001; Kokernot *et al.*, 1956; McIntosh *et al.*, 1962a). For the Kwa-Zulu Natal 23% and for the Eastern Cape 12.5% was observed, again these values are significantly greater than the 1-3% previously observed (Jupp, 2001; Kokernot *et al.*, 1956; McIntosh *et al.*, 1962a). In the Karoo region (Northern Cape) the observed value of 20% correlated well with previous studies where 17-20% of the population had neutralizing antibodies against WNV (Jupp, 2001; Kokernot *et al.*, 1956).

Although less widespread, SHUV values were similar to those observed for WNV, with 6% of veterinarians in Gauteng and 12.5% in the Eastern Cape having neutralizing antibodies. These results are significant when one compares the frequency of acute disease in horses, where for WNV 9-21% is observed annually (Venter *et al.*, 2009; Venter

& Swanepoel, 2010) compared to our findings of 0-4% for SHUV (van Eeden *et al.*, 2012). Additionally WNV infection is well documented in human population of South Africa with outbreaks having occurred in the 1970s and 1980s (Jupp *et al.*, 1986; McIntosh *et al.*, 1976b).

The findings of this study suggest that human exposure to WNV and SHUV, as well as other arboviruses responsible for causing neurological disease, may occur in South Africa in animal handlers whom have a potentially higher risk for zoonotic infection.

The detection of SHUV neutralizing antibodies is comparable to WNV, suggesting that SHUV may be of importance in the human population. To determine the clinical relevance, febrile and neurological human cases should be investigated.

CHAPTER 9
Conclusion

Many zoonotic arboviruses, are capable of causing major outbreaks, sometimes with severe morbidity and high mortality rates and are important emerging and re-emerging diseases. Several mosquito-borne zoonotic viruses in the families *Flavi-*, *Bunya-* and *Togaviridae* have emerged from Africa as new pathogens in previously unaffected regions and caused major epidemics and epizootics. These include West Nile virus (WNV); Rift Valley fever (RVF) and Chikungunya virus (CHIKV) (Hollidge *et al.*, 2010). Horses in particular are highly sensitive to some of these viruses and have thus been targeted as sentinel animals in the identification of zoonotic arboviruses associated with neurological disease in South Africa (Venter & Swanepoel, 2010).

During the seasonal occurrence of more readily recognised vector-borne diseases such as African horse sickness (AHSV) and Equine encephalosis (EEV), many horses exhibit febrile, neurological and fatal infections for which the aetiology remains unsolved. Following the emergence of West Nile virus lineage 1 (WNV1) as an important pathogen in Europe and North America (Ulbert, 2011). The Zoonosis Research unit (ZRU) re-examined the pathogenicity and role of WNV lineage 2 (WNV2) in southern Africa (Venter *et al.*, 2009). They proceeded to identify WNV2 as a cause of unexplained nervous disease of humans and horses in South Africa. There were however numerous residual cases in which no diagnosis could be established. This led to the formulation of the first aim which was to develop an assay for the detection of unknown viral agents in animals which tested negative for the standard requested pathogens. This was achieved through the implementation of an arbitrarily primed PCR method, which proved both efficient and reproducible, allowing for the amplification of viruses from both cell culture and diagnostic specimens. The success of this assay supported its inclusion into our diagnostic facility, where it can now be applied on a routine basis.

Virus discovery techniques led to the identification of Shuni virus (SHUV) in early 2009, this agent was amplified from a cell culture isolate of brain tissue taken from a horse that had succumbed to meningoencephalitis. SHUV, a member of the *Orthobunyavirus* genus of the family *Bunyaviridae* had been previously identified in vector species, livestock and

a febrile human child in Nigeria in the 1960's, though no disease had been described (Causey *et al.*, 1969; Kemp *et al.*, 1973; Lee, 1979; Moore *et al.*, 1975). It was subsequently identified in vector species, asymptomatic livestock in South Africa (McIntosh, 1980; McIntosh *et al.*, 1972). In 1977, SHUV was isolated from the brains of two horses displaying nervous disease, one in South Africa and one in Zimbabwe (Coetzer & Erasmus, 1994; Howell & Coetzer, 1998). Considering the public and veterinary health importance of other members of the orthobunyaviruses, surprisingly little attention had been placed on uncovering the role SHUV may play in neurological disease. The focus of the study thus shifted, to investigating the molecular epidemiology of SHUV within South Africa.

Following the identification of the first SHUV case, both conventional and real-time PCRs were developed for its specific detection. The asymmetric nested real-time PCR that was developed is based on FRET probe technology and proved to be highly sensitive for the detection of SHUV. The specificity of this assay means that sequencing of amplicons is not necessary and that diagnosis can be made in short time, thus this was the method incorporated into our diagnostic panel. This assay was employed in a five year (2008-2012) investigation into the epidemiology of SHUV, 490 horses and 136 other animals were screened, 9/490 (1.8%) horses and 4/136 (2.9%) tested positive. Through this analysis we were able to shed light on both the clinical presentation of SHUV infection as well as its seasonality.

The majority of cases 10/13 (77%) presented with neurological signs, the most prominent of these were paralysis, tremors and leg paddling, with anorexia and depression often featuring as well. Mortality was also high 8/13 (62%), with five of the animals being euthanized on humane grounds. In seven cases SHUV was amplified from either the brain or the spinal cord of the infected animal, this breach into the CNS indicates crossing of the blood-brain barrier, and infers that SHUV is the likely cause of the observed nervous disease. Two co-infections with Middelburg virus were observed.

SHUV was present in 0-3.7% of cases a year, no cases were reported in 2008 or 2012 with a definite peak being observed in 2010. This pattern is well recognised for other members of the *Bunyaviridae* and is largely dependent on high levels of rainfall and the proliferation of vector species, our data suggests that this is also true for SHUV. Annually most cases were observed during and towards the end of the rainy season (January–April). This

observed seasonality has some overlap with AHSV, EEV and WNV outbreaks in South Africa (Quan *et al.*, 2008; Venter *et al.*, 2006; Venter *et al.*, 2009). This may contribute to the under recognition of viruses such as SHUV, due to routine diagnostic investigation being limited to the above mentioned viruses. Phylogenetically there was very little intrinsic variation between the SHUV strains. The wildlife and the equine cases grouped together, with no obvious influence of geographic distribution, seasonality or host species on topology. This observation is largely due to the high level of conservancy of the N gene in orthobunyaviruses, which was the target of the real-time assay employed.

After identifying SHUV in multiple cases of neurological disease, the need to investigate its molecular biology arose. In a recent study the near complete genome for the prototype SHUV isolate (An10107) was determined (Goller *et al.*, 2012). This gave us the opportunity to compare SAE18/09; an isolate which had been associated with neurological disease in an equine, to this isolate from an asymptomatic bovine. Although only a limited number of viral sequences were available for the full segment analyses, the overall topology for the S, M and L segments of SAE 18/09 was uniform and reflected the results seen in other partial genome studies (Goller *et al.*, 2012; Saeed *et al.*, 2001a). The genome of SHUV shared all the features and conserved domains previously identified for the Simbu serogroup, with phylogeny of the viral proteins being similar throughout, no indication of genomic reassortment was observed.

Sequence identity between the two SHUV isolates was high and ranged between 93.5–99.3% at the nt. level and 96.2-100% at the aa level, of all viral proteins. One noteworthy variation was however observed, the prototype SHUV isolate (An10107) has three amino acid changes in the predicted cleavage site for NSm/Gc (VDA-ND → AAS-DK). This results in an NSm and Gc protein of one aa longer and shorter, respectively. Such mutations can lead to changes in the efficiency of signal peptide cleavage, which in turn can affect the production of infectious virions (Lee *et al.*, 2000), and will need to be investigated further. The high level of conservancy between these two isolates; one of neurologic origin and the other asymptomatic origin highlights the need to investigate the factors which influence SHUV pathogenicity. This genome sequence and phylogenetic analysis marks an important step towards the establishment of a reverse genetics system to analyse the biology and pathogenicity of SHUV, as well as further elucidating the molecular relationship between members of the Simbu serogroup.

The fact that both WNV and SHUV are associated with disease in horses, and that the health of these valuable animals is closely monitored by veterinarians, we hypothesized that veterinarians may be at greater risk to exposure, both through contact with vector species and the tissues of infected animals. Many studies have focused on the sero-prevalance of WNV in humans in South Africa and as such it was investigated as a comparative tool in the analysis of SHUV sero-prevalance.

The last aim of this study was thus to develop neutralization assays to test for the presence of both WNV and SHUV antibodies, these assays were used to screen 125 large animal veterinarians. Investigation into the levels of neutralizing antibodies to WNV revealed higher levels (6% in Gauteng - 25% in the Free State) than had been observed in the general population in the 1960s-1980s (Jupp, 2001). Although less widespread, SHUV values were similar to those observed for WNV, with 6% of veterinarians in Gauteng and 12.5% in the Eastern Cape having protective antibodies. These results are significant when one compares the frequency of acute disease in horses, where for WNV 9-21% is observed (Venter & Swanepoel, 2010) compared to our findings of 0-4% for SHUV. These findings suggest that in South Africa the veterinary population may indeed be at a higher risk to zoonotic infection with WNV and SHUV, as well as other arboviruses responsible for causing neurological disease. The clinical significance of such infections will thus need to be investigated further, through the screening of both febrile and neurological cases of human disease.

APPENDICES

APPENDIX A

Establishment of arbitrarily primed PCR technique

Arbitrarily primed PCR on tissue samples (SAE 66/09)

Particle purification and RNA extraction

Tissue samples from an equine who suffered an acute death (SAE 66/09), were made up as 10% suspensions and were freeze-thawed three times to disrupt the tissues, hereafter they were spun down at 11 000g for 10 minutes. For each 500µl supernatant, 0.05g PEG-6000 (Merck, Darmstadt, Germany) and 0.02g NaCl (Merck), were added and the reactions incubated on ice with agitation for one hour and then on ice overnight. Samples were then spun at 11 000g for 20 minutes and the supernatant removed. The pellet was resuspended in 50µl 1x phosphate buffered saline (PBS). A total of 12.5 U DNase (Merck), 42mM $MgCl_2$ and 10µg RNase (Merck) was added and the reaction incubated at 37ºC for 60 minutes. 5mM EDTA was then added to stop the reaction. Samples were extracted with the RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Difference to final method:
10% tissue suspension supernatant was not filtered.
Benzonase enzyme not used.

Reverse transcription

First strand cDNA synthesis was achieved by denaturing 10 µl RNA and 20 pmol of random hexamer primers, at 65°C for 10 minutes. The reaction mixtures where then cooled on ice for 2 minutes, following this 10 mM dNTP mix, 4 µl 5x RT buffer, 100mM DTT solution, 50 U RNase inhibitor (Roche, Mannheim, Germany) and 50 U Expand reverse transcriptase (Roche) were added to each reaction. The reaction mix was then heated to 30°C for 10 minutes, 42°C for 45 minutes and then placed on ice.

Difference to final method:
Random hexamer primers were used.

PCR amplification

PCR reactions were performed as in final method (refer to 2.2.4). Multiple bands were observed, with some being distinct between the mock and sample PCRs (Figure 2.1).

Unique bands in the samples that were distinct from the mock PCRs were cloned and sequenced. Results are discussed in Section 2.3.2.

APPENDIX B

Primers and additional phylogenetic trees for the full genome analysis of SAE 18/09

Table 1. Primers used to amplify the full genome of Shuni virus

SHUNI S SEGMENT PRIMERS				
Primer	**Sequence**	**TM**	**Position**	**Reference**
ORO1A	AGT AGT GTA CTC CAC TAT	45	N/A	(Saeed *et al.*, 2001a)
ShuNR	CGG AAT CTC GCT ACG	49	180 (S) rc	This study
Shu111+	CGA TAC CGT TAG AGT CTT CTT CC	55	111 (S)	(van Eeden *et al.*, 2012)
Shu178+	CCG AGT GTT GAT CTT ACA TTT GGT	56	178 (S)	(van Eeden *et al.*, 2012)
Shu611-	GCT GCA CGG ACA GCA TCT A	57	611 (S) rc	(van Eeden *et al.*, 2012)
Shu688-	CGA ATT GGG CAA GGA AAG T	53	688 (S) rc	(van Eeden *et al.*, 2012)
Bunya1	GTC ACA GTA GTG TAC TCC AC	52	N/A	(Bowen *et al.*, 2001)
Bunya2	CTG ACA GTA GTG TGC TCC AC	55	N/A rc	(Bowen *et al.*, 2001)
SHUNI M SEGMENT PRIMERS				
Primer	**Sequence**	**TM**	**Position**	**Reference**
ShuMF	GGW TGC GAA GAA TGG	46	3114 (M)	This study
ShuMR	GYA TCT GYG GWA TG	40	3344 (M) rc	This study
ShuMF1+	TGY GSM ACA TGG YTA	50	4017 (M)	This study
ShuMR1-	CAT ARC CAT GTK SCR CA	49	4017 (M) rc	This study
ShuMR2-	CAK ATA CAK RTT KTD GG	41	844 (M) rc	This study
ShuMF2+	GGG AWT GYG AAG ATA	44	3112 (M)	This study
ShuMend	AGT AGT GTA CTA CC	36	1 (M)	This study
ShuMp	CTT GTG ATG GTT ATA AAG CAT TAC	51	893 (M)	This study
ShuM+1310	CAC GGT GTT ACA GTC CGA	54	1310 (M)	This study
ShuM3425-	CAG AAT CCG GAG AAT AC	46	3425 (M) rc	This study
ShuM1010-	CCA ATG GGA AGC ATT CAG C	54	1010 (M) rc	This study
ShuM590+	CCG TGT TGC CGG AGA TAA C	56	590 (M)	This study
ShuM2040+	GCA ACA CAA CTT CTA AC	46	2040 (M)	This study
SHUNI L SEGMENT PRIMERS				
Primer	**Sequence**	**TM**	**Position**	**Reference**
BunM+	(GTT TCC CAG TCA CGA TC) A GTA GTG TAC TAC C	44	1-14bp (S, M, L)	This study
G52B	AAT CAA CGC ACT GCC AGY ACT	57	Random primer	(Christensen *et al.*, 1999)
ShuSML	AGT AGT GTG CTC CAC ATA G	51	1-19bp (S, M, L)	This study

1. S segment proteins

Non-structural protein (NSs)

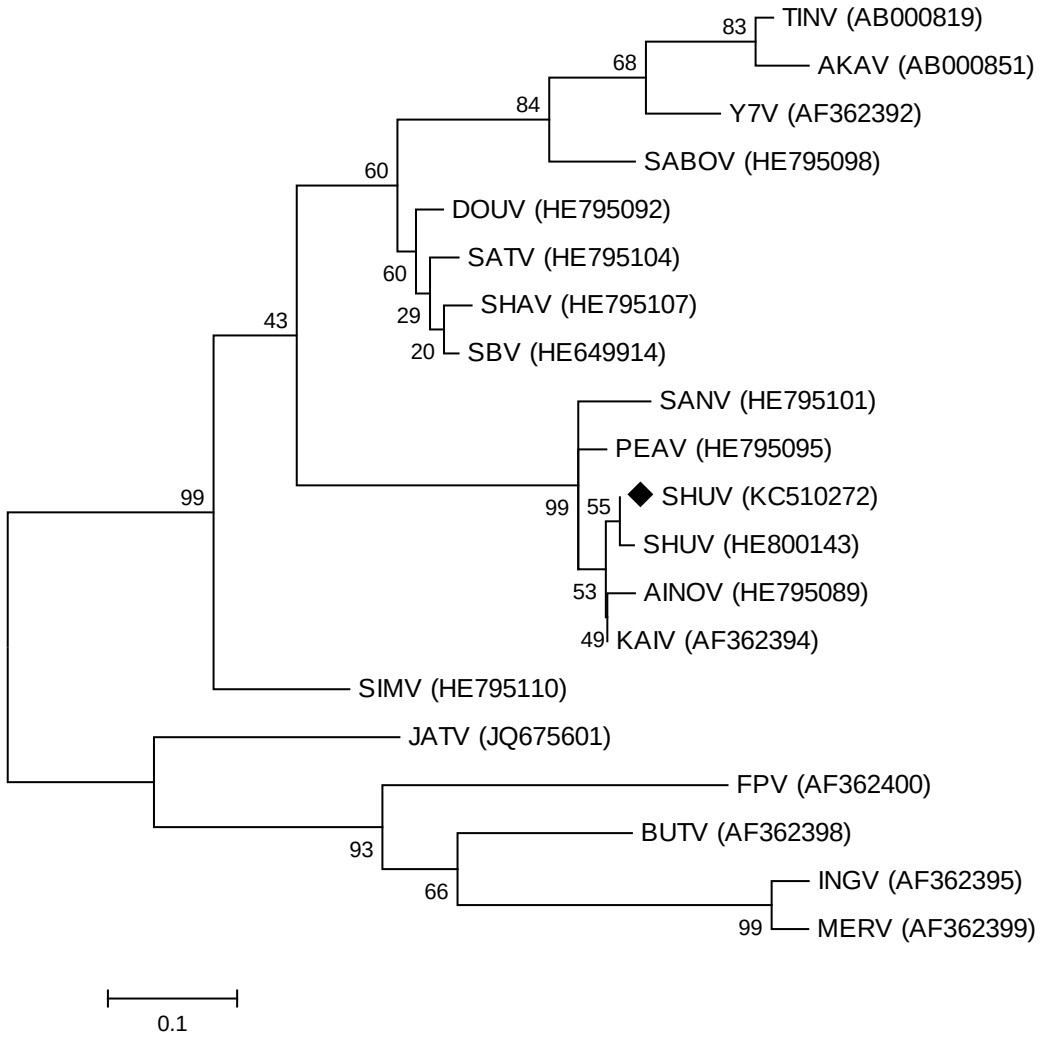


Figure. 1 A maximum likelihood tree constructed under the Jones-Taylor-Thornton (JTT) substitution model using the Mega 5 program, of the amino acid sequence of the NSs protein of SAE 18/09, with representative sequences of selected other Simbu serogroup viruses. The bar indicates 0.1 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1000 replicates. Reference strains GenBank accession numbers are indicated**.**

2. M segment proteins

Glycoprotein precursor

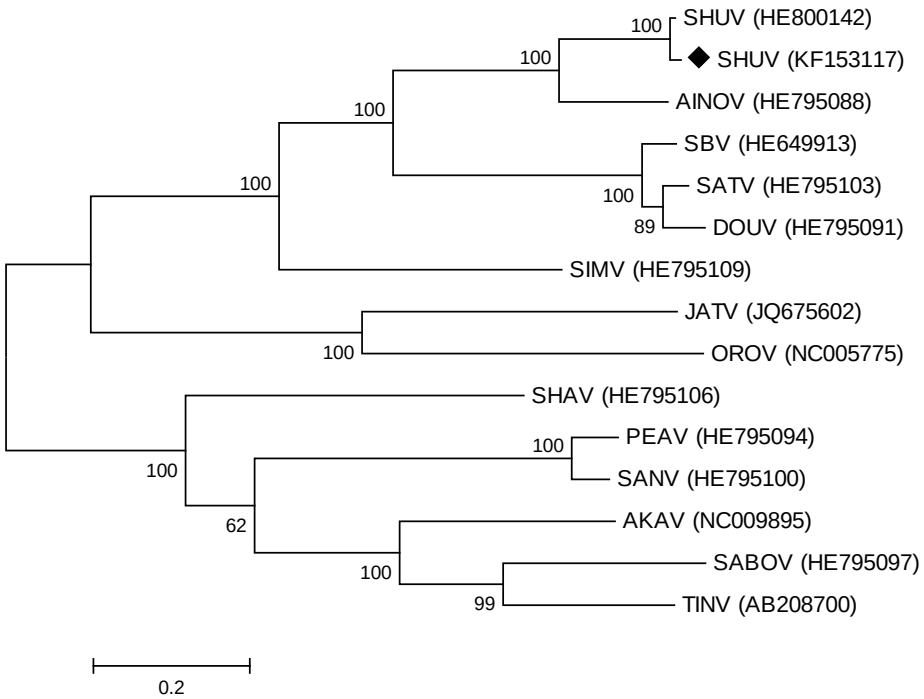


Figure. 2a A maximum likelihood tree constructed under the Jones-Taylor-Thornton (JTT) substitution model using the Mega 5 program, of the amino acid sequence of the GPC of SAE 18/09, with representative sequences of selected other Simbu serogroup viruses. The bar indicates 0.2 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1000 replicates. Reference strains GenBank accession numbers are indicated**.**

Non-structural protein (NSm)

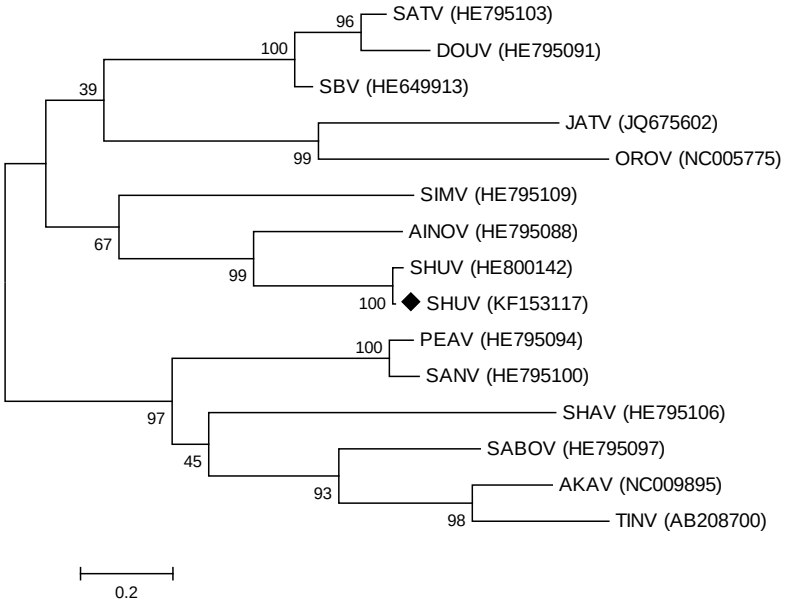


Figure. 2b Maximum likelihood tree constructed under the Jones-Taylor-Thornton (JTT) substitution model using the Mega 5 program, of the amino acid of NSm. Estimates were based on bootstrap resampling carried out with 1000 replicates. Reference strains GenBank accession numbers are indicated.

APPENDIX C

Genomic Sequence Analyses

1. S segment

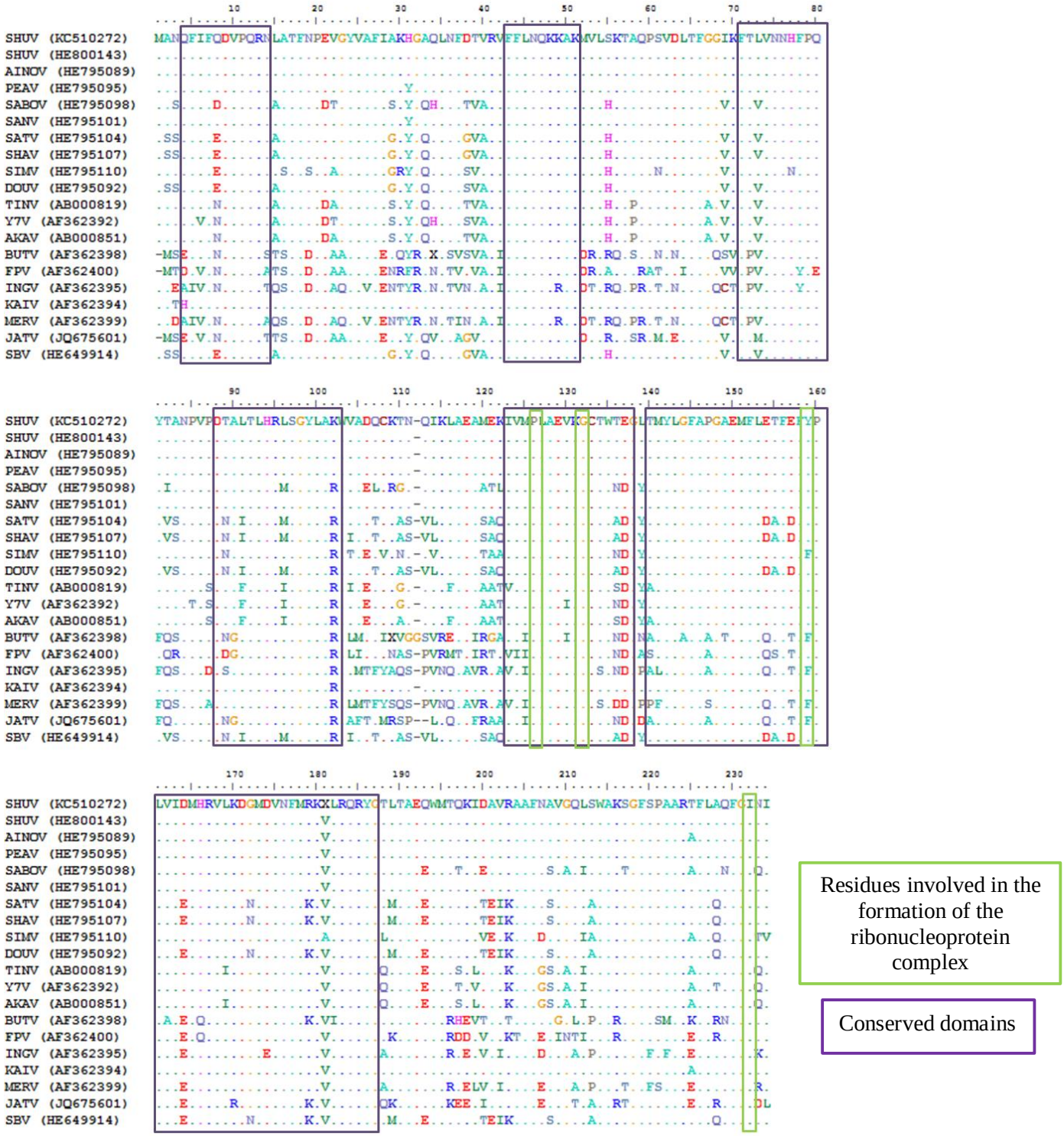


Figure 1. Amino acid alignment of the N protein of members of the Simbu serogroup, indicating both those residues involved in the formation of the ribonucleoprotein complex and the conserved domains.

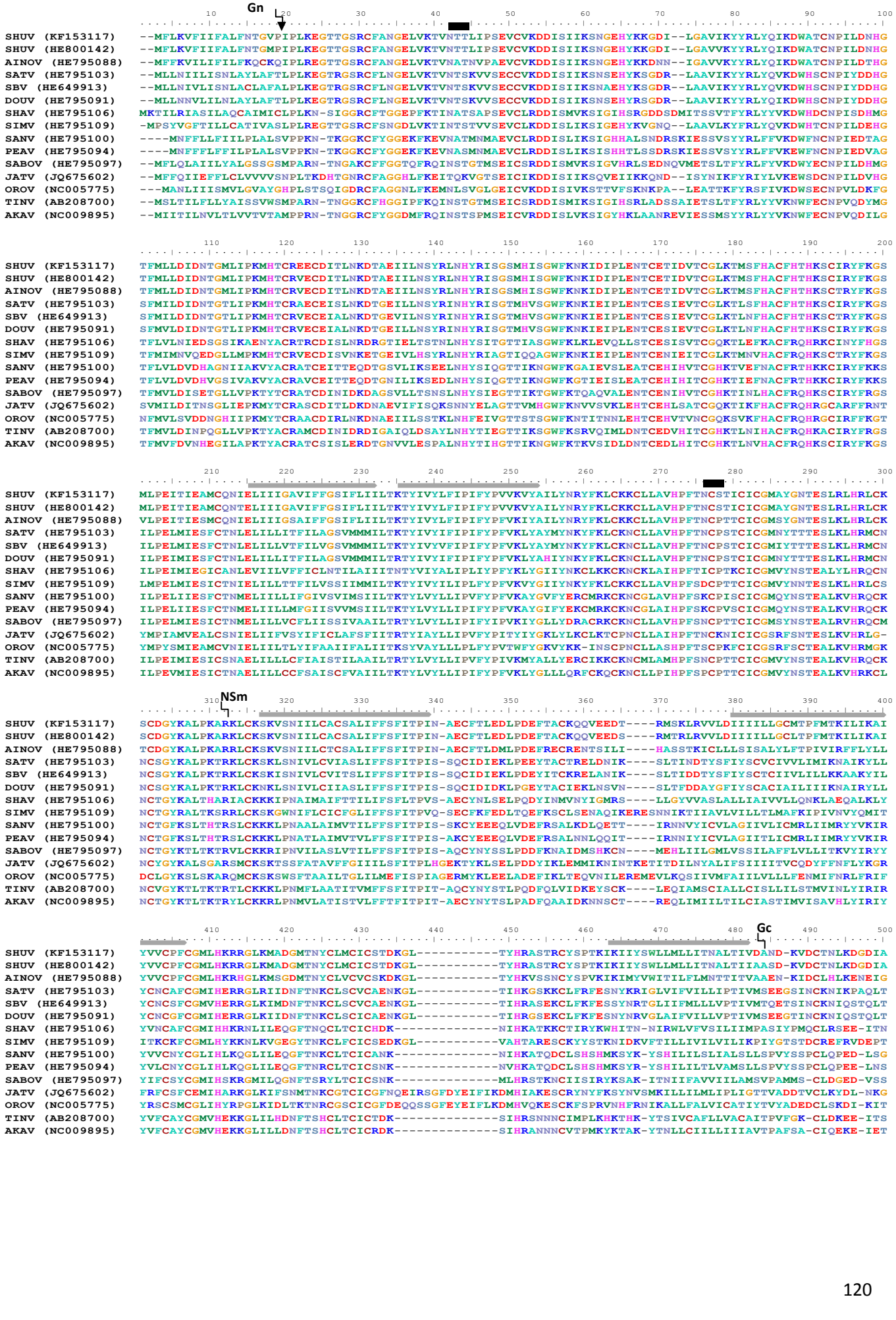
2. M Segment

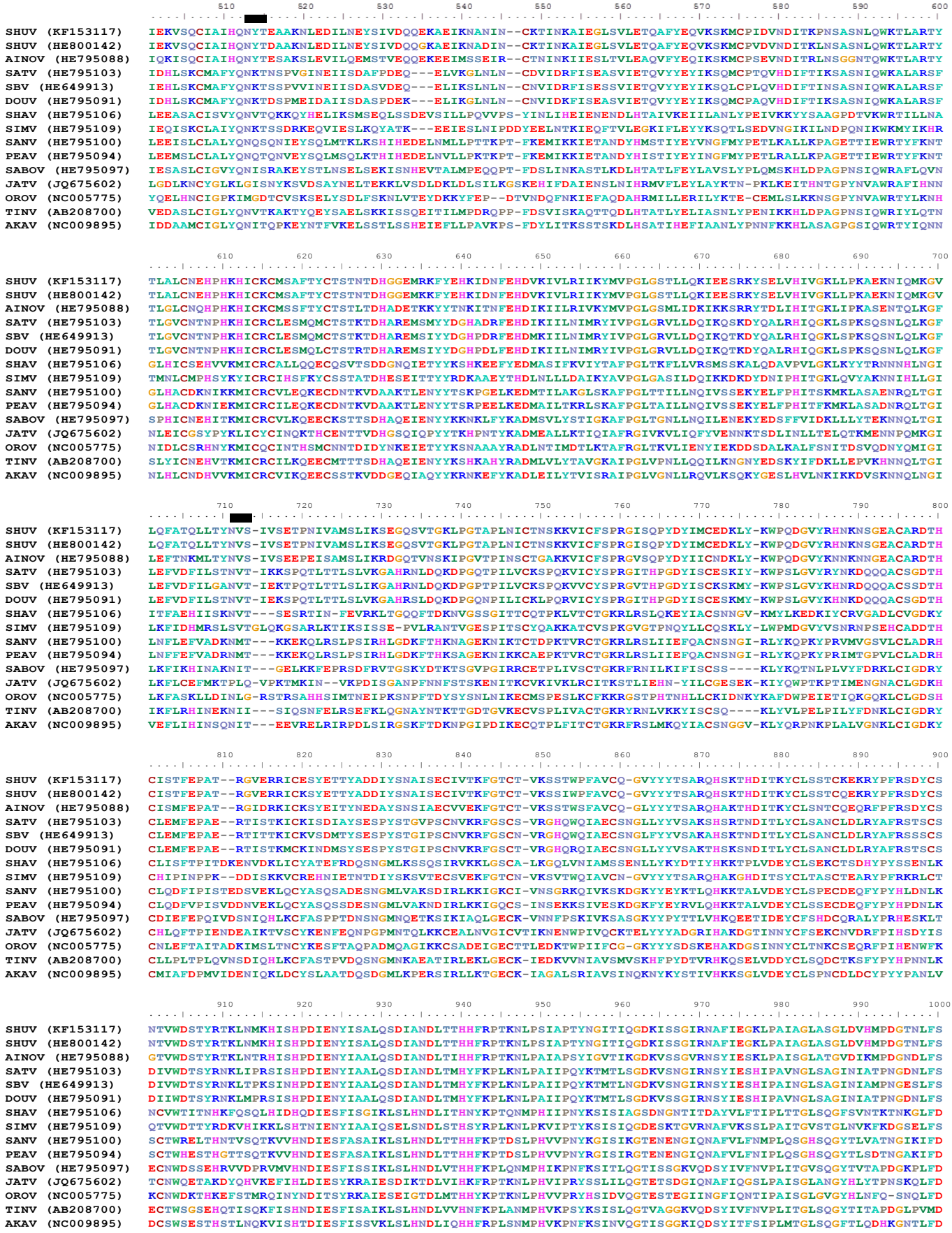
Table 1. N glycosylation sites of various Simbu group viruses, as determined by NetNglyc 1.0

SeqName	Position		Potential agreement	Jury result	N-Glyc
Aino_	40	NATN	0.7136	(9/9)	++
Aino_	355	NTSI	0.5041	(6/9)	+
Aino_	457	NTTI	0.7086	(9/9)	++
Aino_	492	NYTE	0.7148	(9/9)	++
Aino_	688	NVSI	0.6866	(9/9)	++
Aino_	1401	NKSK	0.5888	(8/9)	+
Peaton_	37	NASM	0.6538	(8/9)	+
Peaton_	283	NSTE	0.6662	(9/9)	++
Peaton_	296	NCTG	0.6598	(8/9)	+
Peaton_	316	NATL	0.6767	(8/9)	+
Peaton_	487	NQTQ	0.6783	(9/9)	++
SAE 18/09	40	NTTL	0.7378	(9/9)	++
SAE 18/09	272	NCST	0.6167	(6/9)	+
SAE 18/09	492	NYTE	0.7087	(9/9)	++
SAE 18/09	688	NVSI	0.6748	(8/9)	+
Simbu_	41	NTST	0.5251	(3/9)	+
Simbu_	285	NNTE	0.5890	(8/9)	+
Simbu_	298	NCTG	0.7582	(9/9)	+++
Simbu_	498	NKTS	0.7569	(9/9)	+++
Simbu_	770	NPSE	0.5473	(7/9)	+

Table 2. Comparison of the trans-membrane domains identified by various prediction software programs.

TMHMM	TopPred2	HMMTOP	TMpred	DAS	MEMSTAT	SOSUI
	1-21		6-25	7-13		1-21
212-229	210-230	212-229	217-235	212-247	212-229	219-241
234-253	233-253	236-253			236-253	
	263-283	264-281	268-290		264-283	
315-337	315-335	315-332	320-338	319-333	318-335	314-336
371-393	369-387		372-392	367-380; 387-396	367-388	371-393
445-462	444-464		448-466	446-459	445-462	
	815-835					
1357-1379	1363-1383	1366-1383	1364-1384	1360-1382	1357-1380	1362-1383


Gn
NSm
Gc
SHUV (KF153117)
SHUV (HE800142)
AINOV (HE795088)
SATV (HE795103)
SBV (HE649913)
DOUV (HE795091)
SHAV (HE795106)
SIMV (HE795109)
SANV (HE795100)
PEAV (HE795094)
SABOV (HE795097)
JATV (JQ675602)
OROV (NC005775)
TINV (AB208700)
AKAV (NC009895)


SHUV (KF153117)
SHUV (HE800142)
AINOV (HE795088)
SATV (HE795103)
SBV (HE649913)
DOUV (HE795091)
SHAV (HE795106)
SIMV (HE795109)
SANV (HE795100)
PEAV (HE795094)
SABOV (HE795097)
JATV (JQ675602)
OROV (NC005775)
TINV (AB208700)
AKAV (NC009895)

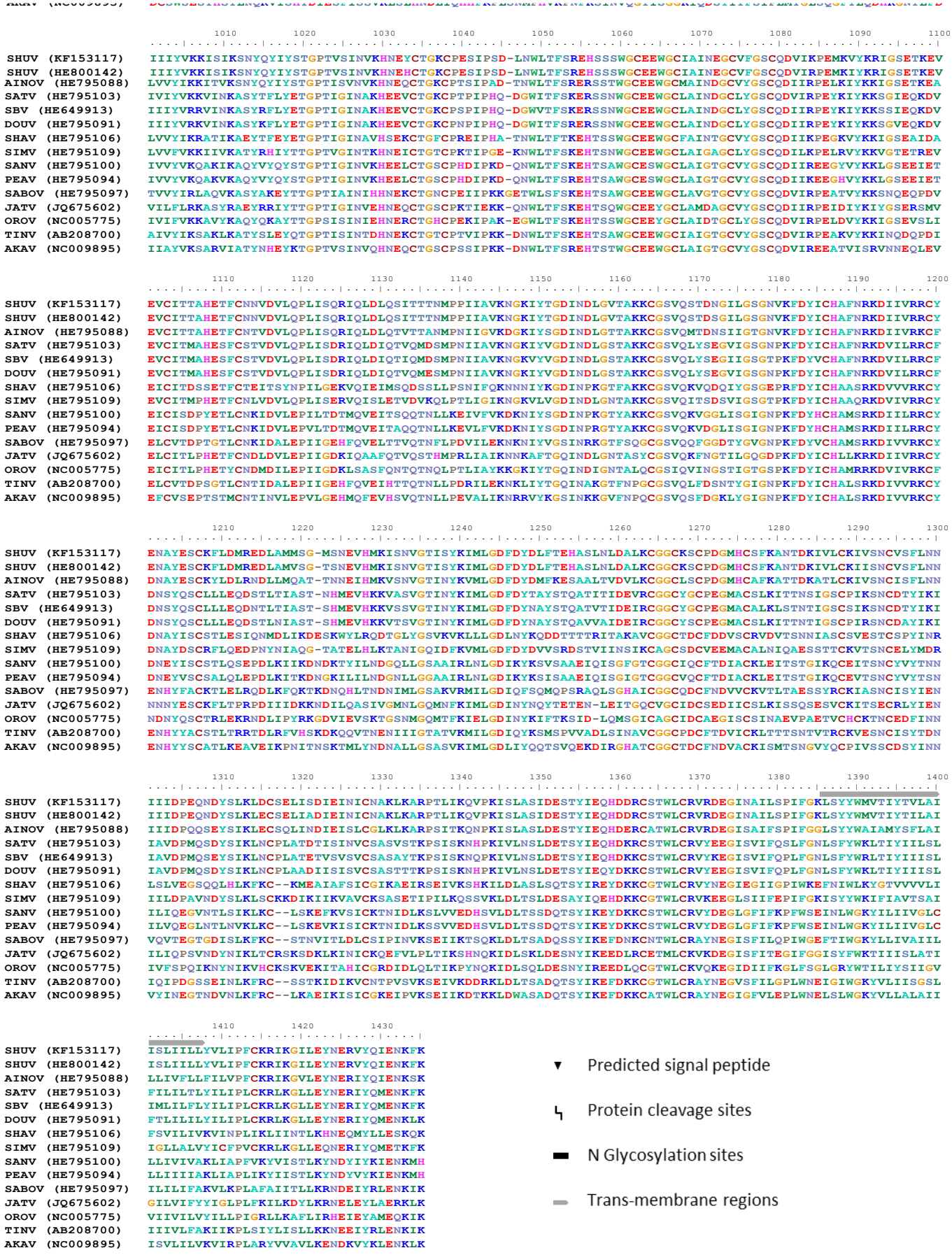
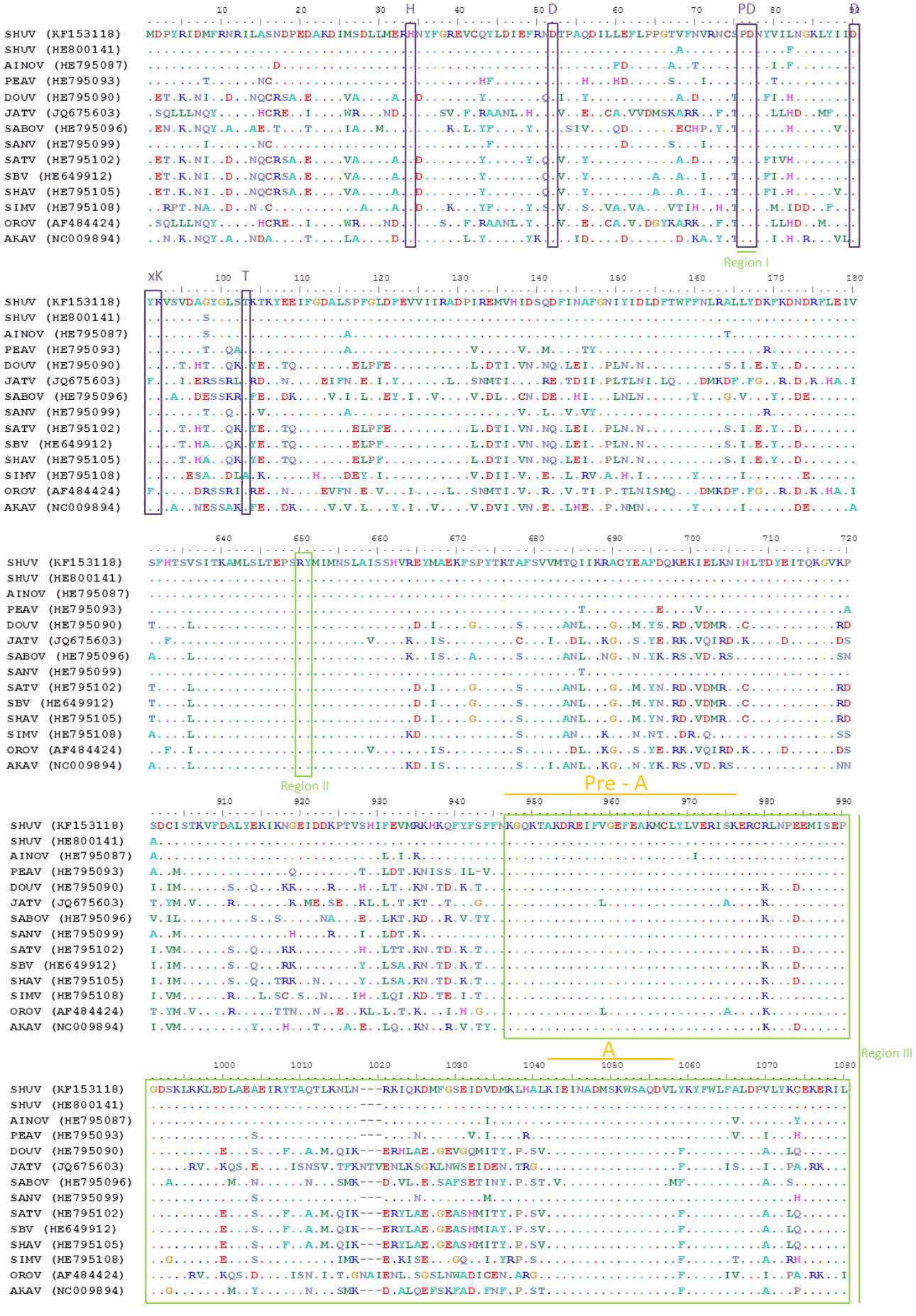


Figure 2. Amino acid alignment of the M polyprotein of members of the Simbu serogroup, indicating the gene layout and functional regions.

3. L Segment



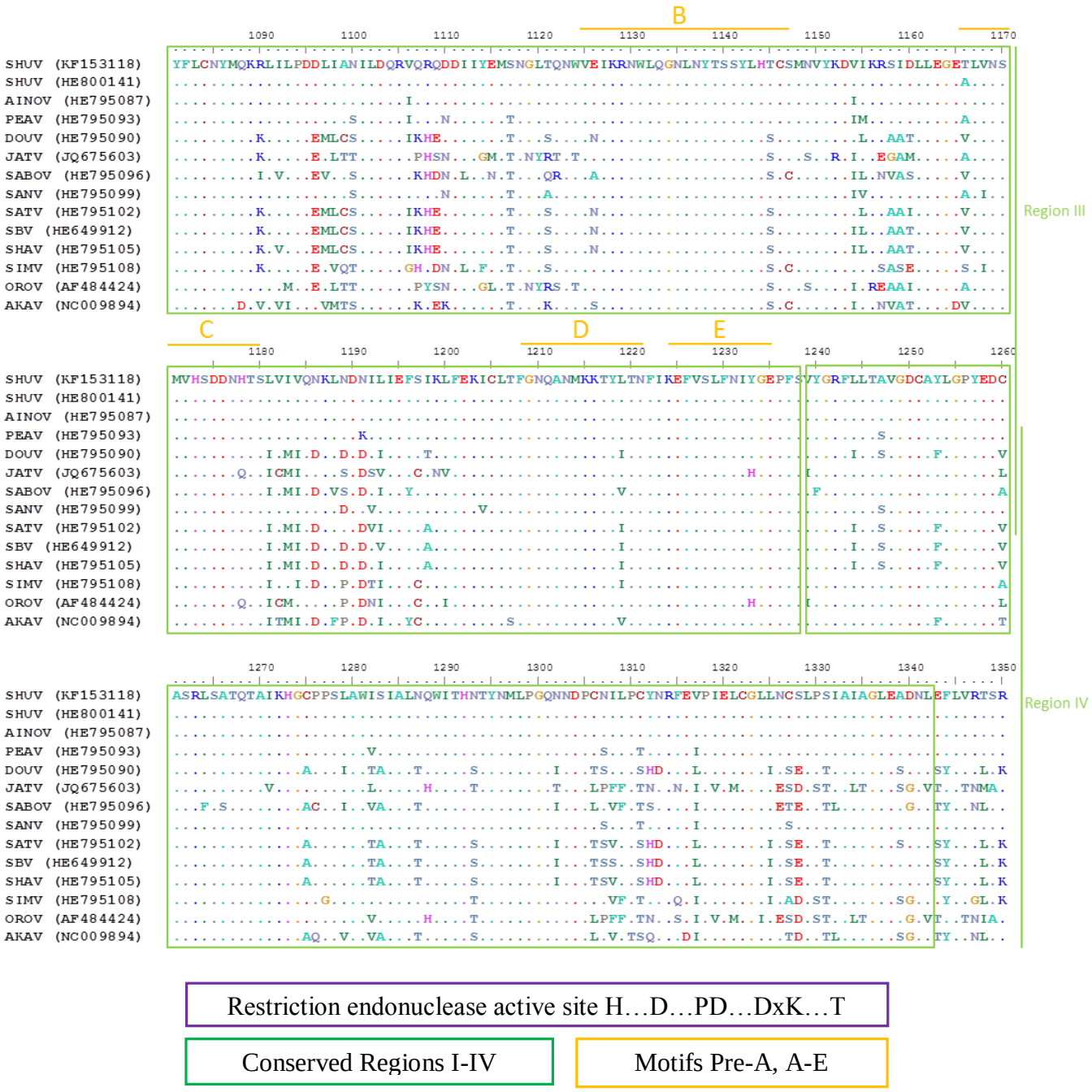


Figure 3. Amino acid alignment of various segments of the L protein of members of the Simbu serogroup, indicating important conserved regions and domains. The orthobunyaviruses restriction endonuclease conserved active site H…D…PD….DxK…..T is also shown.

APPENDIX D

Statistical analysis

Table 1. Factors associated with WNV and SHUV antibodies in the veterinary population

Variable	WNV		SHUV	
	n/N	% positive (95% CI)	n/N	% positive (95% CI)
Gender				
Male	5/79	6.3 (2.1-14.1)	4/79	5.1 (1.3-12.4)
Female	5/46	10.9 (3.6-23.6)	1/46	2.2 (0.05-11.5)
Age (in years)				
20-25	0/4	0.0 (0.0-52.7)	0/4	0.0 (0.0-52.7)
26-50	6/69	8.7 (3.2-17.9)	2/69	2.9 (0.3-10.1)
51-75	4/52	7.7 (2.1-18.5)	3/52	5.7 (1.2-15.9)
Province				
Gauteng	3/50	6.0 (1.2-16.5)	3/50	6.0 (1.2-16.5)
Mpumalanga	0/9	0.0 (0.0-28.3)	0/9	0.0 (0.0-28.3)
Free State	1/4	25.0 (0.6-80.6)	0/4	0.0 (0.0-52.7)
Western Cape	1/18	5.5 (0.1-27.3)	0/18	0.0 (0.0-15.3)
Eastern Cape	1/8	12.5 (0.3-52.6)	1/8	12.5 (0.3-52.6)
Northern Cape	1/5	2.0 (0.05-71.6)	0/5	0.0 (0.0-45.1)
Limpopo	0/7	0.0 (0.0-34.8)	1/7	14.3 (0.7-52.1)
North West	0/6	0.0 (0.0-39.3)	0/6	0.0 (0.0-39.3)
KwaZulu Natal	3/13	23.1 (5.0-53.8)	0/13	0.0 (0.0-20.6)
Total	10/125	8.0 (3.9-14.2)	5/125	4.0 (1.3-9.1)

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