Phylogenetic characterisation of the Palyam serogroup orbiviruses and development of a real-time RT-PCR

by

KAREN EBERSOHN

Submitted in partial fulfilment in accordance with the requirements for the degree of

Magister Scientiae (Veterinary Science)

at the Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science,
University of Pretoria

SUPERVISOR: Prof EH Venter
CO-SUPERVISOR: Dr P Coetzee

NOVEMBER 2018

DECLARATION

I, Karen Ebersohn, student number 4296834 hereby declare that this dissertation, "*Phylogenetic characterisation of the Palyam serogroup orbiviruses and the development of a real-time RT-PCR,*" is submitted in accordance with the requirements for the Magister Scientiae (Veterinary Science) degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

.....

Karen Ebersohn

November 2018

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people:

- My supervisors, Prof EH Venter and Dr P Coetzee, for their guidance
- Dr Louwtjie Snyman, for his assistance with the phylogenetic analysis
- Prof R Swanepoel, for providing the samples and funding
- Prof Luis Neves, for granting me the time to work on this project
- Anna-Mari Bosman, for technical assistance and guidance
- My family and friends, for their support

ABBREVIATIONS

AHSV	African horse sickness virus
BHK21	Baby hamster kidney 21
BLAST	Basic Local Alignment Search Tool
BTV	Bluetongue virus
cDNA	Complementary DNA
CPE	Cytopathic effects
CT	Threshold cycle
DDN	Data display network
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide
dsRNA	Double-stranded RNA
EEV	Equine encephalosis virus
EHDV	Epizootic haemorrhagic disease virus
ELISA	Enzyme-linked immunsorbent assay
FLAC	Full-length amplification of cDNA's
HPLC	High performance liquid chromatography
ICTV	International Committee on Taxonomy of viruses
MCMC	Markov chain Monte Carlo
MMOH	Methyl mercury hydroxide
NCBI	National Centre for Biotechnology
NGS	Next generation sequencing
ORF	Open reading frame
PEG600	Poly ethylene glycol 600
RK13	Rabbit kidney 13
RT-PCR	Reverse transcriptase-polymerase chain reaction
ssRNA	Single-stranded RNA

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Phylogenetic characterisation of the Palyam serogroup orbiviruses and development of a real-time RT-PCR

by

Karen Ebersohn

Supervisor : Prof EH Venter

Co-supervisor : Dr P Coetzee

Department : Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Degree : MSc (Veterinary Science)

The Palyam serogroup of the genus *Orbivirus* and family *Reoviridae* are arthropod-borne viruses that have been isolated in Africa, Australia and Asia. They are associated with abortion and teratogenesis in cattle and other ruminants. There are currently 13 serotypes recognized by the International Committee on Taxonomy of Viruses (ICTV) including Palyam, Kasba, Vellore, Abadina, D'Aguilar, Nyabira, CSIRO Village, Marrakai, Gweru, Bunyip Creek, Petevo, Marondera and Kindia. Although Palyam viruses had been isolated previously, it was only after an outbreak of congenital abnormalities in cattle in Japan from November 1985 to April 1986 that their pathogenic importance began to be investigated.

Of the 13 different serotypes that have been identified, the full genome sequence of only one, Kasba, has been published. Sequences for certain genome segments of the serotypes from Japan, Australia and Zimbabwe are available but not the

complete genome data. The few molecular studies that have been done on the Palyam serogroup viruses, focused mainly on Kasba virus and little is known about the other serotypes. In general, not much is published on Palyam viruses, their occurrence or prevalence, and the impact of their epidemiology in South Africa or elsewhere is unknown. The objective of this project was to perform phylogenetic analysis of the different serotypes of the Palyam viruses to enable a better understanding of the genomic features of the Palyam serogroup of orbiviruses, their relation to each other as well as to other orbiviruses.

The study is presented in two parts. The aim of the first part was to obtain the full genome sequences of the different Palyam serotypes and Apies River virus, as well as selected field isolates from Africa in order to perform phylogenetic analysis. The aim of the second part was to develop a rapid diagnostic test to detect the Palyam viruses.

In the first part, the viruses were propagated and after full-length amplification of cDNA (FLAC) the amplicons were sequenced on an Illumina® Mi-Seq sequencer, using the Nextera XT DNA sample preparation kit and 300-bp paired-end V3 Illumina chemistry. Sequence data generated by Illumina sequencing were analyzed using the CLC Genomics Main workbench, version, 8.0.1. *De novo* assembly of sequence reads was performed and contig sequences prepared. Sequences were aligned and converted into nexus and phylips files. Data-display networks (neighbour-networks) were constructed with SplitsTree 4 and the phylips files were used to initiate model estimation via jModel test2, by using the online portal Cipres Science gateway. Bayesian analyses was performed in MrBayes version 3.

During analysis of the amino acid sequences of the separate genes of the Palyam serogroup serotypes, the gene encoding Viral Protein (VP) 7 (Segment 7) was found to be the most conserved. The amino acid sequences for VP2 and VP5 showed the highest degree of variation, with VP2 being the most variable of the two. Phylogenetic analysis indicated that the Palyam virus group was most closely related to AHSV, and EEV showed the most distant evolutionary relationship to the Palyam viruses. When comparing the different serotypes within the Palyam

serogroup viruses, a high degree of sequence identity was found for isolates from the same geographical region.

The phylogenetic analysis revealed two clades, which were supported by strong bootstrap values of 100 and a posterior probability value of 1. The African serotypes were all very closely related in one clade, with identical sequences for several gene segments. The second clade contained the Australian and Asian serotypes and one African serotype, Petevo. The high percentage sequence identity (85.6% - 77,5%) that exists between the viruses from Australia and Asia may suggest that there has been some gene flow between the serotypes. It was clear from the sequence data that the geographical origin of Palyam serogroup viruses played an important role in the development of the different serotypes.

In the second part of the study, the sequence data obtained in the phylogenetic analysis was used to develop primers and a probe to detect all the Palyam serogroup serotypes in a real-time RT-PCR. The same viruses used in the first part of the study as well as other orbiviruses were propagated and RNA was extracted and tested in a real-time RT-PCR. The real-time RT-PCR was able to detect all the Palyam serogroup serotypes, but further validation is necessary for it to be used as a diagnostic test.

The sequence data generated during this study could enable further investigation into molecular evolution of the Palyam serogroup viruses such as reassortment, genetic drift and intragenic recombination. The developed real-time RT-PCR could be a valuable diagnostic tool for both the detection and exclusion of Palyam serogroup viruses during outbreaks involving relevant symptoms.

CHAPTER 1

1.1 INTRODUCTION

The Palyam serogroup of the genus *Orbivirus* and family *Reoviridae* are arthropod-borne viruses that have been isolated in Africa, Australia and Asia (Swanepoel 2004, Aradaib *et al*. 2009). They are associated with abortion and teratogenesis in cattle and other ruminants (Yamakawa *et al*. 1999, Miura *et al*. 1991, St George 1989, Goto *et al.* 1988). There are currently 13 serotypes recognized by the International Committee on Taxonomy of Viruses (ICTV) including Palyam, Kasba, Vellore, Abadina, D'Aguilar, Nyabira, CSIRO Village, Marrakai, Gweru, Bunyip Creek, Petevo, Marondera and Kindia. Although Palyam serogroup viruses had been isolated previously (Whistler & Swanepoel 1988, Whistler & Swanepoel 1990), it was only after an outbreak of congenital abnormalities in cattle in Japan from November 1985 to April 1986 that their pathogenic importance was initially investigated (Yamakawa & Furuuchi 2001).

Of the 13 different serotypes that have been identified, the full genome sequence of only one, Kasba, has been published (Wang *et al.* 2016, Yamakawa, Furuuchi & Minobe 1999, Yang *et al.* 2016). Sequences for certain genome segments of the serotypes from Japan, Australia and Zimbabwe are available (Yamakawa *et al.* 2000) but not the complete genome data. Obtaining the full genome sequence of all the serotypes will enable a better understanding of the genomic features of the Palyam serogroup viruses and their relation to other orbiviruses.

No serosurveillance for the Palyam viruses is done in South Africa and full genome sequences of the African serotypes are not available. The disease potential of the serotypes that occur in Africa is also unknown (Aradaib *et al.* 2009). Consequently the Palyam serogroup could be of future concern to the cattle and wildlife industry in South Africa and beyond (Attoui & Mohd Jafaar 2015, Whistler *et al*. 1988). The few molecular studies that have been done on the Palyam viruses, focused mainly on Kasba virus and little is known about the other serotypes.

In general, not very much is published on this group of viruses, their occurrence or prevalence and the impact of their epidemiology in South Africa or elsewhere, is unknown.

The availability of full genome sequencing data of the different serotypes of Palyam serogroup viruses will assist with determining the source of viruses in outbreaks, and the reassortment activity of these viruses can be explored. More complete sequence data will also aid in the development of diagnostic assays, particularly rapid molecular tests.

The development of both a molecular and serological assay for the detection of these viruses in South Africa will therefore be of great value. In this study genetic analysis of serotypes of the Palyam serogroup enabled an investigation of sequence diversity between and within serotypes and the relationship of these viruses to other orbiviruses. A real-time reverse transcriptase polymerase chain reaction (RT-PCR) was also developed for future use in the detection of Palyam group viruses in South Africa.

1.2 LITERATURE REVIEW

1.2.1 Aetiological agent

Palyam viruses belong to the genus *Orbivirus* and family *Reoviridae*. Members of the *Orbivirus* genus have non-enveloped, icosahedral virions with triple capsid structures and are approximately 80 nm in diameter (Figure 1.1). Palyam serogroup viruses are similar to the other orbiviruses, with a spherical morphology and are approximately 60 nm in diameter (Kurogi *et al.* 1989).

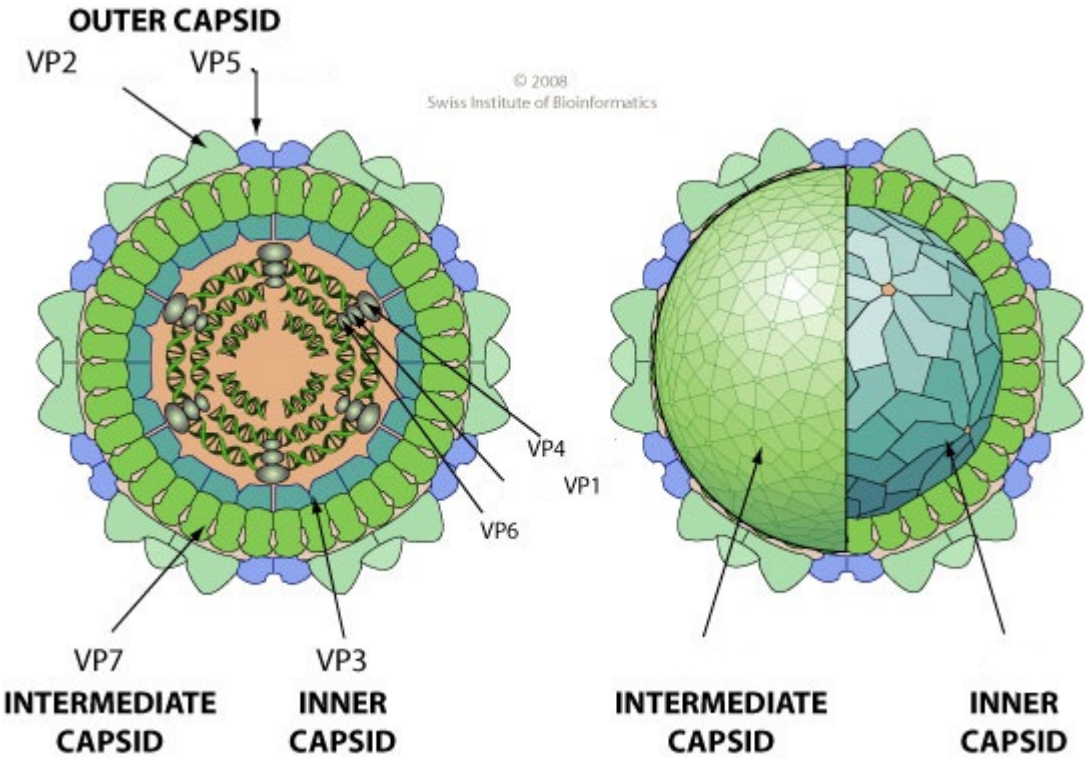


Figure 1.1 The triple capsid orbivirus structure, indicating the location of the seven structural proteins (Roy 1996)

http://viralzone.expasy.org/all_by_species/106.html

The Palyam viral genome is similar to the genome of the other members of the *Orbivirus* genus, which includes notable viruses like bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV). It consists of ten, linear double-stranded (ds) RNA segments that code for seven structural viral proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3a) (Yamakawa *et al.* 1999a, Whistler & Swanepoel 1990, Harasawa *et al.* 1988). Segments 1, 2, 3, 4, 6, 7 and 9 of the Palyam genome encode for VP1, VP2, VP3, VP4, VP5, VP7 and VP6 respectively (Figure 1.2) (Yamakawa *et al.* 1999a). Segment 5 encodes for NS1 and Segment 8 for NS2. NS3 & NS3a are encoded by Segment 10, which is also the smallest gene (Staden & Huismans 1991).

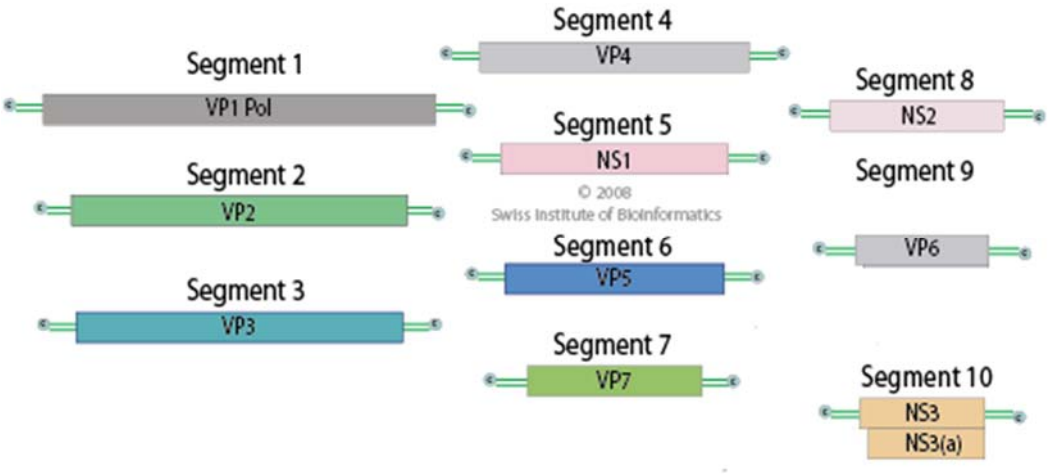


Figure 1.2 The ten segments and translated proteins of the genome of the genus *Orbivirus* and their corresponding translated proteins
http://viralzone.expasy.org/all_by_species/106.html

For the double-layered virus particle VP2 and VP5 form the more diffuse outer capsid and VP3 and VP7 are the sole components of the inner and intermediate parts of the inner capsid respectively (Yamakawa & Furuuchi 2001, Yamakawa *et al.* 1999a). The other three structural proteins, VP1, VP4 and VP6 are closely associated with the dsRNA (Roy 1996). Cell attachment is mediated by VP2 and VP5 as well as penetration during the initiation of infection. Viral Protein 1 is the RNA polymerase, VP4 the capping enzyme and VP6 is the viral helicase that unwinds the dsRNA molecule during transcription (Dilcher & Weidmann 2012). These three proteins act

together to synthesize positive sense messenger RNA. In orbiviruses, an open reading frame (ORF) spans nearly the entire length of the segments but for segment 9, which encodes VP6, a second ORF has also been found in BTV and AHSV which encodes a fourth non-structural protein (NS4) (Zwart *et al.* 2015, Belhouchet *et al.* 2011). This protein might play a role in the virus replication cycle and virus release (Belhouchet *et al.* 2011) and is an interferon antagonist (Ratinier *et al.* 2016). The NS1 (segment 5) forms tubules that are possibly involved in virus transportation processes and is also a regulator of protein expression (Owens *et al*. 2004) and NS2 (segment 8) is a matrix protein responsible for granular viral inclusion bodies that are present in the cytoplasm of virus-infected cells and are involved in virus assembly (Dilcher & Weidmann 2012, Roy 1996). NS3 and NS3a (segment 10) are glycoproteins that are involved in the release of the virus from infected cells (Dilcher & Weidmann 2012, Roy 1996).

The structural proteins of the outer capsid (VP2 and VP5) of Palyam serogroup viruses have variable antigenic structures and are responsible for viral neutralization and serotype specificity (Yamakawa & Furuuchi 2001, Yamakawa *et al.* 1999a, Bodkin & Knudson 1985). Thus, VP2 and VP5 have the most variable genome sequences (Yang *et al.* 2016). The proteins of the inner capsid, VP3 and VP7, are involved with molecular interactions during virus assembly and are greatly conserved (Wang *et al.* 2016). VP7 of BTV can also mediate attachment and penetration of insect cells (Mertens *et al*. 2004). It was reported that genome segment 3, coding for VP3 of Kasba virus was the most conserved when compared to serogroups of other orbiviruses such as BTV, AHSV and EHDV (Yamakawa *et al.* 1999b). It was found that VP7 was also conserved among the different Palyam serotypes with known sequences for Segment 7 (Wang *et al.* 2016, Aradaib *et al.* 2009, Yamakawa *et al.* 1999b). The segments coding for VP6 and NS1 (Segments 9 and 5 respectively) are greatly conserved but do show variability corresponding to the geographic area where the virus was isolated, regardless of the serotype (Wang *et al.* 2016). In general, structural proteins, except for VP2 and VP5 are more conserved than the non-structural proteins (Yamakawa *et al.* 1999b).

A study of the physiochemical properties of an isolate of Kasba virus (Kurogi *et al.* 1989) showed that the virus particles were sensitive to trypsin, stable after freeze-

thawing and inactivated at a pH of 3. Kasba virus also demonstrated haemagglutination when using bovine erythrocytes (Miura *et al.* 1991). Little information is available on the physiochemical properties of the other serotypes.

Currently 13 serotypes have been identified within the Palyam group of viruses (Table 1.1); the serotypes are defined by using the serum neutralization test (Swanepoel 2004). Cross-reaction does occur between serotypes in immunofluorescence as well as neutralization tests and thus the 13 serotypes can be divided into six antigenic complexes (Table 1.2): Kasba (Chuzan), D'Aguilar, Vellore, Palyam, Petevo and Gweru complex (Whistler & Swanepoel 1988). Abadina, Kasba and Marrakai show cross-reactions and are placed in the Kasba complex and D'Aguilar and Nyabira are placed in the D'Aguilar complex. The Vellore complex consists of Bunyip Creek, Vellore and Marondera and the Palyam complex contains Palyam and CSIRO Village. Petevo and Gweru viruses are the sole occupants of two further separate complexes (Whistler & Swanepoel 1988).

Two full genome sequences for the Palyam serogroup viruses have been published, both for one serotype i.e Kasba virus (Wang *et al.* 2016, Yang *et al.* 2016). At least partial sequences for the genes encoding VP1, VP2, VP5, VP6, VP7 and NS1 of various Palyam viruses from Australia and or Zimbabwe are also available (Palacios *et al*. 2011, Ohashi *et al.* 2004, Yamakawa *et al*. 2000)

Table 1.1. Original Palyam virus isolations (Swanepoel 2004)

Virus serotypes	Year of isolation	Country	Source
Palyam	1956	India	Mosquitoes
Kasba	1957	India	Mosquitoes
Vellore	1966	India	Mosquitoes
Abadina	1967	Nigeria	Midges
Apies River (Not recognized by ICTV as a serotype)	1967	South Africa	Febrile cow
D'Aguilar	1968	Australia	Midges
Nyabira	1973	Zimbabwe	Aborted cattle foetus
CSIRO Village	1974	Australia	Midges
Marrakai	1975	Australia	Midges
Gweru	1976	Zimbabwe	Aborted cattle foetus
Bunyip Creek	1976	Australia	Midges
Petevo	1978	Central African Republic	Ixodid ticks
Marondera	1978	Zimbabwe	Cow viscera
Kindia	1983	Guinea	Ixodid ticks

Table 1.2. The six antigenic complexes of the Palyam serogoup of orbiviruses

Kasba complex	D'Aguilar comlex	Vellore complex	Palyam complex	Petevo complex	Gweru complex
Kasba	D'Aguilar	Vellore	Palyam	Petevo	Gweru
Abadina	Nyabira	Bunyip Creek	CSIRO Village		
Marrakai		Marondera			

1.2.2 Replication

As with other orbiviruses, replication of the virus takes place in the cytoplasm of infected cells, followed by cell lysis and the virus is released (Whistler & Swanepoel 1990). Orbiviruses attach to the host cells and enter by endocytosis. Viral cores are released into the cytoplasm and this initiates the transcription of the viral genome into mRNA, using the negative strand of dsRNA segments as templates. The mRNA's are forced out into the cytoplasm through the openings of pores in the VP3 protein and leave the core particles through the central space in the pentameric rings of the VP7 trimers and act as templates for the new dsRNA genome segments (Noad & Roy 2009). NS2 forms viral inclusion bodies which enlist the viral ssRNA and viral protein components needed for genomic packaging, replication and core assembly (Patel & Roy 2014). The new virions assemble within the cytoplasm after which they are released by budding (Noad & Roy 2009).

1.2.2.1 Reassortment

The segmented nature of orbivirus genomes make them prone to reassortment. Reassortment is a process that occurs when two segmented RNA viruses of the same species infect a single host cell and then exchange genome segments and form progeny viruses that are genetically different from the parental strains. This leads to the generation of new virus genotypes and it can have an impact on the phenotypic characteristics of these new viruses. Biological consequences of reassortment include changes in host or vector range, transmissibility, virulence and pathogenicity (Nomikou *et al.* 2015).

Reassortment has been demonstrated in BTV serotypes in Europe, Australia and China (Qin *et al*. 2018, Nomikou *et al*. 2015, Shaw *et al*. 2012). Reassortment between vaccine strains and field strains of AHSV in South Africa has also been revealed (Weyer *et al*. 2016). Genetic reassortment of segment 2, has been detected between Kasba and D'Aguilar which could possibly have caused an antigenic shift within the Palyam serogroup (Yang *et al.* 2016, Ohashi *et al.* 2004). Intragenic recombination also plays a role in the genetic diversity in certain RNA viruses and has been demonstrated in BTV (He *et al.* 2010).

1.2.3 Epidemiology

1.2.3.1 Hosts and vectors

Like other orbiviruses, Palyam serogroup viruses are transmitted by haematophagous arthropod vectors such as mosquitoes, ticks and *Culicoides* biting midges (Kurogi *et al.* 1989, Whistler *et al*. 1989, Doherty *et al.* 1972). Palyam serogroup viruses have been isolated from ixodid ticks and mosquitoes (Swanepoel 2004, Jusa *et al.* 1994) but the greater part of Palyam virus isolations have been from midges (Blackburn *et al*. 1985, Doherty *et al*. 1972). Transmission of the viruses by midges has not been shown but the Nyabira virus has exhibited the ability to replicate in inoculated midges. Therefore *Culicoides* midges are believed to be the most likely natural vectors of the Palyam serogroup viruses in southern Africa (Whistler & Swanepoel 1989). *Culicoides oxystoma*, found mainly in Asia and the Middle East (Morag *et al*. 2012), from which Chuzan (Kasba) virus was isolated in Japan in 1984, was recognized as biological vector rather than a mechanical one. *C. oxystoma* was recently documented in West Africa (Senegal) as a potential vector for other orbiviruses namely BTV and AHSV (Fall *et al.* 2015).

Although cattle are mostly affected, antibodies to Palyam serogroup viruses have been found in sheep, Asian buffalo, goats and humans (Yang *et al.* 2008, Swanepoel 2004, Whistler *et al.* 1989). An antibody survey where sera from domestic animals and humans from ten farms across South Africa were screened for antibodies to the following Palyam serogroup serotypes: Nyabira, Gweru, Abadina, Marondera and Apies River showed a high prevalence of antibody to one or more of the serotypes in cattle (53%) and lower prevalence in sheep (4%) and goats (12.5%) from the same property (Whistler *et al.* 1989). The antibodies found in human sera (14% prevalence) were from one farm and mostly against the Nyabira virus while the cattle sera from the same property mainly contained antibodies against the other four viruses (Whistler *et al.* 1989).

1.2.3.2 Occurrence

Palyam, Kasba and Vellore serotypes were the first Palyam serogroup viruses to be isolated (Table 1.1). These viruses were isolated from mosquitoes in India in 1956, 1957 and 1966 (Myers *et al*. 1971, Dandawate *et al*. 1974, Dandawate *et al*. 1969). In the 1970's D'Aguilar, CSIRO Village, Marrakai and Bunyip Creek viruses were isolated

from midges in Australia (St George 1989). In Africa, Abadina virus, from midges, and Apies River virus, from a febrile cow, were isolated in 1967 (Whistler *et al*. 1989). The Nyabira and Gweru viruses were isolated from aborted cattle foetuses in Zimbabwe in the 1970's (Swanepoel & Blackburn 1976). In 1978 Marondera was isolated from cow viscera in Zimbabwe (Whistler & Swanepoel 1998) and Petevo from ixodid ticks in the Central African Republic (Saluzzo *et al*. 1982). The original isolations of the different Palyam serogroup serotypes are summarized in Table 1.1.

In 1985, an outbreak of bovine disease characterized by congenital abnormalities of calves occurred in the Kyushu district of Japan. Chuzan virus was isolated from a biting midge, *C. oxystoma*, and from sentinel calves. Serological surveys revealed that this virus was the cause of the bovine disease (Miura *et al.* 1991) which was subsequently called Chuzan disease (Miura *et al.* 1991, Yamaguchi *et al.* 1999). A serological comparison of Chuzan and Kasba virus was then carried out by Jusa *et al* (1994). It was found that the two viruses could not be distinguished from each other and Chuzan virus was recognized to be the same virus as Kasba, one of the original members of the Palyam serogroup that had been originally isolated in India in 1957. Kasba (Chuzan) virus has been isolated in Japan from 1985 to 2001 (Ohashi *et al.* 2004) and the first isolate from China was reported (Wang *et al.* 2016). Whole blood samples were collected from sentinel cattle throughout China and the primary isolation was made by using a mosquito cell line (Wang *et al.* 2016).

Palyam serogroup viruses were isolated in southern Africa from the 1970's and the early 1980's. The serotypes that are known to occur in Africa are Abadina, Nyabira, Gweru and Marondera (Whistler & Swanepoel 1988, Whistler *et al.* 1989). The Apies River virus also occurs in Africa and was proposed to be a new serotype but it has not been officially recognized as such by the ICTV according to the species demarcation criteria for this serotype (Whistler *et al.* 1989). The species demarcation criteria for orbiviruses include high levels of serological cross-reactions using polyclonal sera or monoclonal antibodies against conserved antigens, efficient cross-hybridization of conserved genome segments and identification of a common vector or host species and the clinical signs produced. Sequence analysis is also important for example in the conserved sequence of Segment 3, viruses of the same species will have >76% nucleotide identity (https://talk.ictvonline.org/taxonomy/). Currently no surveillance is

done for Palyam serogroup viruses in South Africa, and thus no new isolates or possible serotypes have been reported.

1.2.4 Pathogenesis and clinical signs

The Palyam serogoup viruses are associated with abortion and teratogenesis. Congenital abnormalities such as hydranencephaly and cerebral hypoplasia occur and the placenta is often retained after abortion (Swanepoel 2004). Little information is available on the pathogenesis of the disease in cattle that are not pregnant.

Kasba virus was confirmed as the causative agent of hydranencephaly-cerebellar hypoplasia (HCH) syndrome in calves in Japan on the basis of virological and sero-epidemiological investigations as well as experimental infections with pregnant cows (Miura *et al.* 1991). A transient leukopenia and viraemia without fever was observed when cattle were intravenously inoculated with the Kasba virus (Swanepoel 2004, Miura *et al.* 1991). Calves intracerebrally inoculated showed fever, malaise and impairment of mobility, including ataxia, stumbling and falling, circling and colliding against a barrier (Miura *et al.* 1991). They also developed opisthotonus, stiff gait, tremors and nystagmus. The calves appeared to be fully or partially blind and had difficulty in suckling (Miura *et al.* 1991). Kasba virus affects the brain of the bovine fetus, including the cerebrum and cerebellum (Yamaguchi *et al.* 1999) and necropsy findings showed gross lesions of affected calves were restricted to the brain (Miura *et al.* 1991). Hydranencephaly was observed to be the foremost lesion, with degeneration of white matter and calcification of the nervous tissue (Miura *et al.* 1991). Detection of viral antigens in cytoplasm of necrotic nerve cells with morphologically intact cells around the necrotic area when Kasba virus was inoculated into the brains of suckling mice suggested that the virus spreads by cell-to-cell infection in the brain (Yamaguchi *et al.* 1999). Cases of infected animals can be sporadic or part of an epidemic involving one or more herds over a period of time (Swanepoel 2004).

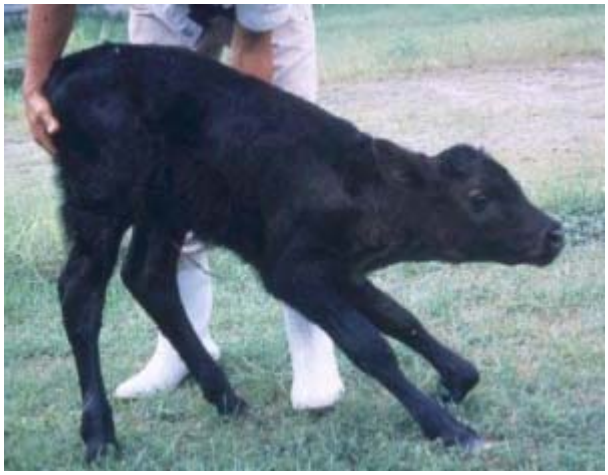


Figure 1.3. A calf with cerebral hypoplasia, characterized by lowered head carriage, a wide-based stance of hind legs and ataxia.
vetbook.org/wiki/cow/index.php?title=Chuzan_virus#cite_ref-1

1.2.5 Diagnosis

1.2.5.1 Samples

Palyam serogroup virus infections are suspected when epidemics of teratogenesis and abortions occur in cattle or other domestic ruminants in the absence of other obvious signs of disease and other known causes of these occurrences have been excluded (Swanepoel 2004). The Simbu serogroup bunyaviruses that cause teratology and bacterial infections as well as Rift Valley fever virus and Wesselsbron disease virus that cause abortions can confound the diagnosis of Palyam serogroup virus infections. Samples that should be submitted for diagnosis are aborted fetuses or tissue samples that include the brain, spleen, liver, lung, kidney and placenta (Swanepoel 2004). Blood or serum from cows that have aborted can also be tested (Goto *et al*. 1988).

1.2.5.2 Virus isolation

Viruses can be isolated from foetal tissue and arthropod vectors, using primary or secondary cell cultures, as well as laboratory animals including suckling mice (Yamaguchi *et al.* 1999). The viruses can be grown in mosquito as well as mammalian cell cultures, obtained from tissue of bovine, porcine, monkey and hamster origin, such as Vero, baby hamster kidney (BHK-21) and rabbit kidney (RK13) cell lines. The viruses produce clear cytopathic effect (CPE) in the mammalian cell lines, the most

distinct in BHK-21 cells (Miura *et al.* 1991). Kasba virus has also been shown to grow well in the suckling mouse brain (Yamaguchi *et al.* 1999) and in hamsters, inducing their death (Miura *et al.* 1991).

1.2.5.3 Serology

Following isolation, viruses can be identified using indirect immunofluorescence with group-specific polyclonal antibodies representative of the serogroup. Neutralization tests with reference antisera to the known serotypes aid in identifying serotypes. Detection of antibodies in foetal sera is largely more successful than virus isolation (Swanepoel 2004). A rapid indirect ELISA for the serogrouping of Australian orbiviruses was developed, which included the four Palyam serotypes that were originally isolated in Australia. Significant inter-serogroup cross-reactions were reported for the Bunyip Creek virus when performing this test (Blacksell *et al.* 1994).

1.2.5.4 Molecular tests

A single-tube nested reverse transcriptase polymerase chain reaction (nRT-PCR) exploiting the Segment 3 gene for the detection of Sudanese and South African isolates of Palyam serogroup viruses was (Aradaib *et al.* 2009), as this had been reported to be the most conserved segment within the viral genome by Yamakawa *et al.* (1999b). The assay was recommended for use in the investigation of outbreaks but the possibility of using the test in practice still needs to be assessed (Aradaib *et al.* 2009). Only the African Palyam serogroup serotypes were included when developing this test and it has not yet been determined whether the other serotypes will also be detected by the assay.

The advent of fluorescent DNA labelling has made the analysis and detection of PCR products in real-time possible. There are fluorescent DNA probes available for the real-time detection of PCR products: TaqMan, Molecular Beacons, and Scorpions. All the probes emit a fluorescent signal that allows for the detection of PCR products. TaqMan probes are oligonucleotides that have a fluorophore attached to the 5' end and a quencher to the 3' end. During the PCR amplification process the probes hybridize to the target sequence and the 5' to 3' exonuclease activity of the Taq polymerase cleaves the fluorescent probe which releases the fluorophore. This breaks the proximity to the quencher and fluorescent signal is emitted. The intensity

of the fluorescent signal increases with the number of cleavage cycles. SYBR Green is also used to quantify amplification of DNA. The fluorescent dye binds to the DNA molecules and the fluorescence is measured at the end of each amplification cycle. The real-time RT-PCR is deemed to be the most sensitive assay for the detection of viral nucleic acid, and currently no data on a real-time assay for the detection of Palyam viruses is available.

The Palyam viruses can also be identified and characterised with the use of sequencing techniques. The demand for accurate high-throughput but low-cost sequencing for diagnostic investigation led to the development of NGS technologies. These technologies have immensely increased the rate of more complete data generation and are therefore broadly used in many different applications, such as whole genome sequencing, transcriptome profiling, DNA-protein interactions and epigenome characterization (Xuan *et al*. 2013).

The Illumina (Solexa) sequencing system is one of the major NGS platforms. It uses an array-based DNA sequencing-by-synthesis technology with reversible terminator chemistry (Xuan *et al*. 2013). During Illumina sequencing the DNA molecules and primers are hybridized to an optically transparent solid surface, such as a slide and amplified with polymerase to form DNA clusters (Xuan *et al*. 2013). Four types of modified nucleotides (reversible terminators), labelled with fluorescent dye, are then added for DNA synthesis. A camera records the labelled nucleotides, after which the dye and terminal 3' blocker is chemically removed in order for the next cycle to start. The DNA chains are extended one nucleotide at a time and are captured by sequential images taken by the camera (Bentley *et al*. 2008).

Full-length amplification of complementary DNA (cDNA) (FLAC) is a technique for the amplification of viral dsRNA genomes without any prior knowledge of sequence information or cloning (Maan *et al*. 2007, Potgieter *et al*. 2009). This technique uses an anchor primer, which is designed to self-prime and thus initiates synthesis of cDNA from a ssRNA template to which it is attached at the 3' end (Maan *et al*. 2007). The cDNA is then amplified using a primer that is complementary to the anchor primer.

NGS has previously been used to generate the full genome of Kasba virus (Wang *et al.* 2016, Yang *et al.* 2016). FLAC was conducted using the purified viral dsRNA, followed by sequencing of the viral genomic cDNA using the Illumina MiSeq platform. The alignment programs BioEdit Sequence Alignment editor (version 6), Mega (version 6) and Clustal X were then used to analyze the sequence data (Wang et al. 2016).

1.2.6 Control

Application of a vaccine would be an effective way of controlling and preventing Palyam serogroup virus infections (Miura *et al.* 1991). An experimental inactivated trivalent vaccine for teratogenic arthropod-borne viruses was developed in Korea in 2011. The vaccine included Aino and Akabane viruses (genus *Orthobunyavirus*, family *Peribunyaviridae*) as well as Chuzan (Kasba) virus (Kim *et al.* 2011, Hechinger *et al.* 2013). Further clinical evaluation of the extent of immunity and prophylactic efficacy of the vaccine in the field still needs to be determined.

The main commercial vaccines available for some of the other orbiviruses, like BTV and AHSV that are used in South Africa are polyvalent, live attenuated vaccines, which include some of the endemic serotypes. The disadvantage of using live attenuated vaccines is that viral serotypes can spread in the field and therefore contribute to reassortment, thereby generating field strains containing sequences were not generated naturally (Nomikou *et al.* 2015). Recombinant vaccines are safer but not commercially available yet (Kim *et al*. 2011).

Currently there are no control strategies in place for Palyam serogroup virus infections in South Africa and new epidemiological studies need to be done to determine the current endemic status of these viruses.

1.3 JUSTIFICATION AND AIM OF THE STUDY

Limited serosurveillance of the Palyam serogroup of viruses has been done in South Africa, the most recent study was by Whistler *et al* in 1989. Therefore the current endemic status and disease potential of many viruses within this serogroup are unknown (Aradaib *et al.* 2009). Consequently, the possible impact these viruses might have on the cattle and wildlife industry in South Africa is undetermined. Since these

viruses have an effect on the reproductive performance of domestic animals and can cause significant loss of cattle (Aradaib *et al.* 2009), it is of economic importance to investigate their genetic and molecular properties. Disease outbreaks could result in the restriction in international trade of livestock (Aradaib *et al.* 2009, Yamakawa & Furuuchi 2001). Palyam virus infections might also be misdiagnosed as many other viruses and bacteria also cause abortions. Although there is molecular data available for certain of the genome segments of the Palyam viruses from Australia and Zimbabwe (Ohashi *et al.* 2004, Yamakawa *et al.* 2000), the full-genome sequence of only one serotype (Kasba) is known (Wang *et al.* 2016, Yang *et al.* 2016).

The main aim of this study was to sequence the full genomes of known members of the Palyam serogroup for which the full genome sequence data is not yet available. This would allow their phylogenetic relationships to be determined as well as their relationship to other members of the *Orbivirus* genus. The second goal was to use the newly generated sequence data to improve on currently available molecular tests by developing a more broadly inclusive generic molecular diagnostic assay to detect Palyam serogroup viruses.

In order to achieve the aims of the study, four specific objectives were formulated.

- To propagate the thirteen Palyam serotypes and selected field isolates of the African serotypes and amplify their genome segments.
- To obtain the full genome sequences of all viruses propagated using NGS.
- To perform phylogenetic analysis of the full genome sequence data that was obtained.
- To confirm the most conserved segment of the virus and design primers to detect the newly characterised Palyam serogroup viruses in a generic real-time RT-PCR assay.

CHAPTER 2

MOLECULAR CHARACTERISATION OF THE SEROTYPES OF THE PALYAM SEROGROUP AND SELECTED FIELD STRAINS

2.1 INTRODUCTION

Sequence data, especially complete genomic data for the Palyam serogroup of orbiviruses are generally lacking, with Kasba being the only serotype for which complete genome sequences are available. Obtaining complete sequence data for all the serotypes will give better insight into the genetic heterogeneity of serotypes and their relationship to other orbiviruses. Sequence data can be used to determine reassortment and recombination, to look at topotypes and the occurrence of the different serotypes in southern Africa and elsewhere and to determine the source of outbreaks. Improving diagnostic assays can make it possible to more accurately determine current prevalence. Control of the disease with the possible development of vaccines, if needed, can thus be established.

Next generation sequencing is commonly used for the whole genome sequencing of orbiviruses. The Illumina platform has been used successfully to obtain complete genome sequences for other orbiviruses such as AHSV (Guthrie *et al*. 2015) and BTV (Qin *et al*. 2018, van den Bergh *et al*. 2016, Caporale *et al*. 2014). For example, the BTV study by van den Bergh *et al*. (2016) obtained full genome sequences for BTV strains used in commercial vaccines (serotypes 3, 8, 9, 10, 11), BTV serotype 4 and a reassortant strain. The sequence data obtained for orbiviruses has demonstrated that this genus is susceptible to reassortment. The full genome sequences for BTV serotype 21 obtained with the Illumina sequencing provided evidence for genetic reassortment with serotype 16 (Qin et al. 2018). Studies have shown that live-attenuated BTV vaccine strains in Europe have reassorted with field strains (Nomikou *et al.* 2015) and the same has been revealed for AHSV field and vaccine strains in South Africa (Weyer *et al.* 2016). Sequence data has also confirmed the reassortment within serotypes of EHDV (Anbalagan *et al.* 2014).

Phylogenetic analysis of the Palyam serogroup of orbiviruses has mainly been focused on the Kasba virus. The Illumina platform was used to obtain sequence reads in the study by Yang *et al.* (2016), where the full sequence of the Chinese Chuzan (Kasba) strain, SZ187 was generated. Genetic diversity has been investigated for serotypes originating from Japan, Australia and Zimbabwe (Palacios *et al*. 2011, Ohashi *et al.* 2004, Yamakawa *et al*. 2000) however these studies focused on segments coding for VP1, VP2, VP6, VP7 and NS1 and further characterisation has been performed for VP2, VP3, VP5 and VP7 of Kasba virus (Wang *et al* 2016, Yamakawa *et al*. 1999a).

The characterisation of VP2, VP3, VP5 and VP7 of Kasba virus clarified the phylogenetic relationship of this virus with the other orbiviruses. The VP3 proteins of Palyam viruses are highly conserved (Yamakawa *et al*. 1999b, Aradaib *et al.* 2009) and phylogenetic analysis of VP7 indicated that the Palyam serogroup viruses can be divided into three groups according to their place of isolation (Wang *et al*. 2016). The viruses that were analysed were Japanese and Taiwanese isolates, Australian isolates and African isolates that were divided into a Japanese/East Asian group, Australian group and Zimbabwean group, respectively.

In this study the aim was to obtain the full genome sequences of the different known Palyam seogroup serotypes, the putative Apies River serotype, as well as selected field isolates from Africa in order to perform genome-wide phylogenetic analysis. Currently only the full genome sequence of the Kasba serotype is available. The phylogenetic analysis would enable a better understanding of the genomic features of the Palyam serogroup of orbiviruses, their relation to each other as well as to other orbiviruses, including inter- and intra-serotype diversity.

2.1 MATERIALS AND METHODS

2.2.1 Samples

The Palyam serogoup viruses used in this study were obtained from the collection of Prof R Swanepoel and the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria. The prototype viruses were originally obtained from the Yale Arbovirus Research Unit, New Haven, Conn., USA, except for Apies River, Gweru, Marondera and Nyabira viruses. All the field isolates had been isolated from Zimbabwe and the Apies River virus from Gauteng Province, South Africa. The viruses had been isolated from various sources and at different times and locations, as indicated in Table 2.1. After initial isolation and identification, the isolates had been freeze-dried and stored at 4°C in DVTD.

Table 2.1. Palyam virus isolates used in the study

Serotype	Strain designation	Date freeze-dried	Country of isolation	Source of isolation	Date of initial isolation
Prototypes					
Abadina	Ib Ar 22388	04/11/1987	Nigeria	*Culicoides* spp.	1967
Bunyip Creek	CSIRO 87	20/10/1987	Australia	*Culicoides schultzei*	05/03/1976
CSIRO Village	CSIRO 11	30/03/1990	Australia	*Culicoides* spp.	20/11/1974
D'Aguilar	B8112	23/10/1987	Australia	*Culex brevitarsus*	02/04/1968
Kasba	GG15534	Unknown	India	*Culex vishnui*	19/07/1957
Marrakai	CSIRO 82	13/07/1989	Australia	*Culicoides* spp.	01/10/1975
Palyam	DVTD collection	27/05/1992	India	Unknown	Not available
Petevo	Ar B 2032	27/06/1989	Central African Republic	*Amblyomma variegatum*	01/07/1978
Vellore	68886	06/07/1989	India	*Culex pseudovishnui*	18/07/1956
Apies River	O/4518	14/11/1990	South Africa	Cow blood	20/03/2005
Gweru	VRL1726/76	29/10/1986	Zimbabwe	Bovine foetus	28/05/1976

Marondera	VRL1070/78	28/05/1980	Zimbabwe	Cow viscera	1978
Nyabira	VRL792/73	28/03/1973	Zimbabwe	Bovine foetus	22/02/1973
Field isolates					
Abadina 2582	2582/78	09/03/1978	Zimbabwe	Bovine foetus	1978
Abadina 2389	2389/76	14/03/1990	Zimbabwe	Bovine foetus	1976
Nyabira 1772	1772/74	01/11/1990	Zimbabwe	Bovine foetus	1974
Nyabira 4646	4646/74	26/05/1980	Zimbabwe	Bovine foetus	1974
Gweru 1828	1828/82	09/03/1990	Zimbabwe	Bovine foetus	1982
Gweru 1832	1832/79	03/11/1986	Zimbabwe	Bovine foetus	1979
Gweru 2038	2038/76	08/05/1989	Zimbabwe	Bovine foetus	1976
Gweru 1856	1856/76	16/03/1990	Zimbabwe	Bovine foetus	1976
Gweru 1619	1619/82	16/06/1989	Zimbabwe	Bovine foetus	1682

2.2.2 Virus propagation

Confluent monolayers of Vero (African green monkey kidney) cells were prepared in 25 cm^2 cell culture flasks (Corning®). Eagles medium (Biochrom®) containing 5% foetal bovine serum (Gibco®, ThermoFisher Scientific) and 1 mg/ml gentamycin (Virbac®) was used to propagate the cells. The virus isolates were reconstituted in 500 µl cell culture medium and 200 µl of the freeze-dried samples were inoculated onto the cell monolayers after which the culture flasks were incubated at 37°C. The cells were observed daily for evidence of CPE. When 100% CPE was observed (all of the cells in the monolayer affected or damaged), a 500 µl aliquot of the supernatant was taken and inoculated onto freshly prepared monolayers of Vero cells. The culture flasks were incubated again and observed daily for CPE. When 100% CPE was visible, the medium and cell debris were harvested and stored at -80°C until used.

2.2.3 Whole genome sequencing

2.2.3.1 RNA extraction

The cell culture material was centrifuged using a Heraeus benchtop centrifuge with rotor 75003607 (Thermo Fisher Scientific®) at 2 000 rpm for 2 minutes to pellet the cell debris. The pellets were collected and dissolved in 100 µl supernatant and 900 µl Trizol™ (Invitrogen®) was added to obtain a final volume of 1000 µl. The samples were mixed, 200 µl chloroform was added and mixed again. To obtain phase separation samples were centrifuged at 14 000 rpm for 10 minutes and the clear supernatant was collected. A volume of 500 µl isopropanol was added to the supernatant and solution was mixed by vortexing. Total RNA was pelleted by centrifugation at 14 000 rpm for 30 minutes at 4°C (Eppendorf 5430 bench-top centrifuge, FA-45-30-11, Merck®). All the liquid was then removed, and the pellet was dissolved in 90 µl of high performance liquid chromatography (HPLC) grade water (Sigma-Aldrich®).

To remove the single-stranded RNA (ssRNA), 30 µl of lithium chloride (LiCl) was added to the samples and incubated at 4°C overnight. The samples were centrifuged at 14 000 rpm for 60 minutes to pellet the ssRNA and the supernatant containing the double-stranded RNA (dsRNA) was transferred to a clean tube.

The dsRNA was purified using the MinElute Gel Extraction Kit (QIAGEN®) according to the manufacturer's instructions. Briefly, 120 µl dsRNA was added to 330 µl of the QG buffer (QIAGEN®), vortexed and placed in spin columns. The columns were centrifuged for 1 minute at 14 000 rpm (Eppendorf 5430 bench-top centrifuge, FA-45-30-11, Merck®). The flow-through was discarded and 750 µl PE buffer (QIAGEN®) was placed in the columns, after which the columns were centrifuged at 14 000 rpm for 1 minute. The flow-through was discarded and the samples were centrifuged for another minute at the same speed. The columns were placed in new tubes, 32 µl EB (QIAGEN®) buffer was added and left at room temperature for 1 minute. The samples were subsequently centrifuged at 14 000 rpm for 2 minutes. A total volume of 5 µl of the eluted RNA was loaded on a 2% agarose (Lasec, South Africa) gel, stained with Ethidium bromide (Sigma-Aldrich®) and visualized for the presence of viral dsRNA. Gels were visualized using the ChemiDoc™ XRS+ molecular imager with Image Lab™ Software (BIO-RAD®, USA).

2.2.3.2 FLAC

Full-length amplification of cDNA (FLAC) was performed as described by Maan *et al*. (2007) and Potgieter *et al*. (2009). The components of the buffers that were prepared for the reactions are listed in Appendix A.

Ligation

Ligation reactions were prepared for all the samples. The reactions contained 3 µl of the prepared ligation buffer (Appendix A), 3 µl dimethyl sulfoxide (DMSO), 10 µl 60% poly ethylene glycol (PEG) 600 (Sigma-Aldrich®), 1 µl of T4-ligase, 1 µl of primer PC3-T7 at 300 ng/ µl and 12 µl of the dsRNA template. The PC3-T7 loop primer (5'p – GGA TCC CGG GAA TTC GGT AAT ACG ACT CAC TAT ATT TTT ATA GTG AGT CGT ATT A – OH3'), manufactured by Roche® (South Africa), served as a primer anchor for the sequence-independent PCR reaction. The reactions were left overnight at 37°C. The ligation products were purified using the MinElute Gel Extraction Kit (QIAGEN®), according to the manufacturer's instructions.

cDNA synthesis

The ligated dsRNA was denatured by adding 2 µl 300 mM methyl mercury hydroxide (MMOH) (Alfa Aesar, Thermo Fisher Scientific®) to 10 µl of each sample and incubated at 37°C for 30 minutes in a Applied Biosystems 2720 thermal cycler. Then 2 µl of 1M β-mercapto-ethanol (Sigma-Aldrich®) was added to reduce the MMOH.

Reactions for the synthesis of cDNA contained 8 µl of the denatured RNA, 3,5 µl cDNA buffer (Appendix A), 16 µl deoxynucleotides (2.5 mM dNTP's), 1 µl 1 M β-mercapto-ethanol, 0,5 µl RNAse inhibitor, and 1 µl (15 U) avian myeloblastosis virus reverse transcriptase (AMV-RT) to make up a total volume of 30 µl. The reactions were incubated in a 2720 thermal cycler (Applied Biosystems®) at 42°C for 60 minutes, followed by 15 minutes at 50°C.

In order to degrade the RNA still present in the reaction tubes, 3 µl 1N NaOH was added and the samples were incubated at 65°C for 20 minutes. A volume of 3 µl 1N Tris was added to buffer the reagents, followed by 3 µl 1N HCl to neutralize the NaOH.

The tubes were then incubated at 65°C for 2 hours to allow for hybridization of single-stranded DNA into double-stranded duplexes.

PCR amplification

PCR reactions were prepared for each sample. Each reaction had a total volume of 45 µl and consisted of 34,5 µl H_2O, 5 µl 10x Ex TAQ buffer (Takara®), 4 µl 2,5 nM dNTP's, 0,5 µl Ex TAQ enzyme (Takara®), 1 µl PC2 primer (5'p – CCG AAT TCC CGG GAT CC – OH3') which is complementary to the PC3-T7 loop primer, and 5 µl cDNA.

The PCR programme used consisted of 4 minutes at 72°C, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 4 minutes, then a final stage of 72°C for 5 minutes.

The PCR products were purified using a MinElute Gel Extraction Kit and eluted in 23 µl HPLC water. The concentration of the DNA was determined using a Qubit™ 2.0 Fluorometer (Life technologies®) with the aid of the DNA broadrange assay kit (Invitrogen®) to confirm sufficient material for sequencing.

2.2.3.3 NGS

The DNA amplicons obtained during the FLAC assay were sent to Inqaba Biotech® (Pretoria, South Africa) for sequencing. The amplicons were sequenced on an Illumina® Mi-Seq sequencer, using the Nextera XT DNA sample preparation kit and 300-bp paired-end V3 Illumina chemistry.

2.2.4 Phylogenetic analysis

2.2.4.1 Nucleotide sequences

Sequence data generated by Illumina sequencing were analyzed using the CLC Genomics Main workbench, version, 8.0.1 (http://www.clcbio.com/). *De novo* assembly of sequence reads was performed and contig sequences prepared. The contigs were aligned with sequences on the National Centre for Biotechnology (NCBI, http://www.ncbi.nlm.nih.gov/) database by the Basic Local Alignment Search Tool (BLAST) for verification of the different gene sequences. The reads were mapped using the contigs as reference sequence. Where sequences were mapped to a

reference sequence, a consensus was extracted. Translation was done for all the sequences to ensure that ORF's were maintained.

The sequences for all 10 genes of all the serotypes were aligned separately, together with an outgroup, AHSV. Concatenated genes were also constructed for all the serotypes, including the field isolates as well as the four other orbiviruses: AHSV, BTV, EEV and EHDV (Accession numbers in Appendix B). The sequences for the other orbiviruses were obtained from GenBank (NCBI nucleotide database, https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned and pairwise comparisons created for each gene, using the CLC Genomics Main workbench. The distance between the sequences was calculated for each gene as well as the percentage sequence identities.

2.2.4.2 Amino acid sequences

The ORF's were identified for the different gene sequences of all the serotypes and the sequences were translated into proteins. The protein sequences were then aligned and pairwise comparisons generated for each gene, using the CLC Genomics Main workbench. The distance between sequences was calculated for each gene as well as the percentage sequence identities.

2.2.4.3 Phylogenetic trees

All sequences were aligned using the online version of MAFFT (https://mafft.cbrc.jp/alignment/server/), using default parameters. The aligned matrix were viewed, edited and truncated using MEGA X (Kumar *et al.* 2018). The alignments were converted into nexus and phylips files using the online Format Converter Tool (www.hiv.lanl.gov). Data-display networks (neighbour-networks) were constructed with SplitsTree 4 (Huson & Bryant, 2005). Support values calculated were based on 1000 bootstrap replicates and the networks were based on uncorrected p-distances using all characters.

The phylips files were used to initiate model estimation via jModel test2 (Darriba *et al*. 2015), by using the online portal Cipres Science gateway (Miller *et al*. 2015). Bayesian analyses was performed in MrBayes version 3 (Ronquist & Huelsenback, 2003). The data for the concatenated sequences was partitioned into gene regions, unlinked with

previously estimated models assigned to the appropriate partitions. The probability density rates were set to a flat dirichlet and using the Markov Chain Monte Carlo (MCMC) method four chains were run simultaneously for 1 million generations. Trees were sampled and saved for every 500th generation and 10% of the trees sampled were discarded as burn-in. Posterior probabilities were calculated from the remaining saved majority rule consensus trees and a value of 0.95 was chosen as statistically significant (Harrison & Langdale 2006). Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer) was used to investigate the effective sample size (ESS) of the Bayesian analysis, where values > 200 were viewed as an appropriate sample size. RaXML was employed for a maximum likelihood analysis of the same partitioned dataset. GTR models were used for analysis including either gamma distribution, or invariable sites, or both, as estimated by jModel Test. The autoMRE function was invoked for bootstrap calculations. Bootstrap values from the ML analysis were superimposed on the phylogeny recovered from the Bayesian analysis.

2.3 RESULTS

2.3.1 NGS

Of the 23 freeze-dried virus isolates, four did not grow in cell culture. These samples included Vellore, Abadina 2389, Gweru 1619 and Gweru 866. In these cases, RNA extraction was done directly form the freeze-dried material. In total, the FLAC amplification protocol was successful for 20 of the 23 samples (Figure 2.1). Nyabira 4646, Gweru 1619 and Gweru 866 were not submitted for sequencing after failed attempts at FLAC and were consequently excluded from the sample panel. The remaining 20 samples were sent for sequencing.

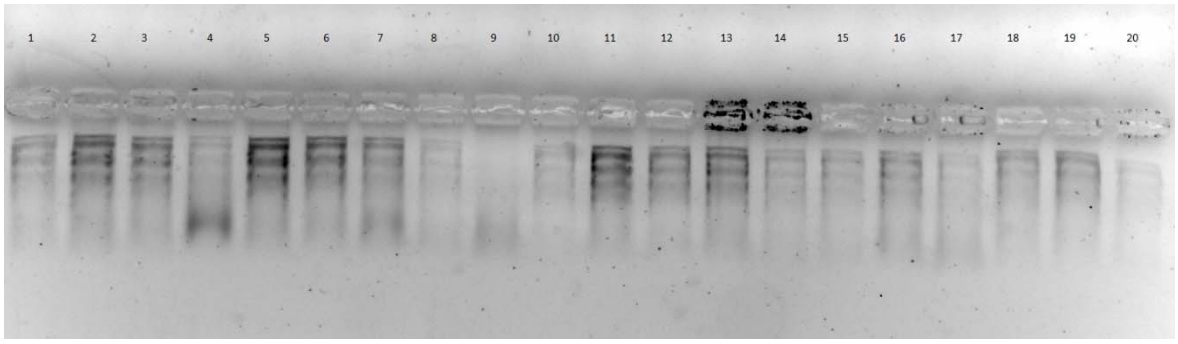


Figure 2.1. Extracted RNA visualized on a 2% agarose gel. In Lanes 1 to 13 are the prototype serotypes and Lanes 14 to 20 are the field isolates.

2.3.2 Phylogenetic analysis

2.3.2.1 Nucleotide sequences

Complete sequences were obtained for all the genes of the viruses submitted, except for Segment 1 of Vellore and Nyabira 1772. The Segment 1 sequence for Vellore had a gap of 46 bp, from position 2351 to 2397, from the total of 3930 bp, in regard to the reference sequence.. There was not sufficient sample left to repeat the sequencing for Vellore as the extraction had to be done directly from the freeze-dried material. The sequence for Nyabira 1772 had 3 large sections in the sequence, with only 2088 bp available of the total 3930 bp. All the Segment 1 sequences except for Nyabira 1772 were submitted to GenBank (NCBI nucleotide database, https://www.ncbi.nlm.nih.gov/genbank/) the accession numbers are given in

Table 2.2. The partial Segment 1 sequence of the Nyabira 1772 was included in the dataset for phylogenetic analysis.

Table 2.2. Accession numbers of Palyam virus sequences submitted to GenBank

Virus isolate	Segment 1 (VP1)	Segment 2 (VP2)	Segment 3 (VP3)	Segment 4 (VP4)	Segment 6 (VP5)	Segment 9 (VP6)	Segment 7 (VP7)	Segment 5 (NS1)	Segment 8 (NS2)	Segment 10 (NS3)
Abadina	MH782454	MH823477	MH817078	MH817097	MH817117	MH823377	MH823397	MH823417	MH823437	MH823457
Bunyip Creek	MH782455	MH823478	MH817079	MH817098	MH817118	MH823378	MH823398	MH823418	MH823438	MH823458
CSIRO Village	MH782456	MH823479	MH817080	MH817099	MH817119	MH823379	MH823399	MH823419	MH823439	MH823459
D'Aguilar	MH782457	MH823480	MH817081	MH817100	MH817120	MH823380	MH823400	MH823420	MH823440	MH823460
Kasba	MH782458	MH823481	MH817082	MH817101	MH817121	MH823381	MH823401	MH823421	MH823441	MH823461
Marrakai	MH782459	MH823482	MH817083	MH817102	MH817122	MH823382	MH823402	MH823422	MH823442	MH823462
Palyam	MH782460	MH823483	MH817084	MH817103	MH817123	MH823383	MH823403	MH823423	MH823443	MH823463
Petevo	MH782461	MH823484	MH817085	MH817104	MH817124	MH823384	MH823404	MH823424	MH823444	MH823464
Vellore	MH782462	MH823485	MH817086	MH817105	MH817125	MH823385	MH823405	MH823425	MH823445	MH823465
Apies River	MH782463	MH823486	MH817087	MH817106	MH817126	MH823386	MH823406	MH823426	MH823446	MH823466
Gweru	MH782464	MH823487	MH817088	MH817107	MH817127	MH823387	MH823407	MH823427	MH823447	MH823467
Marondera	MH782465	MH823488	MH817089	MH817108	MH817128	MH823388	MH823408	MH823428	MH823448	MH823468
Nyabira	MH782466	MH823489	MH817090	MH817109	MH817129	MH823389	MH823409	MH823429	MH823449	MH823469
Abadina 2582	MH782467	MH823490	MH817091	MH817110	MH817130	MH823390	MH823410	MH823430	MH823450	MH823470
Abadina 2389	MH782468	MH823491	MH817092	MH817111	MH817131	MH823391	MH823411	MH823431	MH823451	MH823471
Gweru 1828	MH782469	MH823492	MH817093	MH817112	MH817132	MH823392	MH823412	MH823432	MH823452	MH823472
Gweru 1832	MH782470	MH823493	MH817094	MH817113	MH817133	MH823393	MH823413	MH823433	MH823453	MH823473
Gweru 2038	MH782471	MH823494	MH817095	MH817114	MH817134	MH823394	MH823414	MH823434	MH823454	MH823474
Gweru 1856	MH782472	MH823495	MH817096	MH817115	MH817135	MH823395	MH823415	MH823435	MH823455	MH823475
Nyabira 1172	Not submitted	MH823496	MK007563	MH817116	MH817136	MH823396	MH823416	MH823436	MH823456	MH823476

The nucleotide sequence identity for the concatenated dataset of the Palyam serogroup serotypes and field isolates ranged between 77.14% and 97.22% (Appendix C). The percentage sequence identity for the Gweru prototype and Gweru field isolates ranged between 99.41% and 99.79%, showing that the isolates are closely related. The sequence identity between the Nyabira prototype and the Nyabira field isolate was 96.38% and the range for the Abadina samples was between 99.77% and 99.78%. The nucleotide content of each gene of the Palyam serogroup viruses analysed is presented in Appendix D.

2.3.2.2 Amino acid sequences

The amino acid sequence identities ranged between 77.23% and 100% for all the genome segments of the Palyam serogroup serotypes (Appendix C), with the exception of Segment 2. This segment, coding for VP2, showed the greatest variation, with a percentage sequence identity ranging between 35.26% and 99.9% (Table 2.3). The viruses with the most similar sequences for VP2 were Gweru and CSIRO Village (99.9%), Marondera and Apies River (98.72%) and Vellore and Kasba (98.11%). Palyam and Petevo showed the biggest variation in sequence identity (35.26%).

VP5 (Segment 6) was second to VP2 in extent of variation in sequence identity (77.23 – 100%) (Appendix C). Vellore and Kasba had identical sequences for VP5, as did Marondera and Apies River viruses. The percentage sequence identity for VP6 (Segment 9) ranged between 85.3% and 100%, with Vellore and Kasba displaying the same amino acid sequence. The range was from 89.1% to 100% for NS2 (Segment 8), with Gweru and Nyabira being identical.

The amino acid sequences for VP7 were the most conserved of all the proteins across the serogoup, with percentage sequence identity ranging between 97.75% and 100% (Table 2.4). The sequences for Abadina, Nyabira, Apies River and Marondera were identical. Bunyip Creek, D'Aguilar and CSIRO Village also had identical sequences. The segment coding for VP3 was also highly conserved (96.81 – 100 % sequence identity) (Appendix C). Gweru, Nyabira and Marondera had the same amino acid sequences for VP3.

The percentage sequence identity for VP1 ranged from 92.71% to 99.54% and the range for NS1 was between 89.1% and 100%. Apies River, Marondera and Nyabira had the same amino acid sequence for NS1. VP4 (Segment 4) had a sequence identity ranging from 92.08% to 99.53% and NS3 (Segment 10) had a range between 91.28% and 100%. The identical sequences for NS3 were for Gweru, Nyabira and Abadina.

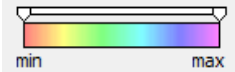
Table 2.3. Pairwise comparison of VP2 proteins of the Palyam serotypes. The top shows distance and the bottom percentage identity.

		1	2	3	4	5	6	7	8	9	10	11	12	13
Kasba_VP2	1		0.02	0.14	0.64	0.64	0.83	0.78	0.89	0.91	0.89	0.90	0.92	0.90
Vellore_VP2	2	98.11		0.16	0.66	0.66	0.85	0.79	0.90	0.92	0.90	0.91	0.93	0.91
Abadina_VP2	3	86.68	85.19		0.65	0.65	0.84	0.79	0.92	0.94	0.91	0.94	0.95	0.94
CSIRO_Village_VP2	4	52.25	51.07	51.95		0.00	0.80	0.80	0.95	0.96	0.96	1.00	1.01	0.98
Gweru_VP2	5	52.25	51.07	51.95	99.90		0.80	0.80	0.95	0.96	0.96	1.00	1.01	0.98
Marrakai_VP2	6	43.01	42.33	42.72	44.80	44.80		0.63	0.95	0.95	0.92	0.95	0.97	0.95
Petevo_VP2	7	44.42	43.84	43.94	44.31	44.31	52.85		0.97	0.98	0.93	0.99	1.01	0.99
Apies_River_VP2	8	40.23	39.85	39.07	38.25	38.25	38.48	37.29		0.01	0.29	0.73	0.75	0.72
Marondera_VP2	9	39.75	39.36	38.59	38.15	38.15	38.48	37.00	98.72		0.29	0.73	0.76	0.73
Bunyip_CreeK_VP2	10	40.33	39.65	39.26	37.86	37.86	39.25	38.45	74.26	74.17		0.71	0.74	0.71
D'Aguilar_VP2	11	39.96	39.57	38.40	36.70	36.70	37.91	36.14	47.37	46.98	47.95		0.03	0.04
Palyam_VP2	12	38.80	38.41	37.63	35.85	35.85	36.83	35.26	46.30	45.91	46.78	95.82		0.07
Nyabira_VP2	13	39.86	39.38	38.30	37.38	37.38	37.91	36.24	47.56	47.08	48.14	95.99	92.23	

Table 2.4. Pairwise comparison table of VP7 proteins of the Palyam serotypes. The top shows distance and the bottom percentage identity.

		1	2	3	4	5	6	7	8	9	10	11	12	13
Marrakai_VP7	1		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01
Nyabira_VP7	2	99.72		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
Apies_River_VP7	3	99.72	100.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
Abadina_VP7	4	99.72	100.00	100.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
Kasba_VP7	5	100.00	99.72	99.72	99.72		0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01
Marondera_VP7	6	99.72	100.00	100.00	100.00	99.72		0.00	0.01	0.01	0.01	0.01	0.01	0.01
Gweru_VP7	7	99.44	99.72	99.72	99.72	99.44	99.72		0.01	0.01	0.01	0.01	0.01	0.02
CSIRO_Village_VP7	8	98.87	98.87	98.87	98.87	98.87	98.87	98.59		0.00	0.00	0.01	0.01	0.02
Bunyip_Creek_VP7	9	98.87	98.87	98.87	98.87	98.87	98.87	98.59	100.00		0.00	0.01	0.01	0.02
D'Aguilar_VP7	10	98.87	98.87	98.87	98.87	98.87	98.87	98.59	100.00	100.00		0.01	0.01	0.02
Petevo_VP7	11	99.72	99.44	99.44	99.44	99.72	99.44	99.15	99.15	99.15	99.15		0.01	0.01
Palyam_VP7	12	99.15	98.87	98.87	98.87	99.15	98.87	98.59	98.87	98.87	98.87	99.44		0.02
Vellore_VP7	13	98.87	98.59	98.59	98.59	98.87	98.59	98.31	97.75	97.75	97.75	98.59	98.03	

Colour key for percentage identity



Colour key for distance

2.3.2.3 Phylogenetic trees

The final concatenated sequence matrix comprised 20 ingroup taxa, four outgroup taxa and 20775 nucleotides from 10 genes (Table in matrix with dimensions of the different gene regions). Similar topologies were recovered from the Splitstree (DDN) and Bayesian (MrB) analysis (Figures 2.2 and 2.3), both with higher support in comparison to the ML analysis. The phylogenetic tree constructed from the concatenated dataset (Figure 2.3) showed a common ancestor between the Palyam serogroup viruses and AHSV. When looking at the other orbiviruses used as outgroups in this study, it was evident that the orbiviruses all shared a common ancestor, but that AHSV was the closest related to the Palyam serogroup viruses and EEV more distantly related. The Palyam serogroup viruses formed a monophyletic clade with strong support from the MrB analysis (pp: 1), with the outgroups in the DDN separated as a clade with a bootstrap value of 100.

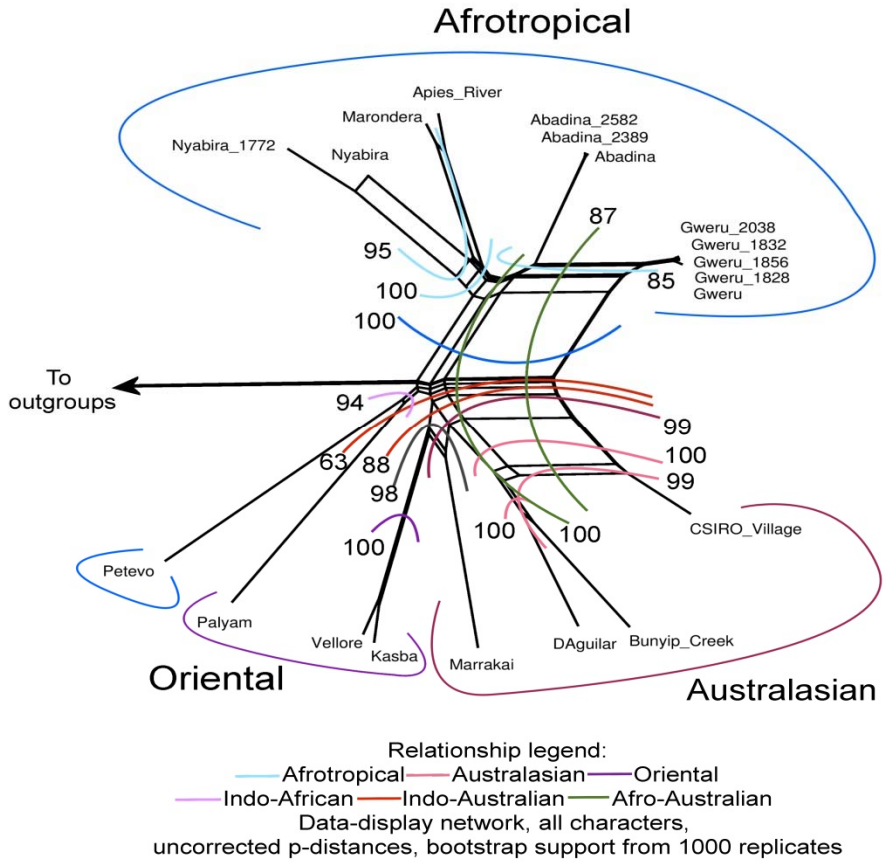
The Afrotropical serotypes, excluding Petevo, formed a well-supported monophyletic clade (Figures 2.3 & 2.3; DDN: 100, MrB: 1). Within the clade, Apies River and Marondera were recovered as closely related sister taxa (DDN: 100, ML: 100, MrB: 1). Nyabira was recovered as either sister to Apies River and Marondera (DDN: 100), or sister to the remaining Afrotropical taxa (ML: 95, MrB: 0.93). Abadina and Gweru represent the most derived sister relationship (DDN: 85, ML: 77, MrB: 1). Together the Oriental and Australasian serotypes formed a second monophyletic clade (DDN: 63, MrB: 1). Petevo, an Afrotropical serotype fell within this clade in both the ML and Bayesian analysis. A monophyletic Australasian clade was recovered with the DDN (bs: 99), in contrast to the topologies recovered from the ML and Bayesian analysis where Kasba, Vellore and Petevo fell within the Australasian group. Kasba and Vellore are closely related sister taxa with strong support across all methods (DDN: 100, ML: 100: MrB: 1). Marrakai seems to be a probable sister group to Kasba and Vellore (DDN: 98, MrB: 1), however, Marrakai was covered as a sister to Petevo virus in the phylogenetic analysis, not supported by the DDN.

Table 2.5: Data characteristics and model estimations of all used gene regions

Gene	Length[1]	Best fit model (AIC)	Gamma value	Invariable site value
NS 1	1941	TIM2 + G	0.3160	NA
NS 2	1272	GTR + G	0.2420	NA
NS 3	869	TPM2uf + G	0.2850	NA
VP 1	4070	GTR + G	0.1730	NA
VP 2	3564	GTR + G	0.7650	NA
VP 3	2802	TIM2 + G	0.1710	NA
VP 4	2044	TIM2 + G	0.2290	NA
VP 5	1667	GTR + I + G	0.5780	0.2510
VP 6	1334	GTR + G	0.2700	NA
VP 7	1212	TVM + G	0.2000	NA

[1] Length of gene region in nucleotide positions in final aligned matrix, including gaps and/or missing data.

The placement of Petevo and Palyam virus remains uncertain with the two isolates forming a sister group in the DDN (bs: 94). Petevo fell within the Oriental and Australiasian clade in the MBayes topology whereas Palyam was a sister to the Oriental and Australasian clade in the DDN. From the DDN, CSIRO Village shared some similarities with the Afrotropical serotypes, with clade support with Gweru (bs: 87) and Gweru and Abadina (bs: 100).



.

Figure 2.2. Data-display network for all the serotypes of the Palyam serogroup used in this study.

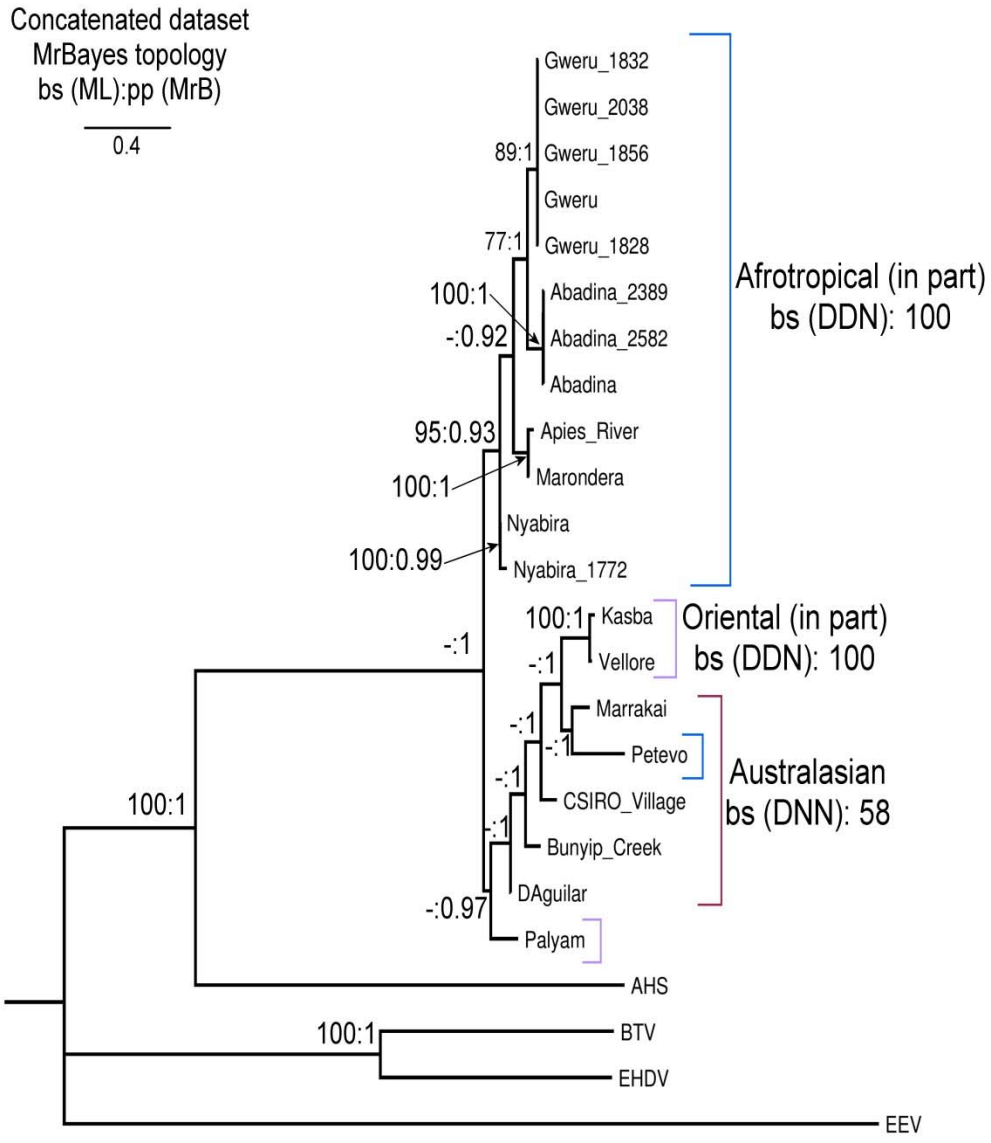


Figure 2.3. Phylogenetic tree for the concatenated dataset of the Palyam serogroup of orbiviruses used in this study

Bootstrap values from the Maximum Likelihood analysis were superimposed on the posterior probabilities gained from Bayesian analysis (bs:pp)

2.4 DISCUSSION

Sequence data for the 13 reference strains of the Palyam serogroup viruses is limited and the complete sequence of only one serotype, Kasba virus, is available. Sequences are available for certain segments of the Australian and African serotypes (Wang *et al.* 2016, Yang *et al.* 2016, Ohashi *et al.* 2004, Yamakawa *et al*. 2000). The availability of sequence data for all the serotypes of the Palyam serogoup viruses will allow the genetic heterogenicity of this group of viruses to be investigated. Reassortment, a characteristic of all orbiviruses, as well as intragenic recombination can be determined which could lead to the identification of phenotypically different viruses.

In this study NGS was used to obtain full genome sequences for 12 of the 13 recognized serotypes of the Palyam serogoup. The newly generated sequences allowed a more complete phylogenetic analysis of the different serotypes and their relationship to the other orbivirusesto be performed. Sequences for field isolates of the Gweru, Abadina and Nyabira serotypes were also obtained and included in the phylogenetic analysis.

The FLAC assay, as described in previous studies on orbiviruses (Maan *et al*. 2007, Potgieter *et al*. 2009). was successfully used to obtain complete sets of cDNA amplicons for the full-length sequences of the Palyam dsRNA genomes without prior knowledge of sequence information or cloning. The specifically designed anchor-primer for orbiviruses (PC3-T7) used in the FLAC method improves the specificity of first-strand cDNA synthesis. Because there are no free primer molecules, mis-priming is prevented, and full-length cDNA's are amplified from all the genome segments simultaneously (Maan *et al*. 2007). This technique has successfully been used for the orbiviruses AHSV, BTV and EEV as reported by Potgieter *et al.* (2009). The method was also used by Yang *et al.* (2016) to successfully obtain the complete sequence for the Kasba serotype of Palyam virus.

Although Illumina sequencing offers a high-throughput platform with 98% accuracy, it is limited to short read lengths (Xuan *et al*. 2013; Bentley *et a*l. 2008). Base calling errors occur, and this increases with read length. Uneven read coverage has also

been reported across AT-rich and GC-rich regions with a bias towards the latter (Xuan *et al*. 2013). The error rates increase toward the ends of the reads (Xuan *et al*. 2013). Even with these disadvantages, sequencing on the Illumina MiSeq sequencer has been used successfully to obtain the full genome sequences for orbiviruses as reported by Guthrie *et al.* (2015) for AHSV sequences and various studies on BTV (Qin *et al*. 2018, van den Bergh *et al.* 2016, Caporale *et al.* 2014). It was also used to obtain a complete genome sequence for a Kasba virus strain (Yang *et al*. 2016).

Since the complete genome sequence of only one serotype was available, several of the genomes had to be reconstructed in the absence of representative reference sequences. Because of this *de novo* assembly of the reads had to be done. During *de novo* assembly all the information in the reads are assembled into simple contig sequences and then the reads are mapped using the contigs as a reference. The same strategy was followed by Guthrie *et al*. (2015) to obtain the complete sequences of AHSV strain from a commercial vaccine. The short length of the Illumina sequence reads (average read lengths of 35 – 301) added greatly to the difficulty of the genome assembly of the Palyam viruses. Reference sequences of segments 9, 7 and 5 were available for most of the Australian and African reference serotypes and assembly of these genes was less complicated. The Palyam, Abadina and Petevo viruses had no sequence data available and sequence assembly proved challenging for the Palyam and Petevo viruses in particular.

After a combination of *de novo* assembly and read mapping complete sequences were obtained for all ten segments of all the viruses used in the study with two exceptions. The VP1 gene sequences for Vellore and Nyabira 1772 were incomplete. Segment 1 coding for VP1 is the longest of the ten segments and thus the most difficult to assemble. The VP1 encoding gene sequence for Vellore had a 46 bp gap which did not influence the translation of the protein. The VP1 encoding gene sequence obtained from Nyabira 1172 had 3 large gaps and translations were not possible for this incomplete sequence. The sequence was therefore not submitted to GenBank.

During analysis of the amino acid sequences of the separate genes of the Palyam serogroup serotypes, VP7 was found to be the most conserved of the genes with a sequence identity of between 97.75% and 100%, with Abadina, Nyabira, Apies River

and Marondera having identical sequences. The sequences for Bunyip Creek, D'Aguilar and CSIRO Village were also the same but defined a second distinguishable group. VP3 was also highly conserved across the serogroup. Sequence identities ranged from 96.81% to 100%, with indistinguishable sequences for Afrotropical serotypes Gweru, Nyabira and Marondera. Sequence homology of these genes is linked to the function of the proteins. VP7 and VP3 are major structural proteins which form the inner capsid, and are involved with molecular interactions during virus assembly (Yamakawa *et al*. 1999a; Wang *et al*. 2016). This is similar to sequence data obtained from other studies on segment 7 of the Palyam serogroup viruses from Japan, Australia and Zimbabwe (Wang *et al*. 2016, Ohashi *et al*. 2004, Yamakawa *et al*. 2000) where sequence identities of 97.3 – 100% were observed for Chuzan, D'Aguilar, Marrakai, CSIRO Village, Marondera, Gweru and Nyabira viruses. Sequence data for other orbiviruses such as BTV has also demonstrated that VP7 and VP3 are more highly conserved between serotypes than the outer capsid proteins, VP2 and VP5 (Maan *et al.* 2011).

The amino acid sequences for VP2 and VP5 showed the highest degree of variation, with VP2 being the more variable of the two. The percentage sequence identities for VP2 ranged between 35.25% and 99.90% (Table 2.3), and for VP5 from 77.23% to 100% (Appendix C). These proteins form the outer capsid of the virus and are responsible for viral neutralization and serotype specificity, hence the variability of the sequences between the serotypes. Studies on BTV have obtained similar results where it was found that segments coding for VP2 and VP5 are the most variable segments of the genome (Maan *et al.* 2010).

Analysis of the nucleotide sequence identity of the concatenated dataset indicated that the identity ranged between 45.91% and 53.52% (Appendix C) when comparing Palyam viruses with the other orbiviruses and between 76.24% and 99.84% within the Palyam serogoup. AHSV was the orbivirus that was the closest related to the Palyam viruses, and had the highest sequence identity value when compared to the Palyam viruses (53.07% – 53.52%). EEV had the lowest sequence identity (46.27% – 46.56%) (Appendix C) and the biggest genetic distance when compared with the Palyam viruses.

Phylogenetic analysis indicated that the Palyam serogroup viruses were the closest related to AHSV and shared a common ancestor with this member of the orbivirus genus. AHSV is closer related to the Palyam serogroup viruses than it is to any of the other orbiviruses included in the study. BTV and EHDV form a sister group and EEV shows the most distant evolutionary relationship from the Palyam serogroup viruses. This agrees with the findings by Yamakawa *et al.* (1999b) concerning the relationship of the Palyam viruses to the other orbiviruses.

When comparing the different serotypes within the Palyam serogroup viruses a high degree of sequence identity was found between isolates from the same geographical region. This was clearly evident from the data obtained for the field isolates; little variation was observed between the field isolates of the same serotype (Figure 2.3). For the Abadina isolates the sequence identity was >99.7% and for Gweru >99.41%. The two Nyabira isolates were the most variable, with a value of 96.38%. Not enough sequence data on isolates of the same serotype is available and therefore more phylogenetic analysis needs to be done before reassortment between and within serotypes can be investigated.

The phylogenetic analysis revealed two clades, which are supported by strong bootstrap values of 100 and posterior probability value of 1. The African serotypes which are in one clade, are all closely related, with identical sequences for several gene segments (Table 2.4 and Appendix B). Of all the African serotypes Apies River and Marondera are the most closely related, with a 97.22% sequence identity. The sequences for Marondera and Apies River virus were the same for VP5, VP7 and NS1 and the sequence identity was >96.42% for all the other segments. This similarity is comparable to the genetic variability within the two Nyabira isolates (96.38%) suggesting a review of the current status of Apies River virus as a separate serotype (Whistler *et al.* 1989). The serotypes of African origin are distinct from the other serotypes of Australian and Asian origin, with the exception of Petevo virus (Figures 2.2 and 2.3).

The second clade contained the Australian and Asian serotypes. Palyam was the first virus to be isolated in 1956 in India and fell as the outgroup to the remaining Australasian and Oriental taxa indicating its ancestral status. The Marrakai serotype

lies outside of the gene pool of the other Australian serotypes (Figure 2.2), concurring with findings in previous RNA hybridization studies where the data indicated that the Australasian gene pool is defined by three of the four Australian serotypes (Bodkin & Knudsen, 1986) and with phylogenetic analysis of segment 7 of Palyam viruses (Wang *et al*. 2016). When comparing the Australian viruses, the sequence identities for CSIRO Village, D'Aguilar and Bunyip Creek were high but Marrakai had lower sequence identity values in this comparison. Marrakai virus was isolated from midges and is also the only Australian serotype that had not been isolated from cattle. Marrakai virus forms a sister group with Petevo (Figure 2.3), the only African (Central African Republic) serotype to group in this clade, and was well supported by the Bayesian analysis. Petevo virus was previously isolated from Ixodid ticks. Interestingly, in the DDN, Petevo virus and Palyam virus had a strong sister relation (bs. 94). The unexpected placement of Petevo outside of the Afrotropical clade is possibly due to translocation of the virus via infected vectors or hosts to Africa from Asia or Australasia after the establishment and diversification of the remaining Afrotropical strains.

Kasba virus and Vellore virus also form sister groups and are very closely related (97.01% sequence identity). The high sequence identity that exists between the viruses from Australia and Asia may suggest that there has been some gene flow between the serotypes, possibly due to the cattle from Indian descent being introduced to Australia during cattle trade. The CSIRO Village serotype shares a curious genetic similarity with the Afrotropical Gweru and Abadina viruses and is worth investigating. The similarity could be due to intermitted gene-flow between the regions, possibly with the trade of infected hosts or may reflect evolutionary convergence.

It is clear from the sequence data that the geographical origin of viruses of the Palyam serogroup played an important role in the development of the different serotypes. Since similar vectors are found in both Australasia and the Afrotropics, the presence of closely related vector species in distant geographic regions could have allowed for the establishment and diversification of introduced viruses due to local episystem factors. A study comparing certain viruses from Japan, Australia and Zimbabwe (Yamakawa *et al*. 2000) concluded that Palyam serogroup viruses evolved independently in separate gene pools, but that isolates from the same geographical

area are closely related at nucleotide and amino acid levels even if they are different serotypes. This was confirmed by findings in this study. The evolution of the different Palyam serotypes could be explained by the spread of the ancestral virus from India to Northern Africa and Australia during cattle trade. Since then, the Afrotropical viruses (excluding Petevo) diversified, whereas gene-flow between the Australasian and Orient seems more complex. It is probable that continued trade of infected hosts or long-distance wind-borne dispersal of infected vectors between Australia and Asia (Eagles *et al*. 2012) resulted in intermitted gene-flow that, for example, may have resulted in the possible close relation between Marakai and the Oriental Kasba and Vellore viruses.

The only known serotype which was not included in this study and for which no sequence data is available is Kindia. Sequence data on this serotype together with the other data gained during the study would lead to a more complete understanding of this serogroup of orbiviruses. The sequence data generated during this study could enable further investigation into the molecular evolution of viruses within the Palyam group with regard to reassortment, genetic drift and intragenic recombination. Divergence time estimation analysis can be investigated and compared to the cattle trade for a more accurate picture of the biogeography of Palyam viruses.

CHAPTER 3
DEVELOPMENT AND INITIAL CHARACTERISATION OF A PALYAM GROUP-SPECIFIC REAL-TIME RT-PCR

3.1 INTRODUCTION

Real-time RT-PCR is a sensitive and reliable assay for the rapid detection and typing of orbiviruses and this has been demonstrated for BTV and AHSV (Lakshmi *et al*. 2018, Maan *et al.* 2016, Bachanek-Bankowska *et al*. 2014). The advantages of real-time RT-PCR over conventional RT-PCR for orbiviruses include high sensitivity and specificity, the reduced risk of contamination and the capability to quantify the viral RNA template (Bachanek-Bankowska *et al*. 2014, Rathogwa *et al*. 2014). The high specificity of the probes and primers can however cause false negatives (Bachanek-Bankowska *et al*. 2014).

Currently there is no rapid diagnostic test available for the reliable detection of all the viruses within the Palyam serogroup. Primers had been developed to amplify sequences from segments 2, 5, 7 and 9 of Palyam viruses from Japan, Australia and Zimbabwe in a RT-PCR (Yamakawa *et al*. 2000, Ohashi *et al*. 2004) for a study to investigate the genetic diversity of these segments. The only recent diagnostic assay published is a single-tube nested RT-PCR for the rapid detection and differentiation of a number of African isolates of the Palyam viruses, and this assay used primers that detect conserved sequences in genome Segment 3 (Aradaib *et al*. 2009). However this assay has not been fully evaluated for use as a diagnostic test.

The Vet-MAX™-Plus One-Step RT-PCR Kit (Applied Biosystems®), together with the StepOnePlus™ Real-Time PCR system (Applied Biosystems®) have been used effectively in diagnostic tests for other orbiviruses such as AHSV (Guthrie *et al*. 2013). One-step RT-PCR kits remove the necessity of preparing cDNA in a separate procedure. Instead the RNA is reverse-transcribed into cDNA and the target is amplified in one step.

The objective of this chapter was to develop a real-time RT-PCR that can adequately facilitate the monitoring of most Palyam serogroup viruses as currently no reliable data on the current distribution of these viruses in South Africa is available

3.2 MATERIALS AND METHODS

3.2.1 Primer and probe design

The different gene sequences of all the Palyam serotypes were aligned in CLC Genomics Main workbench and examined to find conserved segments between serotypes. Sequences of published sets of primers for segment 3 (Aradaib *et al*. 2009) and segments 5, 7 and 9 (Yamakawa *et al*. 2000) were compared to the alignments of these segments. The set of primers that best corresponded with the aligned sequences was selected to be used in the real-time RT-PCR assay. Primers designed to bind to the NS1 gene (Yamakawa *et al*. 2000), were selected and two changes were made to the sequences to better correspond with the alignment of the NS1 encoding gene (Segment 5) obtained in this study. One nucleotide, C, was added to the forward primer (position 13) and one nucleotide was changed from a G to A in position 301 of the reverse primer (Table 3.1). Sequence data was also used to design a probe for the assay. A conserved position was identified between the two primers, at position 196 to 210, and the probe was designed (Figure 3.1). The sequences were sent to IDT (Integrated DNA Technologies, South Africa) to manufacture the probe and primers. The 5' end of the probe was labelled with a FAM fluorophore and a ZEN™/Iowa Black® FQ quencher at the 3' end.

3.2.2 Real-time RT-PCR

3.2.2.1 Samples

The same selection of samples used in the phylogenetic analysis were included as template for the real-time RT-PCR, with the exception of the viruses that did not grow in cell culture (Vellore & Abadina 2389) (Chapter 2, Table 2.1). The viruses were propagated on Vero cells, as previously described (Chapter 2, Section 2.2.2).

3.2.2.2 dsRNA template

Once the viruses had produced 100% CPE in the cell culture, a volume of 100 µl of the cell debris and cell culture medium was collected for RNA extraction . The extraction was performed using Trizol™ reagent (Invitrogen®) according to the manufacturer's instructions. The RNA was eluted in 90 µl HPLC water. The total RNA concentration was determined on the Qubit™ 2.0 Fluorometer (Life technologies®) using the dsRNA broadrange assay kit (Invitrogen®) and aliquoted into smaller volumes and stored at -80°C.

3.2.2.3 Real-time PCR

The VetMAX™-Plus One-Step RT-PCR kit (Applied Biosystems®) was used for the real-time RT-PCR. In this reaction the RNA template is reverse-transcribed into cDNA and then the target sequence is amplified by AmpliTaq Gold® DNA polymerase.

The primers and probe were reconstituted in TE (Sigma-Aldrich®) and working solutions of 10 µM primer and 3 µM probe prepared. A PCR mix was prepared using the VetMAX™-Plus One-Step RT-PCR Kit (Applied Biosystems®) according to the manufacturer's recommendations. The mix, with a total volume of 25 µl, consisted of 12,5 µl of the 2x RT-PCR Buffer, 1 µl of the 25x RT-PCR Enzyme mix, 3 µl of the primer and probe mix (1 µl of each) and 8,5 µl dsRNA. The dsRNA was denatured at 95°C for 5 minutes before being added to the PCR mix.

The real-time RT-PCR was performed in a StepOnePlus™ Real-Time PCR system (Applied Biosystems®), using the recommended programme. In the initial stage, reverse transcription takes place at 48°C for 10 minutes, followed by RT-inactivation and denaturation at 95°C for 10 minutes. The region of interest was amplified during 40 cycles at 95°C for 15 seconds and 60°C for 45 seconds with acquisition of FAM fluorescence.

3.2.2.4 Assay characteristics

The viruses, AHSV, BTV, EEV and EHDV were also tested to determine assay specificity. The RNA was extracted in a similar manner as for the Palyam serogroup viruses and tested in the same runs as the Palyam samples. One of the Palyam samples with a known concentration was selected, and a serial ten-fold dilution range was made of the RNA template. The dilutions were tested in triplicate for an indication of the assay's sensitivity. A standard curve was generated in Microsoft Excel (2010) by plotting the CT values against the corresponding RNA concentration and the slope of the line was calculated between two points using statistical functions. The PCR efficiency was determined from the slope value using an online qPCR Efficiency calculator (https://www.thermofisher.com/za/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html).

3.3 RESULTS

3.3.1 Primers and probe

The primers were designed to detect a conserved sequence on Segment 5 of all the Palyam serotypes, producing a 305 bp product. The forward primer was designed to bind at position 13 - 35 and the reverse primer at 297 – 318 with the probe binding at position 196 – 210. The annealing positions of the primers and probes are illustrated in Figure 3.1. The primers differ from the published primers at 2 positions, C was added to the forward primer at position 1 and an A replaced a G in position 301 of the reverse primer.

Table 3.1 Primer and probe sequences for the Palyam real-time RT-PCR

	Sequence	**bp**	**Tm**
Forward primer	5’ CTG GCT TTC TGA GGC GTT TCA GA 3’	23	70°C
Reverse primer	5’ GGT TAT CAA TAT GCC AAG CGA 3’	21	60°C
Probe	5’6-FAM/TGG CTG TTG/ZEN/ATC ATG C/3’IBFQ	16	

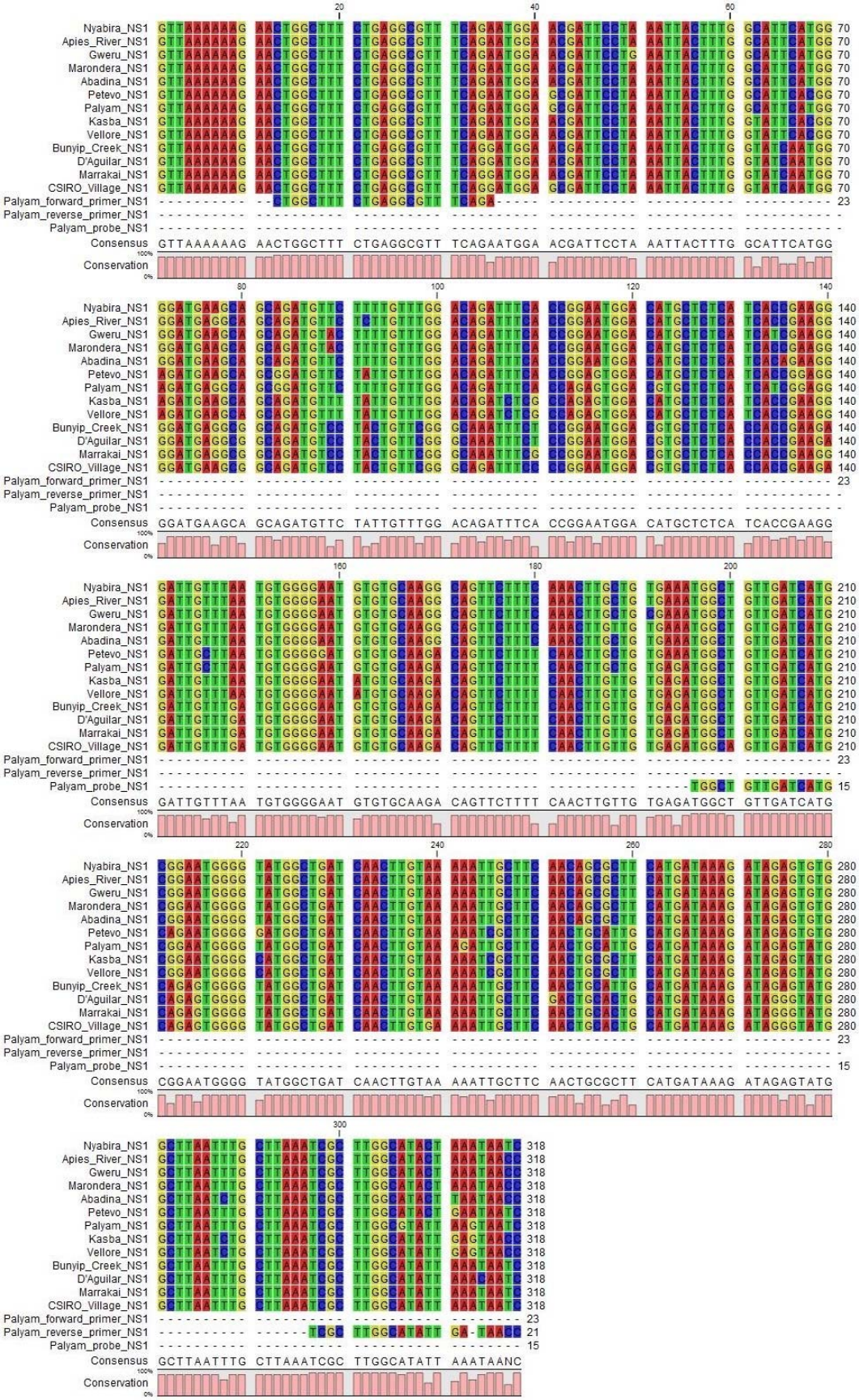


Figure 3.1 Alignment of NS1 sequences of serotypes of the Palyam serogroup and the position of the primers and probe.

3.3.2 Real-time RT-PCR

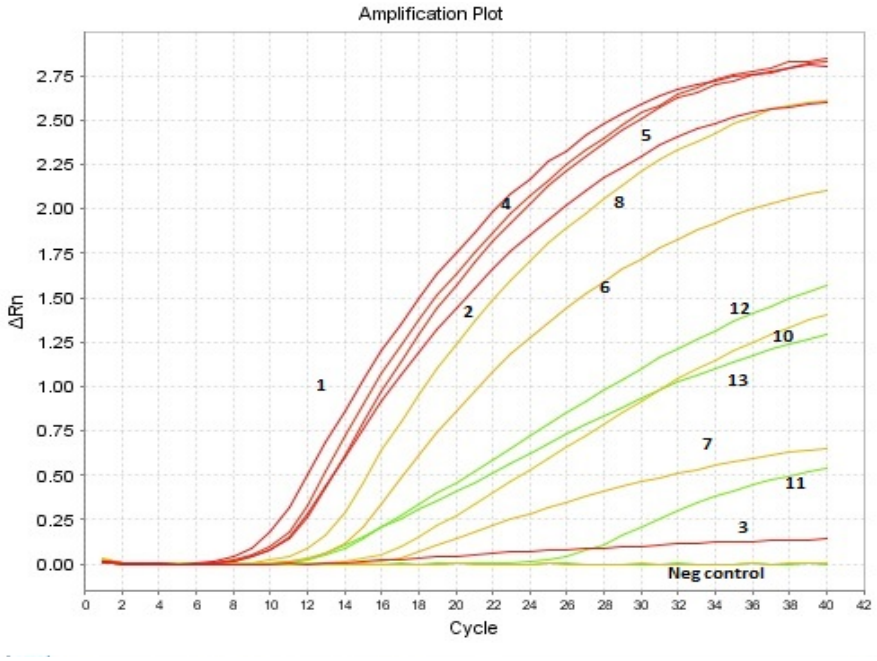
In the RT-PCR assay a positive result was achieved for all the serotypes tested (Figure 3.2A), as well as for the field isolates of Gweru, Abadina and Nyabira (Figure 3.2B). The threshold cycle (CT) values ranged between 9.08 and 29.72, as summarized in Table 3.2. Other orbiviruses tested indicated no amplification.

Table 3.2. CT values obtained during the RT-PCR

Virus	**CT value**
Abadina	9.08
Bunyip Creek	10.28
CSIRO Village	29.72
D'Aguilar	10.43
Kasba	10.05
Marrakai	13.74
Palyam	18.94
Petevo	12.18
Apies River	17.03
Gweru	27.68
Marondera	14.14
Nyabira	13.95
Field isolates	
Abadina 2582	18.63
Nyabira 1772	13.02
Nyabira 4646	13.46
Gweru 1828	13.46
Gweru 1832	14.94
Gweru 1856	14.11
Gweru 2038	15.64
Control viruses	
AHS 1	No CT

BTV 4	No CT
EEV 1	No CT
EHDV	No CT
Negative control (nuclease-free water)	No CT

(A)



(B)

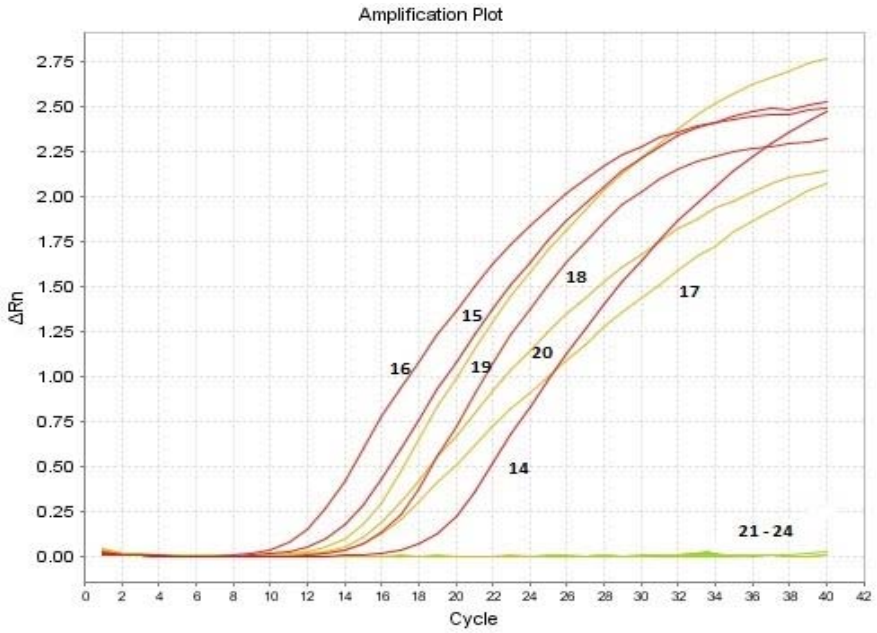


Figure 3.2. Amplification plots for the real-time RT-PCR.

(A) Amplification plot for the Palyam serotypes. 1 = Abadina, 2 = Bunyip Creek, 3 = CSIRO Village, 4 = D'Aguilar, 5 = Kasba, 6 = Marrakai, 7 = Palyam, 8 = Petevo, 10 = Apies River, 11 = Gweru, 12 = Marondera, 13 = Nyabira

(B) Amplification plot for the field isolates and other orbiviruses.

14 = Abadina 2582, 15 = Nyabira 1772, 16 = Nyabira 4646, 17 = Gweru 1828, 18 = Gweru 1832, 19 = Gweru 1856, 20 = Gweru 2038, 21 = AHSV, 22 = BTV, 23 = EEV, 24 = EHDV

The concentration of Bunyip Creek virus RNA was determined, diluted ten-fold and tested with the RT-PCR in triplicate. Amplification was achieved to the 10^{-7} dilution, where a RNA concentration of 0.000 07 ng/ml could still be detected by the assay (Table 3.3). The standard curve had a slope of -3.0 (Figure 3.3) and the PCR efficiency calculated was 115.44%.

Table 3.3. CT values for dilution range of Bunyip Creek virus

Dilution	RNA concentration	CT value Test 1	CT value Test 2	CT value Test 3
10^{-1}	70 ng/ml	11.91	11.71	11.43
10^{-2}	7 ng/ml	14.92	14.68	15.36
10^{-3}	0.7 ng/ml	20.75	20.60	20.39
10^{-4}	0.07 ng/ml	24.28	23.94	23.68
10^{-5}	0.007 ng/ml	26.85	26.87	26.80
10^{-6}	0.000 7 ng/ml	31.28	31.25	31.34
10^{-7}	0.000 07 ng/ml	35.55	37.10	36.36

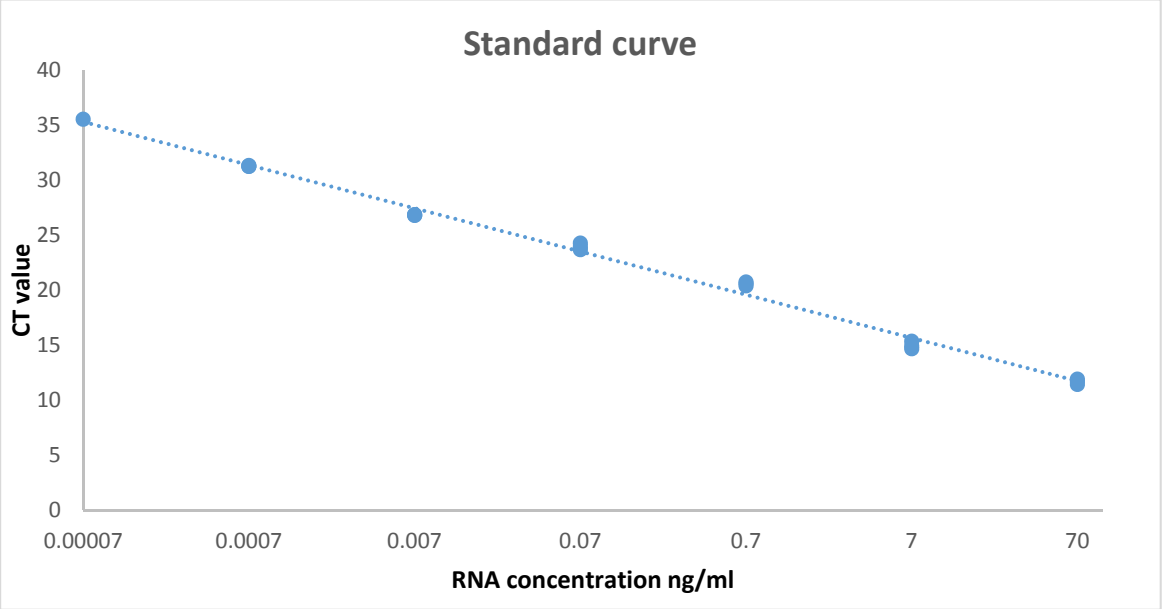


Figure 3.3 Standard curve for CT values against RNA concentration for the dilution range of Bunyip Creek virus.

Non-linearity of the highest dilutions were excluded from the analysis.

3.4 DISCUSSION

Molecular assays have been developed to produce for more rapid and reliable detection of viruses than provided by conventional methods such as serology. Real-time RT-PCR has proven to be an effective method for detection and typing of orbiviruses (Maan *et al*. 2016, Bachanek-Bankowska *et al.* 2014). It allows for quantification of RNA and is able to detect short amplicons, which is advantageous if degradation of the RNA target occurs (Bachanek-Bankowska *et al*. 2014). The risk of contamination is also reduced since the reaction and detection takes place in a closed tube. False negative results can however occur, due to the extremely high specificity of the primers and probes. Care should thus be taken when designing primers and probes that are required to target a wide selection of potentially diverse samples (Bachanek-Bankowska *et al*. 2014).

The availability of sequence data obtained for the Palyam serogroup of orbiviruses in this study has allowed for the development of a specific real-time RT-PCR. The conserved segments within the serotypes, and highly conserved sequences within the segments have been determined. Segment 5 was found to be one of the more conserved segments of the Palyam viruses during the study, and published primers for this segment were aligned with the newly generated Segment 5 sequences of the serotypes (Yamakawa *et al*. 2000). The published primers aligned exactly with all the serotypes in the study from position 13 to 35 and one extra base was added to the forward primer to make increase the specificity. For the reverse primer only one change had to be made to match the alignment, in position 301. A consensus sequence from the alignment from position 196 to 210 was selected for the probe.

The real-time RT-PCR assay was able to detect all of the Palyam serogroup serotypes tested, as well as the field isolates of the Abadina, Gweru and Nyabira serotypes. However, the CT values differ considerably between the different isolates. The viruses were propagated in cell culture, some serotypes growing faster and showing more CPE than others, and thus the virus concentration varied for the different viruses and varying RNA concentrations were obtained after extraction. This accounts for the difference in CT values, viruses from which higher concentrations of RNA were extracted, obtained lower CT values. As this is a one-step assay in which cDNA is made and amplified in one reaction, the quality and concentration of the RNA used is

very important to the performance of the test. Automated extractions, with internal controls such as Xeno™ RNA Control would assist in standardizing the RNA input for the assay. Purifying the total RNA and only using the dsRNA in the assay would also improve the performance of the assay. Viruses that were first propagated on cell culture and for which the RNA was immediately extracted, showed positive PCR results with low CT values. Where the RNA extractions were directly performed from the freeze-dried material (Vellore and Abadina 2389), as had been done in the FLAC assay, the real-time RT-PCR could not detect the Palyam virus nucleic acid, most likely due to the degradation of the sample. Therefore, the Vellore serotype could not be tested with this assay, but the primers would be expected to detect this serotype as well.

When good quality RNA, in sufficient concentrations were used, the assay was able to detect the different Palyam serogroup serotypes. The CSIRO Village serotype obtained much higher CT values than the other serotypes. This is most likely due to some unique characteristics of this virus as this serotype grows slower than the other serotypes in cell culture and would therefore likely have had a lower concentration.

To determine the specificity of the RT-PCR, the assay was used to evaluate the detection of other orbivirus serogroups (AHSV, BTV, EEV and EHDV). The RT-PCR produced no amplification for these viruses, which shows that the assay was specific for the Palyam group viruses.

In order to determine the sensitivity of the assay one of the samples, Bunyip Creek virus, was diluted ten-fold, and tested. The PCR was still able to detect the Palyam viral nucleic acid at a concentration of 7 fg/ml. The published nested RT-PCR (Aradaib *et al*. 2009), using primers for segment 3, reported that the assay was able to detect 0.1 fg of Palyam RNA. The study by Yamakawa *et al*. (2000) concentrated on the phylogenetic analysis of samples and thus did not determine the sensitivity of their assays.

The high efficiency value of this assay is most likely attributed to polymerase inhibition due to contaminants such as ethanol or phenol from the RNA extraction procedure. Even when more template is added, the CT values don't change to earlier cycles thus

the flattening out of the curve and lowering of the slope, resulting in a higher efficiency value. The RNA was not purified during this part of the study and did not include a purification step to remove impurities thus inhibitors could decrease the efficiency value.

Real-time RT-PCR's have been developed for many orbiviruses including AHSV, BTV, EEV and EHDV (Lakshmi *et al*. 2018, Maan *et al*. 2016, Bachanek-Bankowska *et al*. 2014 and Rathogwa *et al*. 2014). All these assays have been reported to be fast, reliable and sensitive tools for the detection of the particular orbiviruses. The assay developed in this study for the detection of Palyam serogroup viruses is sufficiently specific to detect the different serotypes but would have to be validated particularly in regard to sensitivity. The extraction process will have to be automated in order to have comparable RNA concentrations to determine the repeatability of the test and including a purification step may improve assay performance. Other sample matrices need to be tested such as tissue from aborted foetuses or vectors.

This assay should be a valuable diagnostic tool for the detection and or exclusion of Palyam serogroup viruses during disease outbreaks.

CHAPTER 4
CONCLUSION

The techniques used during this study allowed for the achievement of the two aims; the phylogenetic analysis of the Palyam serogroup of orbiviruses and the development of a group-specific diagnostic assay for detection of the majority of serotypes of the serogroup.

The availability of most of the serotypes and the ability to propagate the viruses in cell culture to increase the viral concentration enabled good quality and high concentrations of viral dsRNA to be extracted for use in the phylogenetic analysis. The FLAC assay performed satisfactorily for the amplification of the viral templates. No prior need for specific sequencing data, which in the case of most Palyam serotypes is not available, made this assay ideal for the amplification of the viral genomes.

The Illumina sequence reads were successfully used to obtain the full genome sequences of the samples submitted. Due to the short read lengths obtained by the sequencing platform, high coverage was needed and some samples had to be resubmitted to increase the coverage. It was difficult to obtain the full genome sequences for the bigger Palyam genes, specifically Segment 1. A different NGS technique, for instance single-molecule real-time sequencing (Pacific Biosystems) with longer read lengths, may have been a better sequencing technique to use.

The fact that there were no reference sequences available for many of the Palyam virus genes investigated and specifically some serotypes, made obtaining the full-length genome sequences challenging, even when using suitable sequence assembly software. The CLC Genomics Main workbench functioned effortlessly for the alignment of sequences, finding of ORF's and pairwise comparisons. However, for the construction of the phylogenetic tree other programs produced better results. Bayesian inference produced trees with better support, compared to the maximum likelihood analysis.

The techniques and reagents used in the development of the real-time RT-PCR were all fit for their purpose of detecting the Palyam viruses in a rapid molecular assay. The RNA extraction method using Trizol® reagent however, while very effective, made it difficult to obtain similar RNA concentrations across replicates of the same sample. For validation of the assay, another extraction method might be more suitable such as an automated extraction system. The use of a one-step RT-PCR kit, when used with a real-time PCR System was effective and shortened the PCR time considerably as compared to other PCR methods and kits where cDNA has to be synthesized in a separate procedure. The primers and probes developed were effective in detecting all serotypes of the Palyam serogroup however the CSIRO Village virus did not perform as well as the other serotypes most likely due to low RNA concentration in the prepared sample material. The availability of field isolates of some of the African serotypes also assisted in further testing of the assay.

The sequencing data acquired during this study allowed for the development of a diagnostic assay that could be invaluable for epidemiological studies. There have not been any recent Palyam serogroup virus detection and isolations in South Africa, possibly because when abortions in cattle occur, other causes are investigated rather than Palyam serogroup viruses. With the use of the developed real-time RT-PCR here, Palyam virus infections can be detected more readily. The real-time RT-PCR could also assist in the design of control measures for potential Palyam serogroup virus infections. If this assay is used as a screening tool it could provide opportunities to obtain new virus isolates. Recent isolates that can be included in phylogenetic analysis, together with the newly generated sequences, could assist in determining the full genetic diversity of circulating field strains. Such data would be valuable in the design of a vaccine by helping to select antigenically representative field strains for vaccine development.

This was the first study where full genome sequence data of the vast majority of the different currently known Palyam serogroup serotypes was obtained. Sequence of field isolates of Gweru, Abadina and Nyabira were also reported for the first time. Further phylogenetic analysis should be done to investigate reassortment, genetic drift and intragenic recombination in this serogroup of orbiviruses.

REFERENCES

ANBALAGAN, S., COOPER, E., KLUMPER, P., SIMONSON, R.R. & HAUSE, B.M. 2014. Whole genome analysis of epizootic hemorrhagic disease virus identified limited genome constellations and preferential reassortment. *Journal of General Virology*, 95:434-441

ARADAIB, I.E., MOHAMED, M.E.H. & ABDALLA, M.A. 2009. A single-tube RT-PCR for rapid detection and differentiation of some African isolates of palyam serogroup orbiviruses. *Journal of Virological Methods*, 161:70-74

ATTOUI, H. & MOHD JAAFAR, F. 2015. Zoonotic and emerging orbivirus infections. *Revue scientifique et technique (International Office of Epizootics),* 34(2):353-361

BACHANEK-BANKOWSKA, K., MAAN, S., CASTILLO-OLIVARES, J., MANNING, N.M., MAAN, N.S., POTGIETER, A.C., DI NARDO, A., SUTTON, G., BATTEN, C. & MERTENS, P.P. 2014. Real time RT-PCR assays for detection and typing of African horse sickness virus*. PLoS ONE*, 9(4):e93758

BELHOUCHET, M., MOHD JAAFAR, F., FIRTH, A.E., GRIMES, J.M., MERTENS, P.P. & ATTOUI, H. 2011. Detection of a fourth orbivirus non-structural protein*. PLoS ONE*, 6(10):e25697

BENTLEY, D.R., BALASUBRAMANIAN, S., SWERDLOW, H.P., SMITH, G.P., MILTON, J., *et al*. 2008. Accurate whole human genome sequencing using reversible terminator chemistry*. Nature*, 456:53-59

BLACKBURN,N.K., SEARLE, L. & PHELPS, R.J. 1985. Viruses isolated from *Culicoides* (Diptera:Ceratopogonidae) caught at the veterinary research farm, Mazowe, Zimbabwe. *Journal of the Entomological Society of Southern Africa*, 48:331-336

BLACKSELL, S.D., LUNT, R.A. & WHITE, J.R. 1994. A rapid indirect ELISA for the serogrouping of Australian orbiviruses. *Journal of Virological Methods*, 49:67-78

BODKIN, D.K. & KNUDSON, D.L. 1985. Sequence relatedness of Palyam virus genes to cognates of the Palyam serogroup viruses by RNA-RNA blot hybridization*. Virology*, 143:55-62

BODKIN, D.K. & KNUDSON, D.L. 1986. Genetic relatedness of Colorado tick fever virus isolates by RNA-RNA blot hybridization*. The Journal of General Virology*, 68 (Pt 4):1199-1204

BOIRO, I., LOMONOSSOV, N.N., ALEXIN, A.F., BAH, A. & BALDE, C. 1986. Isolation of a new orbivirus Kindia (Palyam group) from ticks Amblyomma

variegatum in the Republic of Guinea. *Bulletin de la Societe de pathologie exotique et de ses filiales*, 79:187-190

CAPORALE, M., DI GIALLEONORADO, L., JANOWICZ, A., WILKIE, G., SHAW, A., SAVINI, G., VAN RIJN, P.A., MERTENS, P., DI VENTURA, M. & PALMARINI, M. 2014. Virus and host factors affecting the clinical outcome of bluetongue virus infection. *Journal of Virology*, 88:10399-10411

DANDAWATE, C.N. & PAVRI, K.M. 1974. Palyam group: a new serogroup of arboviruses: physicochemical and biological properties of Palyam and Kasba viruses. *The Indian Journal of Medical Research*, 62:317-325

DANDAWATE, C.N., RAJAGOPALAN, P.K., PAVRI, K.M. & WORK, T.H. 1969. Virus isolations from mosquitoes collected in North Arcot district, Madras state, and Chittoor district, Andhra Pradesh between November 1955 and October 1957. *The Indian Journal of Medical Research*, 57:1420-1426

DARRIBA, ,D., TABOADA, G.,L., DOALLO, R. & POSADA, D. 2015. jModelTest 2: more models, new heuristics and high performance computing

DILCHER, M. & WEIDMANN, M. 2012. Confusions in orbivirus protein classification. *Virology Journal*, 9:166-422X-9-166

DOHERTY, R.L., CARLEY, J.G., STANDFAST, H.A., DYCE, A.L. & SNOWDON, W.A. 1972. Virus strains isolated from arthropods during an epizootic of bovine ephemeral fever in Queensland. *Australian Veterinary Journal*, 48:81-86

EAGLES, D., DEVESON, T., WALKER, P.J., ZALUCKI, M.P. & DURR, P. 2012. Evaluation of long-distance dispersal of *Culicoides* midges into northern Australia using a migration model. *Medical and Veterinary Entomology*, 26:334-340

FALL, M., FALL, A.G., SECK, M.T., BOUYER, J., DIARRA, M., BALENGHIEN, T., GARROS, C., BAKHOUM, M.T., FAYE, O., BALDET, T. & GIMONNEAU, G. 2015. Circadian activity of *Culicoides oxystoma* (Diptera: Ceratopogonidae), potential vector of bluetongue and African horse sickness viruses in the Niayes area, Senegal. *Parasitology Research*, 114:3151-3158

GOTO, Y., MIURA, Y. & KONO, Y. 1988. Epidemiological survey of an epidemic of congenital abnormalities with hydranencephaly-cerebellar hypoplasia syndrome of calves occurring in 1985/86 and seroepidemiological investigations on Chuzan virus, a putative causal agent of the disease, in Japan. *The Japanese Journal of Veterinary Science,* 50:405-413

GUTHRIE, A.J., COETZEE, P., MARTIN, D.P., LOURENS, C.W., VENTER, E.H., WEYER, C.T., JOONE, C., GRANGE, M.L., HARPER, C.K., HOWELL, P.G. & MACLACHLAN, N.J. 2015. Complete genome sequences of the three African

horse sickness virus strains from a commercial trivalent live attenuated vaccine. *Genome Announcements*, 3:e00814-15

GUTHRIE, A.J., MACLACHLAN, N.J., JOONE, C., LOURENS, C.W., WEYER, C.T., QUAN, M., MONYAI, M.S. & GARDNER, I.A. 2013. Diagnostic accuracy of a duplex real-time reverse transcription quantitative PCR assay for detection of African horse sickness virus. *Journal of Virological Methods*, 189:30-35

HARASAWA, R., YOSHIDA, T., IWASHITA, O., GOTO, Y. & MIURA, Y. 1988. Biochemical characteristics of Chuzan virus, a new serotype of palyam serogroup. *Japanese Journal of Veterinary Science*, 50:777-782

HARRISON, C.J. & LANGDALE, J.A. 2006. A step by step guide to phylogeny reconstruction. *The Plant Journal : for cell and molecular biology*, 45:561-572

HE, C.Q., DING, N.Z., HE, M., LI, S.N., WANG, X.M., HE, H.B., LIU, X.F. & GUO, H.S. 2010. Intragenic recombination as a mechanism of genetic diversity in bluetongue virus. *Journal of Virology*, 84:11487-11495

HECHINGER, S., WERNIKE, K. & BEER, M. 2013. Evaluating the protective efficacy of a trivalent vaccine containing Akabane virus, Aino virus and Chuzan virus against Schmallenberg virus infection. *Veterinary Research*, 44:114

HUSON, D.,H. & BRYANT, ,DAVID. 2005. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, 23:254-267

ITO, T., AKASHI, H., YAMAKAWA, M., KUROGI, H. & INABA, Y. 1990. Structural polypeptides of a Palyam serogroup orbivirus isolated in Japan. *Australian Veterinary Journal*, 67:153-154

JANOWICZ, A., CAPORALE, M., SHAW, A., GULLETTA, S., DI GIALLEONARDO, L., RATINIER, M. & PALMARINI, M. 2015. Multiple genome segments determine virulence of bluetongue virus serotype 8. *Journal of Virology*, 89:5238-5249

JUSA, E.R., INABA, Y., KADOI, K., KUROGI, H., FONSECA, E. & SHOPE, R.E. 1994. Identification of Kagoshima and Chuzan viruses of Japan as Kasba virus, an orbivirus of the Palyam serogroup. *Australian Veterinary Journal*, 71:57

KARRAR, A.R.E., ABDALLA, M.A., MAJID, A.M., SALIH, M.M.M., ELAMIN, S.M.M., OSBURN, B.I. & ARADAIB, I.E. 2003. Application of RT-PCR for detection of African isolates of Palyam orbiviruses serogroup in cell culture. *Journal of Animal and Veterinary Advances*, 2:671-674

KIM, Y., KWEON, C., TARK, D., LIM, S., YANG, D., HYUN, B., SONG, J., HUR, W. & PARK S. 2011. Development of inactivated trivalent vaccine for the teratogenic Aino, Akabane and Chuzan viruses. *Biologicals*, 39:152-157

KUMAR, ,SUDHIR, STECHER, ,GLEN, LI, ,MICHAEL, KNYAZ, ,CHRISTINA & TAMURA, ,KOICHIRO. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35:1547-1549

KUROGI, H., SUZUKI, T., AKASHI, H., ITO, T., INABA, Y. & MATUMOTO, M. 1989. Isolation and preliminary characterization of an orbivirus of the Palyam serogroup from biting midge *Culicoides oxystoma* in Japan. *Veterinary Microbiology*, 19:1-11

LAKSHMI, I.K., PUTTY, K., RAUT, S.S., PATIL, S.R., RAO, P.P., BHAGYALAKSHMI, B., JYOTHI, Y.K., SUSMITHA, B., REDDY, Y.V., KASULANATI, S., JYOTHI, J.S. & REDDY, Y.N. 2018. Standardization and application of real-time polymerase chain reaction for rapid detection of bluetongue virus. *Veterinary World*, 11:452-458

MILLER, M. A., PFEIFFER, W. & SWARTZ, T. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *2010 Gateway Computing Environments Workshop (GCE),* 2010:1-8

MAAN, N.S., MAAN, S., POTGIETER, A.C., WRIGHT, I.M., BELAGANAHALLI, M. & MERTENS, P.P.C. 2016. Development of real-time RT-PCR assays for detection and typing of epizootic haemorrhagic disease virus. *Transboundary and Emerging Diseases*, 64:1120-1132

MAAN, S., RAO, S., MAAN, N.S., ANTHONY, S.J., ATTOUI, H., SAMUEL, A.R. & MERTENS, P.P.C. 2007. Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. *Journal of Virological Methods*, 143:132-139

MERTENS, P.P., DIPROSE, J., MAAN, S., SINGH, K.P., ATTOUI, H. & SAMUEL, A.R. 2004. Bluetongue virus replication, molecular and structural biology. *Veterinaria Italiana*, 40:426-437

MIURA, Y., KUBO, M., GOTO, Y. & KONO, Y. 1991. Chuzan disease as congenital-hydranencephaly cerebellar hypoplasia syndrome in calves. *JARQ, Japan Agricultural Research Quarterly*, 25:55-60

MORAG, N., SAROYA, Y., BRAVERMAN, Y., KLEMENT, E. & GOTTLIEB, Y. 2012. Molecular identification, phylogenetic status, and geographic distribution of *Culicoides oxystoma* (Diptera: Ceratopogonidae) in Israel. *PLoS ONE*, 7(3):e33610

MYERS, R.M., CAREY, D.E., REUBEN, R., JESUDASS, E.S. & SHOPE, R.E. 1971. Vellore virus: a recently recognized agent of the Palyam group of arboviruses. *The Indian Journal of Medical Research*, 59:1209-1213

NOAD, R. & ROY, P. 2009. 'Bluetongue virus replication and assembly' in Mellor P.S., Bayliss, M. & Mertens P.P.C. (ed.) *Bluetongue*. Academic Press, London, UK

NOMIKOU, K., HUGHES, J., WASH, R., KELLAM, P., BREARD, E., ZIENTARA, S., PALMARINI, M., BIEK, R. & MERTENS, P. 2015. Widespread reassortment shapes the evolution and epidemiology of bluetongue virus following European invasion. *PLoS Pathog*, 11(8):e1005056

OHASHI, S., MATSUMORI, Y., YANASE, T., YAMAKAWA, M., KATO, T. & TSUDA, T. 2004. Evidence of an antigenic shift among Palyam serogroup orbiviruses. *Journal of Clinical Microbiology*, 42:4610-4614

OWENS, R.J., LIMN, C. & ROY, P. 2004. Role of an arbovirus nonstructural protein in cellular pathogenesis and virus release. *Journal of Virology*, 78:6649-6656

PALACIOS, G., COWLED, C., BUSETTI, A.V., SAVJI, N., WEIR, R., WICK, I., TRAVASSOS DA ROSA, A., CALISHER, C.H., TESH, R.B., BOYLE, D. & LIPKIN, W.I. 2011. Rapid molecular strategy for orbivirus detection and characterization. *Journal of Clinical Microbiology*, 49(6):2314-2317

PATEL, A. & ROY, P. 2014. The molecular biology of Bluetongue virus replication. *Virus Research*, 182:5-20

POTGIETER, A.C., PAGE, N.A., LIEBENBERG, J., WRIGHT, I.M., LANDT, O. & DIJK, A.A.V. 2009. Improved strategies for sequence-independent amplification and sequencing of viral double-stranded RNA genomes. *Journal of General Virology*, 90:1423-1432

QIN, S., YANG, H., ZHANG, Y., LI, Z., LIN, J., GAO, L., LIAO, D., CAO, Y., REN, P., LI, H. & WU, J. 2018. Full genome sequence of the first bluetongue virus serotype 21 (BTV-21) isolated from China: evidence for genetic reassortment between BTV-21 and bluetongue virus serotype 16 (BTV-16). *Archives of Virology*, 163:1379-1382

RAMBAUT, A., SUCHARD, M.A., XIE, D. & DRUMMOND, A.J. Tracer v1.6, http://beast.bio.ed.ac.uk/Tracer

RATHOGWA, N.M., QUAN, M., SMIT, J.Q., LOURENS, C., GUTHRIE, A.J. & VAN VUUREN, M. 2014. Development of a real time polymerase chain reaction assay for equine encephalosis virus. *Journal of Virological Methods*, 195:205-210

RATINIER, M., SHAW, A.E., BARRY, G., GU, Q., DI GIALLEONARDO, L., JANOWICZ, A., VARELA, M., RANDALL, R.E., CAPORALE, M. & PALMARINI, M. 2016. Bluetongue virus NS4 protein is an interferon antagonist and a determinant of virus virulence. *Journal of Virology*, 90:5427-5439

RONQUIST, F. & HUELSENBECK, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models*. Bioinformatics (Oxford, England)*, 19:1572-1574

ROY, P. 1996. Orbivirus Structure and Assembly*. Virology,* 216:1-11

SALUZZO, J.F., DIGOUTTE, J.P., CORNET, J.P., HEME, C., HERVE, J.P., GONZALEZ, J.P. & GEORGES, A.J. 1982. Petevo virus, a new arbovirus of the Palyam group isolated in Central African Republic from the tick *Amblyomma variegatum*. *Annales de l'Institut Pasteur/Virologie,* 133:215-221

SHAW, A.E., RATINIER, M., NUNES, S.F., NOMIKOU, K., CAPORALE, M., GOLDER, M., ALLAN, K., HAMERS, C., HUDELET, P., ZIENTARA, S., BREARD, E., MERTENS, P. & PALMARINI, M. 2013. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment*. Journal of Virology*, 87:543-557

STADEN, V.V. & HUISMANS, H. 1991. A comparison of the genes which encode non-structural NS3 of different orbiviruses. *Journal of General Virology*, 72:1073-1079

ST GEORGE,T.D. 1989. An overview of arboviruses affecting domestic animals in Australia. *Australian Veterinary Journal*, 66(12):393-395.

SWANEPOEL, R. 2004. 'Palyam serogroup orbivirus infections' in Coetzer, J.A.W. *Infectious diseases of livestock.* Oxford University Press, Cape Town, South Africa, pp. 1252-1255

SWANEPOEL, R. & BLACKBURN, N.K. 1976. A new member of the Palyam serogroup of orbiviruses*. The Veterinary Record*, 99:360

VAN DEN BERGH, C., COETZEE, P., GUTHRIE, A.J., LE GRANGE, M. & VENTER, E.H. 2016. Complete genome sequences of five bluetongue virus (BTV) vaccine strains from a commercial live attenuated vaccine, a BTV-4 field strain from South Africa, and a reassortant strain isolated from experimentally vaccinated cattle*. Genome Announcements*, 4:10.1128/genomeA.00462-16

WANG F, LIN, J. , CHANG J., CAO Y., QIN S., WU J. & YU L. 2016. Isolation, complete genome sequencing, and phylogenetic analysis of the first Chuzan virus in China. *Virus Genes*, 52:138-141

WEYER, C.T., GREWAR, J.D., BURGER, P., ROSSOUW, E., LOURENS, C., JOONE, C., LE GRANGE, M., COETZEE, P., VENTER, E., MARTIN, D.P., MACLACHLAN, N.J. & GUTHRIE, A.J. 2016. African horse sickness caused by genome reassortment and reversion to virulence of live, attenuated vaccine viruses, South Africa, 2004-2014*. Emerging Infectious Diseases*, 22:2087-2096

WHISTLER, T. & SWANEPOEL, R. 1988. Characterization of potentially foetotropic Palyam serogroup orbiviruses isolated in Zimbabwe. *Journal of General Virology*, 69:2221-2227

WHISTLER, T. & SWANEPOEL, R. 1990. Proteins of Palyam serogroup viruses. *The Journal of General Virology*, 71:1333-1338

WHISTLER, T. & SWANEPOEL, R. 1991. Teratogenicity of the Palyam serogroup orbiviruses in the embryonated chicken egg model. *Epidemiology and Infection*, 106:179-188

WHISTLER, T., SWANEPOEL, R. & ERASMUS, B.J. 1989. Characterization of Palyam serogroup orbiviruses isolated in South Africa and serologic evidence for their widespread distribution in the country. *Epidemiology and Infection*, 102:317-324

XUAN, J., YU, Y., QING, T., GUO, L. & SHI, L. 2013. Next-generation sequencing in the clinic: Promises and challenges. *Cancer Letters,* 340:284-295

YAMAGUCHI, R., NAITOH, Y., UCHIDA, K., HIRANO, N., WADA, T. & TATEYAMA, S. 1999. Encephalopathy in suckling mice infected with Kasba (Chuzan) virus. *Journal of Comparitive Pathology,* 120:247-256

YAMAKAWA, M. & FURUUCHI, S. 2001. Expression and antigenic characterization of the major core protein VP7 of Chuzan virus, a member of the Palyam serogroup orbiviruses. *Veterinary Microbiology*, 83:333-341

YAMAKAWA, M., FURUUCHI, S. & MINOBE, Y. 1999a. Molecular characterization of double-stranded RNA segments encoding the major capsid proteins of a Palyam serogroup orbivirus that caused an epizootic of congenital abnormalities in cattle. *Journal of General Virology*, 80:205-208

YAMAKAWA, M., KUBO, M. & FURUUCHI, S. 1999b. Molecular analysis of the genome of Chuzan virus, a member of the Palyam serogroup viruses, and its phylogenetic relationships to other orbiviruses. *Journal of General Virology*, 80:937-941

YAMAKAWA, M., OHASHI, S., KANNO, T., YAMAZOE, R., YOSHIDA, K., TSUDA, T. & SAKAMOTO, K. 2000. Genetic diversity of RNA segments 5, 7 and 9 of the Palyam serogroup orbiviruses from Japan, Australia and Zimbabwe. *Virus Research*, 68:145-153

YANG H., XIAO L., MENG J., XIONG H., GAO L., LIAO D. & LI H. 2016. Complete genome sequence of a Chuzan virus strain isolated for the first time in mainland China. *Archives of Virology*, 161:1073-1077

YANG, D.K., HWANG, I.J., KIM, B.H., KWEON, C.H., LEE, K.W., KANG, M.I., LEE, C.S. & CHO, K.O. 2008. Serosurveillance of viral diseases in Korean native goats (*Capra hircus*). *The Journal of Veterinary Medical Science*, 70:977-979

ZWART, L., POTGIETER, C.A., CLIFT, S.J. & VAN STADEN, V. 2015. Characterising Non-Structural Protein NS4 of African Horse Sickness Virus. *PLoS ONE*, 10:e0124281

APPENDIX A

Buffers used in the FLAC assay

1. Ligation buffer
    - 500 µl HEPES
    - 180 µl $MgCl_2$
    - 100 µl BSA
    - 100 µl ATP
    - 100 µl DTT

2. cDNA buffer
    - 300 µl Tris
    - 140 µl KCl
    - 60 µl $MgCl_2$
    - 180 µl β-mercapto-ethanol

APPENDIX B

Accession numbers of sequences used in the phylogenetice analysis

	AHS 1 Isolate E160445	BTV Isolate BTV10IND2003k3	EEV Isolate Kimron1	EHDV Isolate CC 304-06
VP1	KX987198.1	KP339244.1	AB811635.1	HM641772.1
VP2	KX987199.1	KP339245.1	AB811636.1	HM641773.1
VP3	KX987200.1	KP339246.1	AB811637.1	HM641774.1
VP4	KX987201.1	KP339247.1	AB811638.1	HM641775.1
VP5	KX987202.1	KP339248.1	AB811639.1	HM641777.1
VP6	KX987203.1	KP339249.1	AB811630.1	HM641780.1
VP7	KX987204.1	KP339250.1	AB811631.1	HM641778.1
NS1	KX987205.1	KP339251.1	AB811632.1	HM641776.1
NS2	KX987206.1	KP339252.1	AB811633.1	HM641779.1
NS3	KX987207.1	KP339253.1	AB811634.1	HM641781.1

APPENDIX C

Pairwise comparisons of amino acids for the different genes of the Palyam serotypes and the nucleotides of the concatenated dataset. The top shows distance and the bottom percentage identity.

VP1

		1	2	3	4	5	6	7	8	9	10	11	12	13
Bunyip_CreeK_VP1	1		0.01	0.02	0.04	0.04	0.03	0.07	0.07	0.07	0.07	0.08	0.07	0.07
D'Aguilar_VP1	2	99.46		0.01	0.04	0.04	0.03	0.07	0.07	0.07	0.07	0.07	0.07	0.07
CSIRO_Village_VP1	3	98.47	98.54		0.04	0.03	0.03	0.06	0.07	0.07	0.07	0.07	0.07	0.07
Marrakai_VP1	4	95.93	96.01	96.24		0.02	0.03	0.06	0.06	0.06	0.07	0.07	0.06	0.06
Kasba_VP1	5	96.39	96.47	96.85	97.62		0.02	0.06	0.07	0.06	0.06	0.07	0.06	0.06
Vellore_VP1	6	97.24	97.31	97.39	96.62	97.54		0.06	0.07	0.06	0.06	0.07	0.06	0.06
Petevo_VP1	7	93.71	93.63	94.09	94.55	94.40	94.47		0.04	0.07	0.07	0.07	0.06	0.06
Palyam_VP1	8	93.40	93.32	93.71	94.01	93.40	93.55	95.70		0.07	0.07	0.07	0.07	0.07
Nyabira_VP1	9	93.17	93.32	93.32	93.86	94.01	94.09	93.71	93.48		0.00	0.01	0.01	0.01
Gweru_VP1	10	92.86	93.02	93.02	93.63	93.78	94.01	93.48	93.40	99.54		0.01	0.01	0.02
Marondera_VP1	11	92.71	92.86	92.86	93.40	93.63	93.71	93.32	93.25	99.31	99.23		0.01	0.02
Abadina_VP1	12	93.32	93.48	93.32	94.17	94.32	94.40	94.09	93.55	99.08	98.93	98.70		0.01
Apies_River_VP1	13	92.86	93.02	92.86	93.78	94.01	94.09	93.94	93.17	98.62	98.47	98.23	99.31	

VP3

		1	2	3	4	5	6	7	8	9	10	11	12	13
CSIRO_Village_VP3	1		0.00	0.00	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.03	0.03
Bunyip_Creek_VP3	2	99.89		0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03
D'Aguilar_VP3	3	99.78	99.89		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.03
Kasba_VP3	4	98.57	98.46	98.35		0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.03
Vellore_VP3	5	98.13	98.02	97.91	99.45		0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03
Marrakai_VP3	6	98.57	98.46	98.35	98.90	98.35		0.02	0.02	0.02	0.02	0.02	0.02	0.03
Nyabira_VP3	7	97.80	97.69	97.58	98.35	97.80	98.13		0.00	0.00	0.00	0.00	0.02	0.02
Gweru_VP3	8	97.80	97.69	97.58	98.35	97.80	98.13	100.00		0.00	0.00	0.00	0.02	0.02
Marondera_VP3	9	97.80	97.69	97.58	98.35	97.80	98.13	100.00	100.00		0.00	0.00	0.02	0.02
Apies_River_VP3	10	97.91	97.80	97.69	98.24	97.69	98.24	99.78	99.78	99.78		0.00	0.02	0.02
Abadina_VP3	11	97.69	97.58	97.47	98.24	97.69	98.02	99.89	99.89	99.89	99.67		0.02	0.02
Petevo_VP3	12	96.92	96.92	96.81	97.58	97.25	97.58	98.24	98.24	98.24	98.02	98.13		0.02
Palyam_VP3	13	97.25	97.25	97.14	97.25	96.81	97.36	97.80	97.80	97.80	97.69	97.69	98.13	

VP4

		1	2	3	4	5	6	7	8	9	10	11	12	13
Gweru_VP4	1		0.00	0.01	0.01	0.01	0.06	0.06	0.06	0.07	0.07	0.07	0.06	0.07
Abadina_VP4	2	99.53		0.01	0.01	0.01	0.06	0.06	0.06	0.07	0.08	0.07	0.06	0.07
Nyabira_VP4	3	99.38	99.22		0.01	0.01	0.06	0.06	0.06	0.07	0.07	0.06	0.06	0.06
Apies_River_VP4	4	98.76	98.60	99.07		0.01	0.06	0.06	0.06	0.06	0.07	0.06	0.06	0.06
Marondera_VP4	5	98.91	98.76	99.22	98.91		0.06	0.06	0.06	0.07	0.08	0.07	0.07	0.07
Petevo_VP4	6	94.25	94.25	94.57	94.41	94.25		0.05	0.06	0.07	0.08	0.07	0.07	0.07
Palyam_VP4	7	93.94	93.94	94.10	94.25	93.94	94.72		0.05	0.06	0.07	0.06	0.06	0.06
Kasba_VP4	8	94.10	93.79	94.10	94.25	93.79	93.79	94.88		0.02	0.05	0.05	0.05	0.06
Vellore_VP4	9	93.63	93.32	93.63	93.79	93.32	93.32	94.25	98.45		0.04	0.06	0.05	0.06
CSIRO_Village_VP4	10	93.01	92.70	93.01	93.48	92.70	92.08	93.17	95.19	96.43		0.05	0.05	0.05
Bunyip_Creek_VP4	11	93.63	93.63	93.94	93.79	93.32	92.86	94.25	95.19	94.57	95.19		0.00	0.02
D'Aguilar_VP4	12	93.79	93.79	94.10	94.10	93.63	93.01	94.41	95.34	94.72	95.03	99.53		0.02
Marrakai_VP4	13	93.48	93.48	93.79	93.79	93.32	93.32	94.57	94.57	94.41	95.50	97.83	97.98	

VP5

	1	2	3	4	5	6	7	8	9	10	11	12	13
Kasba_VP5		0.00	0.02	0.02	0.02	0.07	0.17	0.24	0.25	0.25	0.23	0.24	0.24
Vellore_VP5	100.00		0.02	0.02	0.02	0.07	0.17	0.24	0.25	0.25	0.23	0.24	0.24
Marrakai_VP5	98.48	98.48		0.02	0.03	0.07	0.17	0.25	0.24	0.24	0.23	0.24	0.24
Gweru_VP5	97.91	97.91	97.53		0.01	0.05	0.17	0.25	0.24	0.24	0.23	0.24	0.24
Abadina_VP5	97.72	97.72	97.34	99.05		0.06	0.17	0.24	0.24	0.24	0.23	0.24	0.24
CSIRO_Village_VP5	93.55	93.55	93.55	95.07	94.50		0.21	0.20	0.25	0.25	0.23	0.26	0.26
Petevo_VP5	84.44	84.44	84.06	84.25	84.44	81.02		0.25	0.25	0.25	0.25	0.22	0.23
Palyam_VP5	78.56	78.56	77.99	78.37	78.56	81.97	77.99		0.24	0.24	0.24	0.27	0.26
Apies_River_VP5	78.37	78.37	78.75	78.75	78.75	77.80	78.37	78.94		0.00	0.07	0.17	0.19
Marondera_VP5	78.37	78.37	78.75	78.75	78.75	77.80	78.37	78.94	100.00		0.07	0.17	0.19
Bunyip_Creek_VP5	79.70	79.70	79.32	79.89	79.89	79.32	78.37	78.75	92.98	92.98		0.17	0.17
D'Aguilar_VP5	78.94	78.94	78.75	78.94	78.94	77.61	80.08	76.66	84.63	84.63	84.06		0.03
Nyabira_VP5	78.94	78.94	78.75	78.94	78.94	77.61	79.70	77.23	83.11	83.11	84.25	96.58	

VP6

	1	2	3	4	5	6	7	8	9	10	11	12	13
Bunyip_CreeK_VP6		0.01	0.03	0.06	0.06	0.06	0.12	0.13	0.14	0.15	0.14	0.14	0.15
D'Aguilar_VP6	98.92		0.02	0.06	0.06	0.06	0.11	0.12	0.15	0.16	0.15	0.15	0.16
CSIRO_Village_VP6	97.49	97.85		0.06	0.06	0.06	0.11	0.12	0.15	0.16	0.15	0.15	0.15
Kasba_VP6	94.27	94.62	94.62		0.00	0.03	0.10	0.11	0.14	0.15	0.14	0.13	0.13
Vellore_VP6	94.27	94.62	94.62	100.00		0.03	0.10	0.11	0.14	0.15	0.14	0.13	0.13
Marrakai_VP6	94.27	94.62	94.62	97.13	97.13		0.10	0.10	0.15	0.16	0.16	0.15	0.14
Petevo_VP6	88.89	89.96	89.61	90.32	90.32	90.32		0.08	0.11	0.12	0.12	0.12	0.12
Palyam_VP6	88.17	88.53	88.89	89.96	89.96	90.68	92.47		0.13	0.14	0.14	0.12	0.12
Gweru_VP6	87.10	86.02	86.02	87.10	87.10	86.38	89.25	87.81		0.01	0.03	0.04	0.05
Marondera_VP6	86.38	85.30	85.30	86.38	86.38	85.66	88.53	87.10	99.28		0.04	0.05	0.06
Apies_River_VP6	87.10	86.02	86.02	87.10	87.10	85.66	88.53	87.10	97.13	96.42		0.04	0.05
Nyabira_VP6	86.74	86.38	86.38	87.46	87.46	86.02	88.89	88.89	96.06	95.34	96.06		0.04
Abadina_VP6	86.02	85.66	86.38	87.46	87.46	86.74	88.53	88.53	95.34	94.62	95.34	95.70	

NS1

	1	2	3	4	5	6	7	8	9	10	11	12	13
Nyabira_NS1		0.00	0.00	0.00	0.00	0.03	0.03	0.04	0.04	0.04	0.04	0.06	0.06
Apies_River_NS1	100.00		0.00	0.00	0.00	0.03	0.03	0.04	0.04	0.04	0.04	0.06	0.06
Abadina_NS1	99.82	99.82		0.00	0.01	0.03	0.04	0.04	0.04	0.04	0.04	0.06	0.06
Marondera_NS1	100.00	100.00	99.82		0.00	0.03	0.03	0.04	0.04	0.04	0.04	0.06	0.06
Gweru_NS1	99.64	99.64	99.46	99.64		0.04	0.04	0.04	0.04	0.04	0.04	0.06	0.06
Kasba_NS1	96.76	96.76	96.58	96.76	96.40		0.00	0.05	0.05	0.05	0.05	0.06	0.07
Vellore_NS1	96.58	96.58	96.40	96.58	96.22	99.82		0.05	0.05	0.05	0.05	0.06	0.07
Bunyip_CreeK_NS1	96.22	96.22	96.04	96.22	95.86	95.32	95.14		0.01	0.01	0.02	0.07	0.08
D'Aguilar_NS1	96.22	96.22	96.04	96.22	95.86	95.32	95.14	99.46		0.01	0.02	0.07	0.08
CSIRO_Village_NS1	96.22	96.22	96.04	96.22	95.86	95.50	95.32	98.92	99.10		0.02	0.07	0.08
Marrakai_NS1	96.04	96.04	95.86	96.04	95.68	95.14	94.96	97.84	98.02	98.20		0.07	0.08
Petevo_NS1	94.42	94.42	94.60	94.42	94.06	94.06	93.88	93.71	93.71	93.71	93.35		0.07
Palyam_NS1	94.24	94.24	94.06	94.24	93.88	93.17	92.99	92.45	92.27	92.27	92.45	93.71	

NS2

		1	2	3	4	5	6	7	8	9	10	11	12	13
Bunyip_CreeK_NS2	1		0.01	0.03	0.07	0.12	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.10
D'Aguilar_NS2	2	99.41		0.03	0.07	0.12	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.10
CSIRO_Village_NS2	3	97.35	97.35		0.07	0.11	0.11	0.11	0.11	0.08	0.08	0.09	0.08	0.09
Petevo_NS2	4	93.22	93.22	92.92		0.08	0.08	0.09	0.07	0.07	0.07	0.08	0.08	0.08
Kasba_NS2	5	89.09	89.09	89.68	92.04		0.00	0.03	0.05	0.07	0.07	0.08	0.08	0.08
Vellore_NS2	6	89.38	89.38	89.97	92.33	99.71		0.02	0.05	0.07	0.07	0.07	0.08	0.08
Marrakai_NS2	7	89.38	89.38	89.68	91.15	97.35	97.64		0.07	0.09	0.09	0.09	0.09	0.09
Palyam_NS2	8	89.38	89.38	89.38	93.22	95.28	94.99	93.22		0.10	0.10	0.11	0.11	0.11
Nyabira_NS2	9	90.56	90.56	91.74	92.63	92.63	92.92	91.45	89.97		0.00	0.00	0.01	0.01
Gweru_NS2	10	90.56	90.56	91.74	92.63	92.63	92.92	91.45	89.97	100.00		0.00	0.01	0.01
Marondera_NS2	11	90.27	90.27	91.45	92.33	92.33	92.63	91.15	89.68	99.70	99.70		0.01	0.01
Apies_River_NS2	12	90.56	90.56	91.74	92.04	92.04	92.33	90.86	89.38	99.41	99.41	99.11		0.01
Abadina_NS2	13	89.97	89.97	91.15	92.04	92.04	92.33	90.86	89.38	99.41	99.41	99.11	99.41	

NS3

		1	2	3	4	5	6	7	8	9	10	11	12	13
Nyabira_NS3	1		0.00	0.00	0.01	0.00	0.06	0.06	0.07	0.06	0.08	0.08	0.07	0.06
Gweru_NS3	2	100.00		0.00	0.01	0.00	0.06	0.06	0.07	0.06	0.08	0.08	0.07	0.06
Abadina_NS3	3	100.00	100.00		0.01	0.00	0.06	0.06	0.07	0.06	0.08	0.08	0.07	0.06
Apies_River_NS3	4	99.08	99.08	99.08		0.01	0.06	0.06	0.07	0.06	0.09	0.09	0.07	0.07
Marondera_NS3	5	99.54	99.54	99.54	98.62		0.07	0.06	0.07	0.07	0.09	0.08	0.08	0.07
Bunyip_CreeK_NS3	6	94.04	94.04	94.04	94.04	93.58		0.00	0.01	0.05	0.09	0.09	0.08	0.08
D'Aguilar_NS3	7	94.50	94.50	94.50	94.50	94.04	99.54		0.01	0.04	0.09	0.08	0.07	0.07
CSIRO_Village_NS3	8	93.58	93.58	93.58	93.58	93.12	98.62	99.08		0.05	0.09	0.09	0.07	0.08
Palyam_NS3	9	94.04	94.04	94.04	94.04	93.58	95.41	95.87	94.95		0.08	0.07	0.06	0.08
Vellore_NS3	10	92.20	92.20	92.20	91.28	91.74	91.28	91.74	91.28	92.66		0.00	0.03	0.03
Petevo_NS3	11	92.66	92.66	92.66	91.74	92.20	91.74	92.20	91.74	93.12	99.54		0.04	0.04
Marrakai_NS3	12	93.12	93.12	93.12	93.12	92.66	92.66	93.12	93.12	94.50	96.79	96.33		0.05
Kasba_NS3	13	94.04	94.04	94.04	93.12	93.58	92.66	93.12	92.20	92.20	96.79	96.33	94.95	

Concatenated dataset

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Abadina	1		0.00	0.00	0.64	0.13	0.72	0.26	0.23	0.26	0.79	0.73	0.09	0.10	0.09	0.09	0.09	0.18	0.14	0.23	0.13	0.15	0.25	0.24	0.18
Abadina_2389	2	99.77		0.00	0.64	0.13	0.72	0.26	0.23	0.26	0.79	0.73	0.09	0.10	0.09	0.09	0.09	0.18	0.14	0.23	0.13	0.15	0.25	0.24	0.18
Abadina_2582	3	99.78	99.70		0.64	0.13	0.72	0.26	0.23	0.26	0.79	0.73	0.09	0.10	0.10	0.09	0.09	0.19	0.14	0.23	0.13	0.15	0.25	0.24	0.18
AHS	4	53.13	53.07	53.08		0.63	0.76	0.64	0.64	0.64	0.81	0.77	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.58	0.63	0.64	0.64
Apies_River	5	87.27	87.21	87.18	53.52		0.73	0.21	0.27	0.24	0.79	0.73	0.14	0.14	0.14	0.14	0.14	0.26	0.03	0.25	0.12	0.14	0.24	0.25	0.26
BTV	6	49.69	49.66	49.69	47.27	49.32		0.73	0.73	0.73	0.80	0.52	0.73	0.73	0.73	0.73	0.73	0.72	0.73	0.72	0.73	0.65	0.73	0.73	0.72
Bunyip_Creek	7	77.26	77.18	77.19	53.38	81.74	49.42		0.14	0.10	0.79	0.74	0.26	0.26	0.26	0.26	0.26	0.23	0.21	0.20	0.25	0.24	0.24	0.25	0.22
CSIRO_Village	8	79.94	79.84	79.85	53.26	76.97	49.24	86.29		0.15	0.79	0.73	0.14	0.14	0.14	0.14	0.14	0.21	0.27	0.19	0.26	0.26	0.26	0.25	0.19
DAguilar	9	77.21	77.12	77.17	53.17	78.82	49.30	89.81	85.83		0.79	0.73	0.26	0.26	0.26	0.26	0.26	0.23	0.24	0.20	0.15	0.15	0.16	0.25	0.22
EEV	10	46.42	46.41	46.41	45.24	46.42	45.46	46.39	46.45	46.27		0.82	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.70	0.80	0.79	0.79
EHDV	11	49.44	49.43	49.38	47.33	49.23	59.21	49.10	49.22	49.29	44.91		0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.72	0.72	0.64	0.73	0.72	0.73
Gweru	12	90.67	90.58	90.54	53.27	86.81	49.35	77.16	87.00	77.02	46.36	49.22		0.00	0.00	0.00	0.00	0.23	0.12	0.23	0.12	0.15	0.25	0.24	0.22
Gweru_1828	13	90.41	90.37	90.33	53.24	86.60	49.30	77.11	86.85	76.96	46.31	49.21	99.50		0.01	0.00	0.00	0.23	0.12	0.23	0.12	0.15	0.25	0.24	0.22
Gweru_1832	14	90.60	90.55	90.51	53.25	86.74	49.34	77.14	86.91	77.00	46.33	49.23	99.64	99.41		0.00	0.00	0.23	0.12	0.23	0.12	0.15	0.25	0.24	0.22
Gweru_1856	15	90.65	90.61	90.56	53.25	86.82	49.35	77.19	86.95	77.02	46.37	49.24	99.75	99.53	99.69		0.00	0.23	0.12	0.23	0.12	0.15	0.25	0.24	0.22
Gweru_2038	16	90.68	90.64	90.59	53.29	86.82	49.35	77.18	86.98	77.04	46.36	49.22	99.78	99.56	99.79	99.84		0.22	0.12	0.23	0.12	0.14	0.25	0.24	0.22
Kasba	17	83.64	83.58	83.55	53.12	77.61	49.76	79.60	81.56	79.30	46.41	49.26	80.03	79.95	79.97	80.00	80.05		0.26	0.18	0.26	0.25	0.25	0.23	0.03
Marondera	18	86.76	86.68	86.65	53.44	97.22	49.34	81.56	76.81	78.76	46.41	49.21	88.18	88.01	88.10	88.20	88.20	77.39		0.25	0.10	0.13	0.24	0.25	0.26
Marrakai	19	79.74	79.67	79.64	52.99	77.94	49.52	81.75	82.50	81.48	46.56	49.57	79.82	79.65	79.80	79.79	79.83	83.42	77.89		0.25	0.25	0.24	0.21	0.18
Nyabira	20	87.03	86.96	86.91	53.22	88.58	49.36	78.54	76.92	86.16	46.27	49.52	87.96	87.80	87.90	87.97	87.98	77.48	89.84	77.93		0.03	0.17	0.25	0.25
Nyabira_1772	21	84.31	84.28	84.23	55.28	85.81	51.81	77.81	76.24	85.23	48.93	52.04	84.85	84.81	84.85	84.89	84.93	76.77	86.78	77.03	96.38		0.17	0.25	0.26
Palyam	22	77.81	77.75	77.75	53.32	78.94	49.22	78.69	77.45	85.56	45.91	49.32	77.86	77.73	77.82	77.84	77.90	78.25	79.08	78.11	84.85	83.41		0.22	0.25
Petevo	23	78.79	78.75	78.71	53.22	77.78	49.32	77.41	78.20	77.49	46.47	49.40	78.74	78.70	78.73	78.72	78.74	79.16	77.82	81.30	77.79	76.86	79.99		0.23
Vellore	24	83.75	83.68	83.66	53.20	77.81	49.95	80.30	82.87	80.06	46.46	49.23	80.19	80.10	80.14	80.17	80.20	97.01	77.58	83.37	77.68	76.71	78.08	79.52	

APPENDIX D

The nucleotide content of each genome of the Palyam viruses in the study

VP1

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	1479	0.376	2451	0.624
Bunyip Creek	1527	0.389	2403	0.611
CSIRO Village	1520	0.387	2410	0.613
D'Aguilar	1523	0.388	2407	0.612
Kasba	1511	0.384	2419	0.616
Marrakai	1428	0.363	2502	0.637
Palyam	1440	0.366	2490	0.634
Petevo	1481	0.397	2449	0.623
Vellore	1504	0.383	2378	0.605
Apies River	1474	0.375	2456	0.625
Gweru	1476	0.376	2454	0.624
Marondera	1480	0.377	2450	0.623
Nyabira	1489	0.379	2441	0.621
Abadina 2582	1473	0.375	2457	0.625
Abadina 2389	1478	0.376	2452	0.625
Gweru 1828	1479	0.376	2451	0.624
Gweru 1832	1479	0.376	2451	0.624
Gweru 2038	1475	0.375	2455	0.625
Gweru 1856	1475	0.375	2455	0.625

VP2

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	1161	0.380	1031	0.620
Bunyip Creek	1226	0.400	1838	0.600
CSIRO Village	1203	0.388	1894	0.612
D'Aguilar	1146	0.379	1876	0.621
Kasba	1165	0.381	1890	0.619
Marrakai	1178	0.382	1903	0.618
Palyam	1150	0.380	1875	0.620
Petevo	1247	0.410	1794	0.590
Vellore	1176	0.385	1879	0.615
Apies River	1184	0.385	1889	0.615
Gweru	1203	0.388	1895	0.612
Marondera	1153	0.382	1869	0.618
Nyabira	1172	0.381	1901	0.619
Abadina 2582	1161	0.380	1894	0.620
Abadina 2389	1161	0.380	1894	0.620
Nyabira 1772	1199	0.397	1821	0.603
Gweru 1828	1199	0.387	1899	0.613
Gweru 1832	1200	0.387	1898	0.613
Gweru 2038	1200	0.387	1898	0.613
Gweru 1856	1201	0.388	1897	0.612

VP3

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	1109	0.400	1665	0.600
Bunyip Creek	1105	0.398	1669	0.602
CSIRO Village	1093	0.394	1681	0.606
D'Aguilar	1106	0.399	1668	0.601
Kasba	1080	0.389	1694	0.611
Marrakai	1063	0.383	1711	0.617
Palyam	1092	0.394	1682	0.606
Petevo	1105	0.398	1669	0.602
Vellore	1087	0.392	1687	0.608
Apies River	1097	0.395	1677	0.605
Gweru	1103	0.398	1671	0.602
Marondera	1104	0.398	1670	0.602
Nyabira	1105	0.398	1669	0.602
Abadina 2582	1113	0.404	1645	0.596
Abadina 2389	1112	0.401	1659	0.599
Nyabira 1772	1113	0.404	1645	0.596
Gweru 1828	1107	0.399	1664	0.601
Gweru 1832	1107	0.399	1664	0.601
Gweru 2038	1107	0.399	1664	0.601
Gweru 1856	1110	0.401	1661	0.599

VP4

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	780	0.397	1187	0.603
Bunyip Creek	745	0.379	1222	0.621
CSIRO Village	791	0.402	1176	0.598
D'Aguilar	748	0.380	1219	0.620
Kasba	730	0.371	1237	0.629
Marrakai	750	0.381	1217	0.619
Palyam	749	0.381	1218	0.619
Petevo	772	0.392	1195	0.608
Vellore	765	0.389	1202	0.611
Apies River	787	0.400	1180	0.600
Gweru	776	0.395	1191	0.605
Marondera	784	0.399	1183	0.601
Nyabira	788	0.401	1179	0.599
Abadina 2582	780	0.397	1187	0.603
Abadina 2389	780	0.397	1187	0.603
Nyabira 1772	788	0.400	1180	0.600
Gweru 1828	777	0.395	1190	0.605
Gweru 1832	775	0.394	1192	0.606
Gweru 2038	775	0.394	1192	0.606
Gweru 1856	777	0.395	1190	0.605

VP5

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	661	0.411	949	0.589
Bunyip Creek	650	0.404	960	0.596
CSIRO Village	658	0.409	952	0.591
D'Aguilar	664	0.412	946	0.588
Kasba	641	0.398	969	0.602
Marrakai	641	0.398	969	0.602
Palyam	649	0.403	961	0.597
Petevo	653	0.406	957	0.594
Vellore	642	0.399	968	0.601
Apies River	655	0.407	955	0.593
Gweru	657	0.408	953	0.592
Marondera	656	0.407	954	0.593
Nyabira	654	0.406	956	0.588
Abadina 2582	659	0.409	951	0.591
Abadina 2389	660	0.410	950	0.590
Nyabira 1772	684	0.423	932	0.577
Gweru 1828	655	0.407	955	0.593
Gweru 1832	657	0.408	953	0.592
Gweru 2038	657	0.408	953	0.592
Gweru 1856	656	0.407	954	0.593

VP6

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	374	0.426	503	0.574
Bunyip Creek	362	0.413	515	0.587
CSIRO Village	368	0.417	509	0.583
D'Aguilar	360	0.410	517	0.590
Kasba	361	0.412	516	0.588
Marrakai	357	0.407	520	0.593
Palyam	360	0.410	517	0.590
Petevo	363	0.414	514	0.586
Vellore	361	0.412	516	0.588
Apies River	366	0.417	511	0.583
Gweru	362	0.413	515	0.587
Marondera	365	0.416	512	0.584
Nyabira	374	0.426	503	0.574
Abadina 2582	374	0.426	503	0.574
Abadina 2389	376	0.429	501	0.571
Nyabira 1772	374	0.426	503	0.574
Gweru 1828	362	0.413	515	0.587
Gweru 1832	364	0.415	513	0.585
Gweru 2038	362	0.413	515	0.587
Gweru 1856	362	0.413	515	0.587

VP7

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	518	0.450	633	0.550
Bunyip Creek	521	0.453	630	0.547
CSIRO Village	516	0.448	635	0.552
D'Aguilar	516	0.448	635	0.552
Kasba	518	0.450	633	0.550
Marrakai	520	0.452	631	0.548
Palyam	500	0.434	651	0.566
Petevo	517	0.449	634	0.551
Vellore	518	0.450	633	0.550
Apies River	521	0.453	630	0.547
Gweru	523	0.454	628	0.546
Marondera	521	0.453	630	0.547
Nyabira	521	0.453	630	0.547
Abadina 2582	521	0.453	630	0.547
Abadina 2389	520	0.452	631	0.548
Nyabira 1772	523	0.546	628	0.546
Gweru 1828	524	0.455	627	0.545
Gweru 1832	522	0.454	629	0.546
Gweru 2038	524	0.455	627	0.545
Gweru 1856	524	0.455	627	0.545

NS1

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	685	0.387	1087	0.613
Bunyip Creek	688	0.388	1084	0.612
CSIRO Village	698	0.394	1074	0.606
D'Aguilar	692	0.391	1080	0.609
Kasba	671	0.379	1101	0.621
Marrakai	682	0.385	1090	0.615
Palyam	688	0.388	1084	0.612
Petevo	691	0.390	1081	0.610
Vellore	672	0.379	1100	0.621
Apies River	693	0.391	1079	0.609
Gweru	688	0.388	1084	0.612
Marondera	683	0.385	1089	0.615
Nyabira	684	0.386	1088	0.614
Abadina 2582	686	0.387	1085	0.613
Abadina 2389	682	0.385	1089	0.615
Nyabira 1772	690	0.390	1077	0.610
Gweru 1828	684	0.386	1087	0.614
Gweru 1832	684	0.386	1087	0.614
Gweru 2038	684	0.386	1087	0.614
Gweru 1856	684	0.386	1087	0.614

NS2

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	438	0.415	617	0.585
Bunyip Creek	436	0.412	622	0.588
CSIRO Village	434	0.41	624	0.590
D'Aguilar	441	0.417	617	0.583
Kasba	430	0.406	628	0.594
Marrakai	426	0.403	632	0.597
Palyam	421	0.398	637	0.602
Petevo	414	0.391	644	0.609
Vellore	432	0.408	626	0.592
Apies River	434	0.411	621	0.589
Gweru	429	0.407	626	0.593
Marondera	430	0.408	625	0.592
Nyabira	429	0.407	626	0.593
Abadina 2582	437	0.414	618	0.586
Abadina 2389	438	0.415	617	0.585
Nyabira 1772	432	0.409	623	0.591
Gweru 1828	432	0.409	623	0.591
Gweru 1832	431	0.409	624	0.591
Gweru 2038	428	0.406	627	0.594
Gweru 1856	427	0.405	628	0.595

NS3

Virus	C + G		A + T	
	Count	Frequency	Count	Frequency
Abadina	302	0.415	426	0.585
Bunyip Creek	309	0.424	419	0.576
CSIRO Village	310	0.426	418	0.574
D'Aguilar	307	0.422	421	0.578
Kasba	308	0.423	420	0.577
Marrakai	303	0.416	425	0.584
Palyam	307	0.422	421	0.578
Petevo	305	0.419	423	0.581
Vellore	304	0.418	424	0.582
Apies River	303	0.416	425	0.584
Gweru	303	0.416	425	0.584
Marondera	305	0.419	423	0.581
Nyabira	306	0.420	422	0.580
Abadina 2582	302	0.415	426	0.585
Abadina 2389	302	0.415	426	0.585
Nyabira 1772	307	0.422	421	0.578
Gweru 1828	302	0.415	426	0.585
Gweru 1832	306	0.420	422	0.580
Gweru 2038	305	0.419	423	0.581
Gweru 1856	304	0.418	424	0.582

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Characterization of Palyam serogroup orbiviruses**
PROJECT NUMBER	**V139-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**K Ebershon**

STUDENT NUMBER (where applicable)	**UP_4296834**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL ANIMALS	**No animals involved**	
NUMBER OF ANIMALS	**No animals involved**	
Approval period to use animals for research/testing purposes		**November 2016-November 2017**
SUPERVISOR	**Prof. EH Venter**	

KINDLY NOTE:

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

APPROVED	Date 28 November 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Characterization of Palyam serogroup orbiviruses**
PROJECT NUMBER	**V139-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**K Ebershon**

STUDENT NUMBER (where applicable)	**U_4296834**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL ANIMALS	**No animals involved**
NUMBER OF ANIMALS	**No animals involved**
Approval period to use animals for research/testing purposes	**March 2018 – March 2019**
SUPERVISOR	**Prof. EH Venter**

KINDLY NOTE:

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

APPROVED	Date 5 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Henry Golobi • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za

K Ebersohn
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
Private bag x04
Onderstepoort
0110

Email: Karen.ebersohn@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Ms Ebersohn

Your application, received on 13 February 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Only freeze-dried viruses currently stored at the DVTD may be used in this research. No new field samples are permitted to be collected or utilised;
4. Amplicons may be sent for next generation sequencing at Inqaba Biotech.