DEVELOPMENT AND EVALUATION OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR EQUINE ENCEPHALOSIS VIRUS

by

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DEDICATION

To myself, Ntungufhadzeni Maclaughlin Rathogwa for commitment, patience and hardwork.

DECLARATION

I, **NTUNGUFHADZENI MACLAUGHLIN RATHOGWA** (student number 29615195) declare that this dissertation has not been submitted previously by me for a degree at this or any other institution and that all the information referred to and contained herein have been duly acknowledged.

Signature**…………………………..**   Date**………………………………..**

ACKNOWLEDGEMENTS

ABBREVIATIONS

βME	2-mercaptoethanol
AA	amino acid
AGID	agarose gel immune diffusion
AHS	African horse sickness
AHSV	African horse sickness virus
BHK	baby hamster kidney
bp	base pair/s
BT	bluetongue
BTV	bluetongue virus
cELISA	competitive enzyme-linked immunosorbent assay
CFT	complement fixation test
CLP	core-like particles
CPE	cytopathic effect
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double-stranded
dsRNA	double-stranded ribonucleic acid
DTT	dithiothreitol
EB	elution buffer
EDTA	ethylenediaminetetraaceticacid
EEE	eastern equine encephalitis
EEV	equine encephalosis virus
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
EtOH	ethanol
EVA	equine viral arteritis
h	hour/s
IFA	immunofluorescent antibody
MGB	minor groove binder
min	minute/s
MMOH	methyl mercury (II) hydroxide
NS	non-structural
OIE	World Organisation for Animal Health
PALV	Palyam virus
PBS	phosphate buffered saline

PCR   polymerase chain reaction

RNA   ribonucleic acid

rpm   revolutions per minute

RSA   Republic of South Africa

RT   reverse transcription

RT-PCR   reverse transcription polymerase chain reaction

rtRT-PCR   real-time reverse transcription polymerase chain reaction

s   second/s

sELISA   sandwich enzyme-linked immunosorbent assay

ss   single-stranded

TAE   tris-acetate-EDTA

UV   ultraviolet

VIB   virus inclusion bodies

VP   viral protein

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ABSTRACT

Equine encephalosis virus (EEV) is the cause of equine encephalosis. The disease is similar to mild forms of African horse sickness (AHS) and the two diseases are easily confused. Laboratory identification and serotyping of EEV is based on viral isolation in BHK-21 cells and a viral plaque inhibition neutralization test (Erasmus *et al.*, 1970). These procedures require long durations to confirm results and it was desirable that a rapid diagnostic assay was developed to distinguish EEV from African horse sickness virus (AHSV). A PCR test developed for AHSV (Quan *et al.*, 2008) formed the basis for development of a similar assay for EEV. The aim was to develop and evaluate a real time PCR assay for the detection of EEV in the blood and organs of horses.

FastPCR software was used to design primers to amplify and sequence the EEV S7 (VP7) gene. RNA was extracted from EEV tissue culture isolates, representing all seven serotypes, using a MagMax^{TM} Express Particle Processor and MagMax^{TM}-96 Total RNA Isolation kits. A one step reverse transcription PCR (RT-PCR) was carried out to amplify the EEV S7 gene using a GeneAmp Gold RNA PCR core kit. Sequence reactions were carried out using a BigDye terminal v3.1 sequencing kit and analyzed with an ABI 3130xl Genetic Analyzer. After sequences alignment using BioEdit software, conserved regions were identified and Primer Express 3.0 software was used to design EEV primers and TaqMan$^{®}$ MGB^{TM} hydrolysis probes for real-time RT-PCR assay.

The EEV real-time RT-PCR assay was specific and did not detect AHSV nor bluetongue virus (BTV). The real-time format was selected because of its convenience, sensitivity and ability to produce results rapidly. Validation of the assay is the next step in establishing it as a routine diagnostic assay.

CHAPTER

1. GENERAL INTRODUCTION

The concept of biological disease agents has existed since the nineteenth century, with clear differences between various harmful agents. Some were poorly understood and were called "virus", the Latin word for poisoning. Today viruses are well known and understood as infectious agents. Viruses can be pathogenic for their hosts, while others cause inapparent infections, but can be opportunistic in certain circumstances. Viruses are important causative agents of disease but are also of interest as tools in the research field for investigating the cellular and molecular biology of the host organism.

The first isolation of equine encephalosis virus (EEV) was accomplished in South Africa in 1967 from a 13-year-old Thoroughbred mare named Cascara from Mauritzfontein stud farm in the Kimberley district. The clinical signs included a temperature of 39.5 °C and a pulse rate of 44/min about 24 hours before death. Systemic signs included general venous congestion, fatty liver degeneration and brain oedema. Two additional mares became ill on the same stud farm from an apparently similar condition during the following few days. One of those died, while the second recovered after a convalescence of 14 days. The virus isolated from the first horse was characterized as an orbivirus and subsequently named equine encephalosis virus. The virus was also recovered from blood samples taken from other horses which had exhibited no clinical signs of disease except a febrile reaction.

In mid-1967, serum tested from horses in various localities in South Africa, showed very high antibody titres against Cascara virus, indicating a widespread occurrence of the viral infection. Most EEV infections are subclinical in nature and easily confused with AHSV infections because both virus infections exhibit similar clinical signs. This makes it very difficult to diagnose the disease clinically without using laboratory test methods to confirm the results. There are procedures available which are used in the laboratory for the diagnosis of EEV. Isolation of the virus is performed in baby hamster kidney (BHK) cells, suckling mice brains, or embryonated hen's eggs. The virus is also serotyped by the commonly used method of plaque inhibition neutralization. Other tests for antibody detection include complement fixation (CF), agar gel immunodiffusion (AGID) or indirect immunofluorescent antibody (IFA) test. Currently, there is a serological group-specific, indirect sandwich enzyme-linked immunosorbent assay (ELISA) available for the detection of equine encephalosis antigen. The disadvantages of these methods or procedures are that four to

seven days are required to obtain results and the methods only provide a retrospective diagnosis.

There is no polymerase chain reaction-based assay for the detection of EEV nucleic acid that has been described. It is desirable that a real-time polymerase chain reaction (PCR) assay to detect EEV is developed. It will provide several advantages over the use of ELISA and a conventional PCR assay. In real-time PCR assays, the PCR products are detected as they are produced and the advantages over gel-based PCR and ELISA assays are an increased analytical specificity, sensitivity and a more rapid assay with reduced risk for contamination since PCR products are produced in a single tube during the cycling process, eliminating the need for post-PCR product manipulation.

It is known that both AHSV and EEV affect equine species, and there are similarities that exist regarding their cytopathic effect and virus morphology. The clinical signs of both viruses are easily confused and a problem may be cases where it is not possible to distinguish them clinically. It would be advantageous if a rapid and reliable diagnostic assay for EEV were available to distinguish EEV and AHSV infections. It is important to detect and identify a virus infection at an early stage for rapid disease control. The aim of this research project was to develop and characterize a rapid and robust diagnostic real-time PCR assay for the detection of EEV nucleic acid in the blood and tissues of horses or identification of virus isolated in cell cultures.

The specific objectives of this project were to:

- To determine the VP7 gene sequence of EEV.

- To design specific primers and probes for development of a real-time polymerase chain reaction assay.

- To ensure the specificity of the assay to be able to differentiate between AHSV and EEV infected horses.

CHAPTER

2. LITERATURE REVIEW

2.1. Classification and characterization of EEV

Viruses can be classified based on different criteria e.g. the nature of the nucleic acid, host range, physiochemical properties and synthesis of its genomic nucleic acid within the host cell (life cycle). The genus *Orbivirus* in the family *Reoviridae,* subfamily *Sedoreovirinae* (Mertens *et al.,* 2005) consists of viruses with similar structural morphology and functional properties. It consists of 22 species and is the largest genus within the family *Reoviridae* (Table 2.1) (Calisher *et al.,* 1998; Van Niekerk *et al.,* 2003).

Table 2.1. Classification of EEV within the family *Reoviridae.*

Family	*Reoviridae*
Subfamily	*Sedoreovirinae*
Genus	*Orbivirus*
Species	African horse sickness virus
	Changuinola virus
	Bluetongue virus
	Chenuda virus
	Chobar Gorge virus
	Corriparta virus
	Epizootic hemorrhagic disease virus
	Equine encephalosis virus
	Eubenangee virus
	Great island virus
	Ieri virus
	Lebombo virus
	Orungo virus
	Palyam virus
	Peruvian horse sickness virus
	St Croix River virus
	Umatilla virus
	Wad Medani virus
	Wallal virus
	Warrego virus
	Wongorr virus
	Yunnan orbivirus

Orbiviruses are named after their characteristic doughnut shaped capsomers. The genus name, *Orbivirus* was derived from the Latin word *orbis*, meaning ring or circle (Borden *et al.,*

1971). They are non-enveloped particles that are between 70-80 nm in diameter. The virus particles are spherical in appearance and have icosahedral symmetry. They have an outer and an inner capsid layer that surround the genome. Orbiviruses can infect and replicate within a wide range of arthropod and vertebrate hosts. The genus contains several viruses pathogenic for domestic animals [e.g. bluetongue virus (BTV) in sheep and African horse sickness virus (AHSV) in equids], man, wildlife [e.g. epizootic haemorrhagic disease virus (EHDV)] and many other viruses not yet linked with disease (e.g. St. Croix River virus) but classified within orbivirus species (Gorman, 1979). The genus is divided into serogroups based on the cross reactivities in complement fixation tests, and serotypes within serogroups are recognized by specific reactions in serum neutralization tests (Gorman, 1979; Gorman *et al.*, 1983).

Equine encephalosis virus (EEV), an orbivirus, is the aetiological agent of a disease known as equine encephalosis. The genome of EEV is similar to those of other orbiviruses and consists of ten double stranded (ds) RNA segments. It is closely related to both BTV (the prototype virus for the genus *Orbivirus*) and AHSV (Howell *et al.,* 2008; Quan *et al.,* 2008). All orbiviruses are transmitted by *Culicoides* midges.

The first EEV serotype was isolated in 1967, and this lead to the subsequent identification and isolation of another three serotypes between 1972 and 1976 (Gorman *et al.,* 1983). There are seven known serotypes of EEV (Table 2.2). The serotype numbers were assigned in alphabetical order with the first serotype Cascara as the prototype virus. The seven EEV serotypes are not equally prevalent in the field, suggesting that some serotypes are better adapted to either the vector (*C. Imicola* or *C. bolitinos*) or host environment. (Barnard & Paweska, 1993; Venter *et al.,* 1999; Howell *et al.,* 2002). Complement fixation tests have failed to demonstrate a serological relationship within any of the EEV serotypes or relatedness with any other Orbivirus such as BTV or AHSV (Erasmus *et al.,* 1970; Gorman *et al.*, 1983; Viljoen & Huismans, 1989).

Table 2.2. **EEV serotypes with numerical serotype designation (Howell *et al.,* 2002).**

Name of virus	Numerical serotype
Bryanston	EEV-1
Cascara	EEV-2
Gamil	EEV-3
Kaalplaas	EEV-4
Kyalami	EEV-5
Potchefstroom	EEV-6
E21/20	EEV-7

2.2. Structural characteristics

Little information is available about the structural or functional characteristics of EEV, but much work has been done on the viruses of the genus *Orbivirus* in general and has concentrated much on the type species BTV. EEV possesses a similar structure to that seen and described for both BTV and AHSV (Verwoerd *et al.,* 1970). EEV is non-enveloped and the size of the virion is 73 nm in diameter (60 nm when negatively stained). It consists of a dsRNA genome enclosed by a capsid composed of 32 hexamer morphological subunits with icosahedral symmetry or a dimer (or trimer) arrangement of the capsomers (Erasmus *et al.,* 1970).The capsid shell of the virions is composed of two layers. The capsomers have a flattened, hollow cylindrical or prismatic shape. The nucleocapsid structure (core particle) consists of five types of protein, two major (VP3 and VP7) and three minor components (VP1, VP4 and VP6) (Verwoerd *et al.,* 1970; Huismans & Howell, 1973; Bremer, 1976; Mertens *et al.,* 1984; Roy 1989 and 1996). The icosahedral core particle is surrounded by an outer capsid structure (or outer shell) composed of two proteins (VP2 and VP5). In addition to the seven structural proteins, there are four non-structural proteins, NS1, NS2 and NS3/NS3a. Each of the major structural and non-structural proteins is encoded on a different genome segment (Mertens *et al.,* 1984; Van Dijk & Huismans, 1988).

Depending on the experimental conditions, EEV can either be resistant or sensitive to chloroform, is relatively sensitive to sodium deoxycholate and sensitive to trypsin. Unlike BTV and AHSV, it can either be slightly sensitive or resistant to either chloroform or deoxycholate, while AHSV is resistant to the action of trypsin. EEV is labile at pH 3.0 (like BTV) and pH 5.0, as well as temperature-sensitive as it is inactivated at 56°C (stable at temperatures lower than 56 °C). The virus is also susceptible to actinomycin D (Erasmus *et al.,* 1970).

2.3. Viral genome composition

EEV contains ten segments of linear dsRNA (Roy, 1989). The size of the ten genome segments for EEV Cascara (EEV-1) has been estimated by agarose gel electrophoresis using BTV segments for comparison (Table 2.3). EEV has the interesting feature that all ten genomic dsRNA segments possess 5' and 3' end terminal protein sequences which are conserved and genus specific. The conserved sequences are in bold: 5'**GUU**WAD…….HSU**UAC**3' (Potgieter *et al.,* 2002).

Table 2.3. Genome segments of EEV-2 and their estimated sizes in base pairs (Viljoen & Huismans, 1989), compared to the estimated sizes of BTV-10 (Roy, 1989). L – large, M – medium, S – small.

Genome segment	EEV-2	BTV-10
L1	3 900	3 954
L2	3 220	2 926
L3	2 750	2 772
M4	2 020	2 011
M5	1 750	1 638
M6	1 570	1 769
S7	1 080	1 156
S8	1 080	1 124
S9	1 080	1 046
S10	710	822

Other orbiviruses such as AHSV and BTV have an additional feature of inverted repeats which differs in sequence for each segment and which is present next to the conserved termini (Roy, 1989). However, no inverted repeats were identified for EEV S10, although repeats have been identified in the genes that encode VP6 and VP7 (Potgieter *et al.,* 2002). In the *Reoviridae* family, the conserved terminal features of the RNA segments of viruses is thought to play a role in transcription initiation, RNA replication, ribosome binding and translation of mRNAs, as well as in the determination of mRNA secondary structure, which could be important in the sorting and assembly of the genome during viral replication (Huismans*,* 1971).

2.4. Structural proteins

The genome of EEV consists of ten segments of double stranded ribonucleic acid (dsRNA) encapsulated by a double-layered icosahedral shell (Huismans, 1979; Mertens *et al.,* 1984). Each of the segments codes for a viral protein, namely seven structural proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3a).

The genome is surrounded by inner and outer capsid proteins. The inner capsid, which forms the virus core, has two distinct layers of proteins, namely the inner layer or 'subcore' and the outer layer. The inner layer or 'subcore' is composed of VP3 arranged as 12 pentamers and associated with it are three minor structural proteins (the core-associated enzymes) VP1, VP4 and VP6 (Figure 2.1), which are thought to be involved in virus replication and transcription processes. These proteins are more conserved than the proteins of the outer capsid and they show no serological cross-reaction within EEV serotypes.
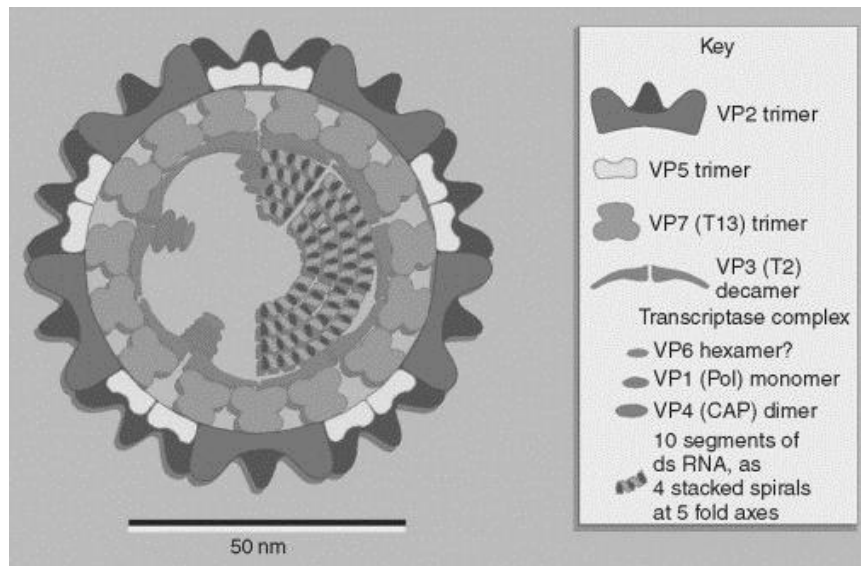
Figure 2.1. Schematic diagram of the structure of an orbivirus particle showing the location of various proteins within the virus particles (Mertens *et al.,* 2005).

The VP3 protein is hydrophobic, plays a major role in the structural integrity of the virus core and can bind ssRNA in a non-specific manner, probably during the virus assembly process (Roy, 1992). VP1 is the largest protein of all the minor proteins. It is present in a low molar ratio in the core. Based on its size, and the presence of a motif common to all polymerases in the gene encoding this protein, it is the prime candidate for the virion RNA polymerase (Mertens *et al.,* 1984; Roy *et al.,* 1996; Huismans & Van Dijk, 1990; Roy, 1992). VP4 binds GTP and may act as a guanyltransferase which catalyses the capping and methylation of the 5' end of viral mRNA (Roy, 1992; Roy *et al.,* 1994). VP6 is a highly basic protein and the smallest structural protein. It is closely associated with the virus genome, as it has a strong binding affinity for ss- and dsRNA. It also binds ATP and exhibits RNA dependent ATPase activity and a helicase activity that catalyses the unwinding of dsRNA substrates prior to the synthesis for the encapsidation of RNA (Roy, 1992; Roy *et al.,* 1994).

The outer layer of the inner capsid is composed of VP7. It forms the outer layer of the core and is made up of five or six membrane clusters of VP7 trimers (Eaton *et al.,* 1991). VP7 forms a knob-like protrusion and is located at the three-fold axes position of the core structural proteins. In between the VP7 structures are aqueous channels or holes of which some penetrate the inner VP7 layer. The inner layer makes a smooth bed of foundation layer upon which the VP7 trimers are located. The VP7 structures are close to the position of the two surface proteins, VP2 and VP5. VP7 is a highly immune-dominant antigen and antibodies against VP7 are detected in many EEV serogroup-specific serology assays (Anthony *et al.,* 2007). It is highly conserved among serotypes and serogroups (Mertens *et al.,* 1984; Bremer *et al.,* 1990) as it has limited exposure on the surface of the intact virion.

VP7 has at least two epitopes exposed on the virus surface, unlike BTV, where only half of VP7 reactive epitope in the amino terminal is accessible on the surface layer.

Surrounding the inner capsid protein is the outer capsid protein composed of VP5 and VP2. The VP5 protein exists as a globular and almost spherical structure. It is located on the channels formed by each of the six-membraned clusters of VP7 trimers. It is more conserved than VP2 among serotypes but not as conserved as the core proteins. It does not appear to have any direct role in binding neutralizing antibodies. However, studies with BTV have demonstrated that VP5 enhances neutralization and protective immune response (Roy *et al.,* 1990). The protein however, may also make some contribution to virulence as was found in one case which the most obvious difference between a virulent and a virulent strain of BTV was in the mobility of the VP5 genome segment. A possible role of the VP5 protein may be to facilitate the interaction between the highly variable VP2 protein with the more highly constrained structure of the virus core, probably by imposing conformational constraints on VP2 (Huismans & Howell, 1973).

The VP2 protein exists as a sail shaped spike which projects beyond the globular proteins and is located above the VP7 trimers. It contains the major serotype-specific antigenic epitope that segregates particular serogroups into distinct serotypes (Huismans & Erasmus, 1981; Roy *et al.,* 1994). It is also the most variable protein (Huismans & Howell, 1973; Roy *et al.,* 1990) and exhibits low conservation between serotypes and serogroups. It is involved in eliciting a protective immune response. Studies done with BTV showed that VP2 is a haemagglutinin and is directly involved in attachment of the virus to cells (Huismans & Van Dijk, 1990) and penetration to the host cell during the initiation of the virus infection (Burrage *et al.,* 1993; Stone-Marschat *et al.,* 1996; Anthony *et al.,* 2007). There is also evidence that VP2 plays a role in the virulence of certain virus strains (Huismans & Howell, 1973; O'Hara *et al.,* 1998).

2.5. Non-structural proteins

There are three non-structural proteins (NS1, NS2 and NS3). However, segment 10 encodes not only NS3, but also a second closely related polypeptide designated NS3a. Non-structural viral proteins are known to be involved in the replication and morphogenesis of virus, specifically assembly and release. Non-structural viral proteins are also responsible for the formation of the characteristic structures in virus infected cells. NS3 has also been shown to influence virulence (O'Hara *et al.,* 1998). The two non-structural proteins, NS1 and NS2, are also highly conserved (Huismans, 1971; Huismans *et al.,* 1987; Owens *et al.,* 2004; Anthony *et al.,* 2007) while the smallest viral protein NS3 is more variable and within some orbivirus

species (e.g. AHSV) is the most variable protein after VP2 (Van Niekerk *et al.,* 2001 and 2003).

NS1 has a unique non-specific structure. In the orbivirus infected cells, NS1 is synthesized in large quantities. Accumulation of NS1 in infected cells results in the formation of unique non-specific tubular structure (Mertens *et al.,* 1984).Little is known about the function of NS1 in orbivirus replication, but it has been suggested that unique non-specific tubular structures of NS1 is found throughout the infection cycle of the virus. They are attached to the intermediate filaments of the cell cytoskeleton and are associated with virus inclusion bodies (VIB) and virus particles (Eaton *et al.,* 1987) prior to the stage of virus morphogenesis. NS1 may be involved in the translocation of virus particles to the host cell membrane.

NS2 is also synthesized in large quantities and is responsible for the formation of virus inclusion bodies (VIB). In BTV studies, NS1 has been shown to exist as multimers with ssRNA binding properties. It may be involved in the selection and condensation of the RNA segments for encapsidation (Huismans *et al.,* 1987a). The N-terminal half of NS2 is more conserved among BTV, AHSV and EHDV than the C-terminal half which has a hydrophilic character and is predicted to have a high content of alpha helix conformation (van Staden *et al.,* 1991). NS2 has the unique property of being the only virus-specific phosphoprotein in BTV and AHSV infected cells, but the functional significance of NS2 phosphorylation has not been determined yet (Huismans *et al.,* 1987b).

Unlike NS1 and NS2, the two closely related non-structural proteins NS3 and NS3a are synthesized in small amounts in orbivirus infected cells. The S10 gene is the smallest segment of the EEV genome and contains two overlapping in-phase open reading frames that encode the non-structural protein NS3 and NS3a (Mertens *et al.,* 1984; van Staden & Huismans, 1991). NS3 is larger than NS3a as a result of an additional 10 amino acids at the N-terminal end. It is an integral membrane protein (Hyatt *et al.,* 1991; Wu *et al.,* 1992; Bansal *et al.,* 1998) and it has been suggested that the protein is involved in facilitating the final stage of viral morphogenesis and release of the virions from infected cells (Hyatt *et al.,* 1993; Stoltz *et al.,* 1996; Martin *et al.,* 1998; Wirblich *et al.,* 2006). It is cytotoxic to insect cells *in vitro* (van Staden *et al.,* 1995; van Niekerk *et al.,* 2001a) and it may play a role in the determination of virulence (O'Hara *et al.,* 1998) of the virus. Sequence data on the S10 gene of a number of AHSV serotypes indicate that these genes are not as conserved as the cognate genes within the BTV serogroup (Quan *et al.,* 2008).

2.6. Epidemiology

Equine encephalosis is endemic to southern Africa (Barnard, 1997; Venter *et al.,* 1999). The first confirmed outbreak of equine encephalosis outside southern Africa was reported in Israel, in 2008 and involved approximately 150 cases with no reported mortalities (Aharonson-Raz *et al.,* 2011). EEV is found more widely throughout southern Africa than AHSV which is restricted to certain areas (Barnard, 1997). This may be due to routine immunisation of many horses against AHSV, or as a result of more than one species involved in the transmission of the disease (Venter *et al.,* 1999).

EEV was isolated from organ specimens collected from Cascara, as well as from other cases where AHSV was suspected, and from the blood of horses which showed no other clinical signs other than fever. Several serological surveys were conducted immediately after the first isolation of the Cascara serotype. The results indicated a widespread occurrence of infection with EEV during first three months of 1967. It was observed that unstabled horses were more likely to have antibodies against EEV than stabled horses, suggesting that biting midges or insects played a role in the transmission of the virus.

EEV is transmitted between equid hosts by the bites of *Culicoides* spp. Midges (*Diptera:Ceratopogonidae*), specifically *C. imicola,* which is regarded as the main vector of EEV (Paweska *et al.,* 1999; Venter *et al.,* 1999). *C. imicola* is the most abundant vector of the *Culicoides* species associated with livestock in the summer rainfall region of southern Africa. It is also the biological vector of BTV and other orbiviruses (Venter *et al.,* 1999; Venter *et al.,* 2002). The first isolation of an EEV strain from *Culicoides* species in South Africa was done by Theodoridis *et al.* (1979), during an unsuccessful attempt to isolate bovine ephemeral fever virus. Subsequently, *C. bolitinos* has been confirmed as another vector for EEV (Paweska & Venter, 2004).

C. imicola and *C. Bolitinos* are widely distributed in sub-Saharan Africa, some parts of North America and southern Europe and southern Asia (Venter *et al.,* 1999). They are also vectors for BTV and AHSV and the potential exists for these diseases to spread to the same region when conditions are favourable for the insect vector and even to persist within those regions.

Infections of horses by EEV occur usually during the late summer and autumn seasons (Coetzer *et al.,* 2004), due to climatic conditions that favour the replication of C*ulicoides* midges. Although the disease has been recognized for many years, it has not been established conclusively how the virus maintain its infectivity and survives between outbreaks (William *et. al.,* 1993).

The prevalence of infection with individual serotypes of EEV may vary substantially from year to year in South Africa. Currently, serotype 4 is the most common isolate. Serotypes 1, 6 and 7 have been associated with extensive outbreaks in recent years while serotypes 2, 3 and 5 are relatively rare (Howell *et al.*, 2002; Howell *et al.*, 2008). The predominant serotype of EEV that circulates in a specific area can change in different years (Howell *et al.*, 2008). It has also been found that there is a continuous circulation or maintenance of the virus between its host and vector in the case of zebra, which act or function as a maintenance host that ensure the persistence of the infection at the population level (Barnard & Paweska, 1993). The prevalence of equine encephalosis is high, with more than 75% of horses and 85% of donkeys tested having antibodies against EEV (Venter *et al.,* 1999). In studies done by Howell *et al.,* (2002), horse serum samples from a ten year period (1990-2000) were tested and a 56.9% prevalence of neutralizing antibodies against one or more serotypes of the EEV was found (Howell *et al.,* 2002).

EEV, like AHSV, infects horses. Antibodies against EEV have also been found in zebra, donkeys and African elephant (Williams *et al.,* 1993; Barnard, 1997). EEV and AHSV share the same vectors and a host, so dual infections with both viruses is possible (Williams *et al.,* 1993).

2.7.   Clinical signs and pathogenesis

EEV was first isolated in 1967 from a 13 years old thoroughbred mare named Cascara at Mauritzfontein stud farm in the Kimberly district in South Africa. Signs were first observed 24 hours before death. It included listlessness and an anxious facial expression with tightening of the face, mouth and ocular commissures. Clinical examination revealed a slightly elevated temperature of 39.5 °C and a pulse rate of 44 beats per minute. Necropsy revealed marked venous congestion, particularly of the liver, kidney and subcutaneous tissue. The liver showed signs of fatty degeneration and sharply demarcated areas of catarrhal enteritis in the distal half of the small intestine. Over the next few days, two other adult mares became ill at the same stud. One of them died acutely, whereas the other one recovered after about 14 days (Erasmus *et al.,* 1970). In other parts of South Africa, there were reports of horse mortalities including the Police Academy in Pretoria. All of those animals had similar clinical signs and lesions.

The incubation period of equine encephalosis is approximately three to six days. Animals infected experimentally have only shown very mild clinical signs. Equine encephalosis is usually mild or subclinical, but most of the infected horses show either no signs of infection or slightly elevated rectal temperatures (39 ^{0}C) for one or two days, or a higher fever (40 ^{0}C to

41 ^{0}C) between one and five days. Fever may be accompanied by varying degrees of listlessness and inappetence. Some horses develop a slight reddish brown discolouration of the mucous membrane of the conjunctiva as a result of congestion and mild icterus (Erasmus *et al.,* 1978). Swelling of the eyelids, heads and supraorbital fossae may be observed. Central nervous system involvement, respiratory distress, abortion and acute heart failure have rarely been observed in some animals from which EEV was isolated (Coetzer *et al.,* 2004).

Most of the clinical and fatal cases were seen in animals older than seven years. Two young horses were infected experimentally with liver and spleen suspensions from the horse Cascara, and showed marked febrile responses that started three days post infection and lasted for four days, with a maximum of 41°C, after which the horses recovered fully. There were no other clinical signs observed. This suggests that age seems to play a role in the pathogenesis of the disease caused by EEV.

The great majority of EEV infections are subclinical in nature, as antibodies against EEV are found often in horses that had never showed any clinical signs of equine encephalosis (Erasmus *et al.,* 1978). There is high morbidity (60% to 70%) but a low mortality (5%) of the disease (Bremer & Viljoen, 1998; Howell *et al.*, 2004). Previous sero-epidemiological studies of EEV performed in South Africa have revealed a seroprevalence of between 77% (Paweska & Venter, 2004) and 84% (Howell *et al.,* 2008) in horses. The higher prevalence of EEV in Thoroughbred foals is thought to be caused by a loss of maternally-derived antibodies at four to seven months of age at a time when the risk of the disease (late summer) is greatest.

Due to the subclinical nature of the disease, no control measures are implemented in endemic areas. The stabling of horses from an hour or two before the sun has set until an hour or two after dawn (the period during which *Culicoides* midges vectors are most active) is a useful control method (Coetzer & Tustin, 2004). Screens on the windows and doors of stables can be used to minimize the number of *Culicoides* midges that enter the stables and no lights should be left on to avoid attracting insects. Insect repellents can be sprayed on horses and in stables and affected animals are treated symptomatically. Non-steroidal, anti-inflammatory drugs can be used to reduce the fever. Currently, there is no vaccine available for the control of EEV.

Little is known on the pathogenesis of EEV, as most orbivirus studies have been done on BTV. Orbiviruses infect vascular endothelial cells preferentially. The first interaction occurs between the virus outer capsid proteins and the host cellular receptors, resulting in viral entry

into the host cell by endocytosis. In the process, the outer capsid of the virus is removed. Viral replication takes place within the cytoplasm of the host cell (Bansal *et al.*, 2008).

Transcription of the viral genome occurs within the core particles and translation makes use of the host cell ribosomes. The speed of viral replication influences the pathogenesis of the virus. Viral proteins are synthesized from two days after initial infection and the new virions self-assemble within the cytoplasm and are released from the host cell by budding. This stage has a major impact on pathogenesis, as it determines the spread of infection within and between the organs (Schneider, 2000).

Some viral proteins are thought to play an important role as determinants of pathogenicity of the virus, viz VP2, VP5 and NS3. These proteins are involved in the interaction with cellular receptors, uncoating and the spread of viral particles in and between cells (Hassan & Roy, 1999; Huismans *et al.,* 2004).

2.8. Diagnosis

Since the majority of EEV infections are subclinical, confirmation is now based on seroconversion in paired sera. Where disease is suspected on clinical grounds, diagnosis is confirmed by several techniques including virus isolation in baby hamster kidney (BHK) cells or suckling mice, demonstration of EEV antigen by antigen capture ELISA, and seroconversion by cELISA, indirect ELISA, CFT, AGID and serum-virus neutralization test for serotyping the virus (Coetzer *et al.*, 2004; Howell *et al.,* 2002; Crafford *et al.,* 2003). To date, there is no PCR assay for diagnostic purposes.

CHAPTER

3. MATERIALS AND METHODS

3.1.   Primer design

FastPCR software V6.1.47 (www.primerdigital.com) (Kalendar *et al.,* 2009) was used together with an EEV S7 (VP7) gene sequence obtained from GenBank® (www.ncbi.nlm.nih.gov/genbank, accession number FJ183391), to design primers for amplification and sequencing of the S7 gene.

3.2.   EEV Isolates

The EEV isolates used for sequencing are listed in Table 3.1 and include all the reference strains and seven serotypes.

Table 3.1 EEV isolates used for sequencing. A dash represents an unknown passage number or isolation date.

Name	Serotype	Passage number	Isolation date
Reference strain	1 (Bryanston)	1	2004/11/26
E080099	1 (Bryanston)	6	2008/07/03
E080243	1 (Bryanston)	6	2008/08/14
E080340_1	1 (Bryanston)	3	2009/02/19
E080341_2	1 (Bryanston)	3	2009/02/19
E080342	1 (Bryanston)	3	2009/02/12
E090039	1 (Bryanston)	4	2009/04/03
E090047	1 (Bryanston)	3	2009/04/03
E090059	1 (Bryanston)	1	2009/04/09
Reference strain	2 (Cascara)	3	2002/09/09
E100043	2 (Cascara)	-	2010/05/06
Reference strain	3 (Gamil)	-	1998/08/06
Reference strain	4 (Kaaplaas)	-	1998/08/06
E080010_5	4 (Kaaplaas)	2	2008/01/31
E080013_2	4 (Kaaplaas)	2	2008/02/07
E080067	4 (Kaaplaas)	2	2008/03/20
E080129	4 (Kaaplaas)	2	2008/04/10
E080210_3	4 (Kaaplaas)	-	-
E080229_2	4 (Kaaplaas)	-	-
E080229_5	4 (Kaaplaas)	3	2008/05/29
E080260_10	4 (Kaaplaas)	4	2008/07/17
E100058	4 (Kaaplaas)	3	2010/05/06
Reference strain	5 (Kyalami)	3	2002/01/28
E090010_6	5 (Kyalami)	5	2009/04/09
E090010_15	5 (Kyalami)	3	2009/03/05

E090010_19	5 (Kyalami)	3	2009/03/05
E090011	5 (Kyalami)	4	2009/03/05
Reference strain	6 (Potchefstroom)	-	2002/02/25
E100020	6 (Potchefstroom)	4	2010/04/15
Reference strain	7 (E21/20)	6	2003/05/23
E080026	7 (E21/20)	2	2008/02/21
E080038	7 (E21/20)	2	2009/03/07
E080070	7 (E21/20)	7	2008/11/27
E080089_3	7 (E21/20)	-	2008/07/31
E080146	7 (E21/20)	9	2008/10/02
E080181	7 (E21/20)	-	-
E080186_1	7 (E21/20)	8	2008/08/07
E080207	7 (E21/20)	-	-

3.3. RNA Extractions

Viral dsRNA was extracted from EEV cell culture isolates. The contents of a flask were agitated and 500 µl transferred to a 1.5 ml eppendorf tube. Samples were spun at 11 000g for 5 min in a 5417C centrifuge (Eppendorf). The supernatant was discarded and the cell pellet mixed with 50 µl of phosphate buffered saline (PBS). Total nucleic acid extractions from the cell pellet were performed using a MagMax™-96 Total RNA Isolation kit (Lifetech), according to the manufacturer's instructions. The sample was mixed with 10 µl RNA Binding Beads, 10 µl Lysis/Binding Enhancer, 65 µl isopropanol and 65 µl Lysis/Binding Solution. The samples were placed in a MagMax™ Express Particle Processor (Lifetech) and a custom protocol run (Appendix). RNA Binding Beads were washed twice with Wash Solution 1 and twice with Solution 2 before elution of the RNA in 50 µl Elution Buffer. The RNA was stored at -20 °C until used.

3.4. Reverse transcription polymerase chain reaction (RT-PCR)

Extracted viral nucleic acid was denatured by adding 1 µl 0.2 M of methyl mercury (II) hydroxide (MMOH) (Alfa Aesar) per 5 µl RNA and incubated for 10 min at room temperature. The reaction was reduced by the addition of 1 µl of 1.0 M of 2-mercaptoethanol (βME) (Sigma) per 5 µl RNA (Quan *et al.,* 2008).

One-step RT-PCR reactions were performed using a GeneAmp Gold RNA PCR core kit (Lifetech) according to the manufacturer's instructions. Two reactions were performed per sample, each containing 5 µl 5X RT-PCR buffer, 1.75 µl 25 mM MgCl$_2$, 2 µl 10 mM dNTPs, 1.25 µl 100 mM DTT, 0.25 µl RNase Inhibitor, 0.25 µl AmpliTaqGold®DNA polymerase, 0.15 µl Multiscribe™reverse transcriptase, 3 µl denatured EEV, 0.5 µl of each 20 µM forward and reverse primers, made up to 25 µl with RNAse free water (Ambion). The RNA was reverse

transcribed at 42 ^{0}C for 12 min and then heated at 94 ^{0}C for 10 min, using a GeneAmp PCR system 9700 (Lifetech). This was followed by 40 amplification cycles consisting of 94 ^{0}C for 20 s, 55^{0}C for 30 s and 72 ^{0}C for 60 s, before a terminal extension step at 72 ^{0}C for 7 min and a hold cycle at 4 ^{0}C.

PCR products were visualized on a 1.5% agarose gel prepared with TAE buffer. Five µl PCR products were mixed with 1 µl loading dye (Fermentas) containing SYBR Gold nucleic acid gel stain (Lifetech) and run at 120 V for 1 h prior to visualization with UV-transillumination.

ExoSAP-IT (Affymetrix) was used according to the manufacturer's instructions to purify the PCR products. The reaction mixtures were incubated at 37 °C for 15 min and then at 80 °C for 15 min.

3.5.   Sequencing

Sequencing reactions were prepared using a BigDye Terminator v3.1 cycle sequencing kit (Lifetech). Reactions consisted of 2 µl Ready Reaction Premix, 1 µl of BigDye Sequencing Buffer, 1 µl of nuclease free water (Ambion), 1 µl 3.2 pmol primer and 5 µl PCR products. A standard sequencing protocol (Lifetech) was followed: denaturation at 96 ^{0}C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.

The sequencing products were purified by ethanol/NaOAc/EDTA precipitation: 25 µl 100% ethanol (Sigma), 1 µl 3M NaOAc and 1 µl 125 mM EDTA (Ambion) were added to each sample and incubated for 15 min at room temperature. The sequencing plate was centrifuged at 2 500g for 30 min using a Sorvall$^®$ RC6 centrifuge (Kendro Laboratory Products) and then inverted immediately and spun gently at 1 000g for 1 min to remove the supernatant; 35 µl 70% ethanol were added to each reaction and the plate centrifuged at 1 650g for 15 min and then inverted immediately and spun gently at 1 000g for 1 min to remove the supernatant. Residual ethanol was allowed to evaporate and 10 µl Hi-Di™ formamide (Lifetech) were added to each sample, heated for 2 min at 94 °C and analyzed with an ABI 3130xl Genetic Analyzer (Lifetech) using POP-7 polymer and a 36 cm capillary.

3.6.   Sequence analyses

The Staden software package v1.5. (Staden, 1996; Staden *et al.,* 2000) was used for sequence assembly, ClustalX v2.0.5 (Larkin *et al.,* 2007) to align sequences and BioEdit Sequence Alignment Editor v7.0.9 software (Hall, 1999) to edit sequences. Conserved regions within the S7 gene were identified and used to design a real-time RT-PCR assay with a TaqMan$^®$minor groove binder (MGB^{TM}) hydrolysis probe, using Primer Express 3.0 software (Lifetech) and FastPCR software v6.1.47 (Primer digital Ltd)(Kalendar *et al.,* 2009).

3.7. Assay characterization

Five µl RNA were added to 1 µl 25X primer/probe mixture (400 nM/120nM final concentrations) and 4 µl nuclease-free water. The mixture was denatured by heating at 95 °C for 1 min, using a StepOnePlus Real-Time PCR^{TM} system (Lifetech), and then cooled quickly on ice. VetMAX^{TM}-Plus One-Step RT-PCR kit (Lifetech) reagents were added (12.5 µl 2X RT-PCR buffer, 1.5 µl nuclease free water and 1 µl enzyme). The samples were placed in a StepOnePlus Real-Time PCR^{TM} system (Lifetech) and a standard protocol run: 48 ^{0}C for 10 min, 95^{0}C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 45 s. The assay was tested on tissue culture reference isolates of 24 serotypes of BTV, 9 serotypes of AHSV and 7 serotypes of EEV.

CHAPTER

4. RESULTS

4.1. Primer design

The EEV S7 gene was amplified in two overlapping segments 581 bp and 656 bp long, using the primers listed in Table 4.1.

Table 4.1 Primers used to amplify and sequence the EEV S7 (VP7) gene. The nucleotide position of the primer is indicated in the name.

Name	Orientation	Sequence (5'-3')
EEV_VP7_F0007_0027	Forward	TTTGGCCAACAAGATGGATGC
EEV_VP7_F0495_0516	Forward	TTCAGGTGAGCCTTACGCCGA
EEV_VP7_R0588_0609	Reverse	CTCGTGTACATTGCAAAAGG
EEV_VP7_R1151_1175	Reverse	GTAACACGTTTGGCCTCAGACGTTT

4.2. Reverse transcription polymerase chain reaction (RT-PCR)

All EEV S7 isolates, representing all seven serotypes, were amplified (Figure 4.1).
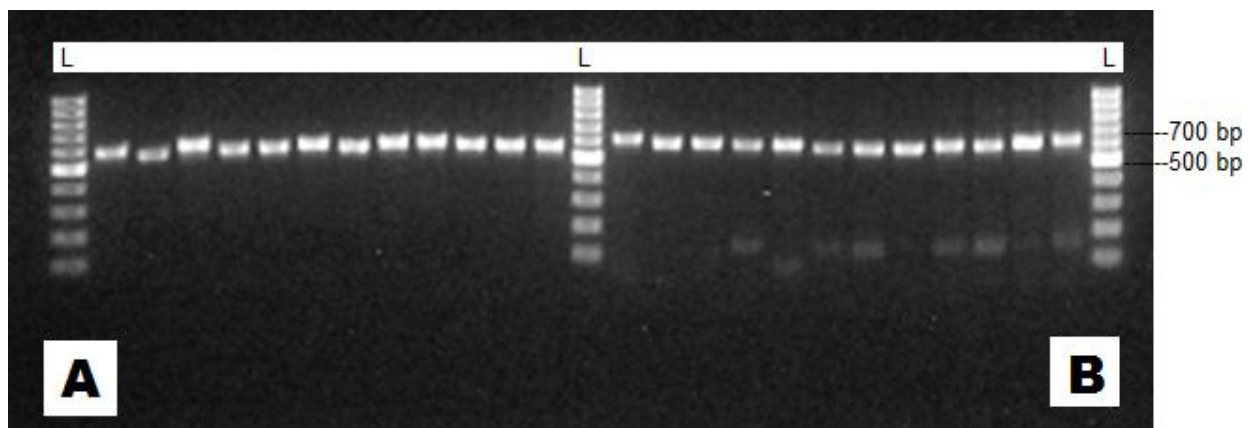
Figure 4.1 Amplification of the EEV S7 gene by conventional RT-PCR using terminal forward and interior reverse primers (A) targeting a 581 bp fragment and a terminal reverse & interior forward primers (B) targeting an overlapping 656 bp fragment. L - DNA molecular marker.

4.3. Sequencing

Conserved regions within the EEV S7 gene were identified (Figure 4.2) from the EEV S7 gene sequences (Figure 4.3).
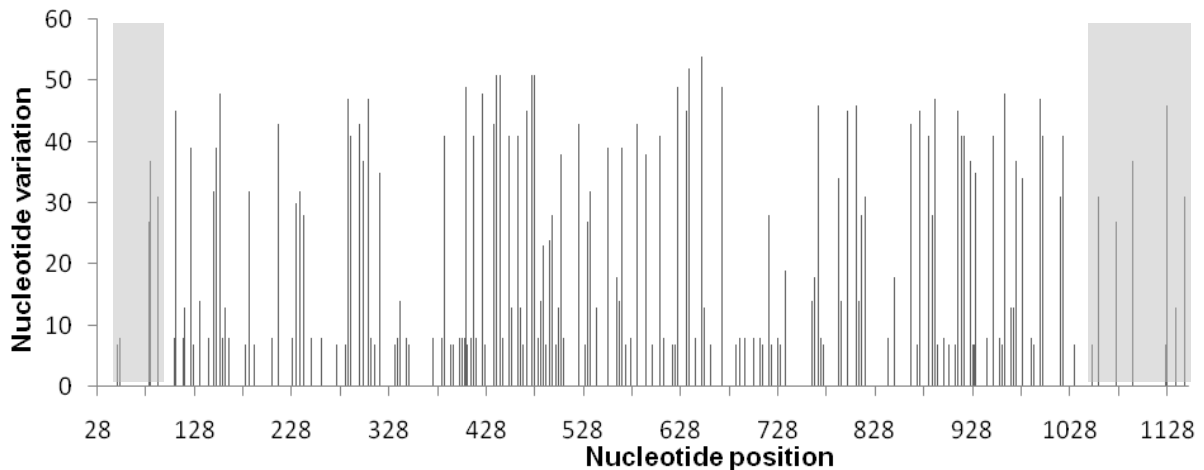
Figure 4.2 Plot of conservation sites on the VP7 gene. No column at a nucleotide position indicates 100% conservation, the higher the column, the greater the nucleotide variation at that site. Conserved regions are indicated by grey blocks.

4.4. Primers andTaqMan® MGB™hydrolysis probe

A conserved domain at the 5'-end of the EEV S7 (nucleotides 1-149) was identified and a 78 nucleotide TaqMan® MGB™ real-time RT-PCR developed in this region (Table 4.2). There was 100% identity of the forward primer and TaqMan®MGB™ hydrolysis probe with all the EEV S7 sequences. A redundant reverse primer was designed as there was one nucleotide mismatch between the primer and the EEV S7 sequences. A BLAST analysis of the primers and probe showed specificity for EEV sequences. An alignment of EEV, BTV and AHSV S7 sequences showed very little identity between the viruses.

Table 4.2. Real-time RT-PCR assay targeting the S7 (VP7) gene of EEV. *R=A/G.

Name	Type	Position	Sequence (5'-3')
EEV_VP7_F0028_0048	Forward primer	28-48	GATAGCGGCTAGAGCCCTTTC
EEV_VP7_P0054_0072	MGB™ probe	54-72	TAAGAGCATGTGTTACTGC
EEV_VP7_R0085_0106	Reverse primer	85-106	AACTTGAGGAGCCATR*GTAGCT

Position ruler: 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170

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4_2009_DVTD_E080010_05_VP7      GAUAGCGGCUAGAGCCCUUUCAGUUGUAAGAGCAUGUGUUACUGCACAGGAUCCACGAGCUACAAUGGCUCCCUCAAGUUUUGGAAAUUUUGGGAUUAGGAAUAAACAGAUACAACGCCAUUACAGGUAGUAGAGUCACGAUGCGCCCGGG
4_2010_DVTD_E100058_01_VP7      ..............................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     .....................................................................................................A..........................................................
EEV5_Kyalami_VP7                ....................C...............................................................................A.......G.....................G..........................
EEV2_Cascara_VP7                ....................................................................................A..........A.......G........................G..........................
1_2009_DVTD_E090047_VP7 (5)     .......................................................A..........A...........A.......G.................................G..C...............................
1_2009_DVTD_E090059_VP7         .......................................................A..........A...........A.......G...........U...........G..C.......................................
7_2008_DVTD_E080026_VP7         .......................................................A..........A...........A.......G.................................G..C...............................
6_2010_DVTD_E100020_VP7         .......................................................A.......C.A.........A...........G.......U..............C..G..C....................................
7_2008_DVTD_E080038_VP7 (4)     ...........................................................A...........A.......G.................................G..C.......................................
5_2009_DVTD_E090019_06_VP7 (3)  ...........................................................A...........A.......G.................................G..C.......................................
7_2008_DVTD_E080089_VP7         ...........................................................A...........A.......G.................................G..C.......................................
7_2008_DVTD_E080186_VP7 (2)     ...........................................................A...........A.......G.................................G..C.......................................
EEV4_Kaalplaas_VP7              ...........................................................A...........A.......G.................................G..C.......................................
2_2010_DVTD_E100043_VP7 (3)     ..................................G..............G.........A...........A.......G..............A..G..C......................................................
5_2009_DVTD_E090010_19_VP7      ..................................G..............G.........A...........A.......G..............A..G..C......................................................
EEV7_E2120_VP7                  ....................C....................G................A...........A.......G................A..G..C......................................................
EEV1_Bryanston_VP7              ...........................................................A...........A...G..G...............G...........G...............................................
EEV3_Gamil_VP7                  ...........................................................C.A..........G.....................G....C..G.....................................................
EEV6_Potchefstroom_VP7          ..................................G..............G.........A...........A...G...............U................G..C........U.................................
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Position ruler: 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320

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4_2009_DVTD_E080010_05_VP7      GACAGUUCCAGAGAGGAAUGAUAUGAUGUUCAUGUGUAUAGAUAUGGUAUGUGCCGCGUUGAACAUUCAGUUAGGAAAUGUUUCUCCAGAUUAUAUACGCAGAAAUUUAGAAACGAUAGGGGUGUUGGCGACAAGUGAAAUACCCUACACUGA
4_2010_DVTD_E100058_01_VP7      ..................................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     ..................................................................................................................................................................
EEV5_Kyalami_VP7                ..........................................................................................................................................G.......................
EEV2_Cascara_VP7                ..........................................................................................U.........................................................................
1_2009_DVTD_E090047_VP7 (5)     ...........................................................................................................G.........U..A..........U.............................
1_2009_DVTD_E090059_VP7         ...........................................................................................................G.........U..A..........U.............................
7_2008_DVTD_E080026_VP7         ..........................................C................................................................G.........U..A...........................................
6_2010_DVTD_E100020_VP7         ...........................................................................C...............................G.........U...............................................
7_2008_DVTD_E080038_VP7 (4)     ...........................................C..............................................................G..A.......U.........G...................................
5_2009_DVTD_E090019_06_VP7 (3)  ...........................................C..............................................................G..A.......U.........G...................................
7_2008_DVTD_E080089_VP7         ...........................................C..............................................................G..A.......U.........G...................................
7_2008_DVTD_E080186_VP7 (2)     ...........................................C..............................................................G..A.......U.........G...................................
EEV4_Kaalplaas_VP7              ...........................................................................................................G.........U...............................................
2_2010_DVTD_E100043_VP7 (3)     .......C.....................................A...C...U................................................G.........U.....G...........................................
5_2009_DVTD_E090010_19_VP7      .......C.....................................A...C...U................................................G.........U.....G...........................................
EEV7_E2120_VP7                  .......C.....................................A...C...U............................................G..G...............................................................
EEV1_Bryanston_VP7              .........................................................................................................U.........................................................
EEV3_Gamil_VP7                  .......A.....................U................U.......................................................U.............................................................
EEV6_Potchefstroom_VP7          ...G.......A.............................C.............C......................A..............U..A.......C.......U...................................................
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Position ruler: 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470

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4_2009_DVTD_E080010_05_VP7      UGAGGCGAUUAACGCGAUUGUGAGGGUGCAGGAGAGACGCAGACCUGGGGUCCAGUCGAUUCGCCGAUGCCGCCGUAUCUGGGGUGCGGUUGCGGUAGAAGCGCCAGGGCACUACUUUAUGCCGUUGGGAAGACAAACACAUUCAGCUUA
4_2010_DVTD_E100058_01_VP7      ....................................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     ....................................................................................................................................................................
EEV5_Kyalami_VP7                ..................................................A.............................U..................................G.......C.
EEV2_Cascara_VP7                ..............................U..A..............................U.......C.........U.......A............C.
1_2009_DVTD_E090047_VP7 (5)     .................................................U.............G.........A..U..U............C.
1_2009_DVTD_E090059_VP7         .................................................A.............G.........A..U..U............C.
7_2008_DVTD_E080026_VP7         .................................................A.............G..G......A..U..U............C.
6_2010_DVTD_E100020_VP7         .............C...............U.......A.............CU.............G.........A..U.......................C.
7_2008_DVTD_E080038_VP7 (4)     .................................................A.......U....A..G.........A..U...........A.......G.....C....C.
5_2009_DVTD_E090019_06_VP7 (3)  .................................................A.......U....A..G.........A..U...........A.......G.....C....C.
7_2008_DVTD_E080089_VP7         .................................................A.......U....A..G.........A..U...........A.......G.....C....C.
7_2008_DVTD_E080186_VP7 (2)     .................................................A.......U....A..G.........A..U...........A.......G.....C....C.
EEV4_Kaalplaas_VP7              .................................................A.............U.........A..U..U.....................G.......C.
2_2010_DVTD_E100043_VP7 (3)     .................................................A.............U.........A..U.......................C.
5_2009_DVTD_E090010_19_VP7      .................................................A.............U.........A..U.......................C.
EEV7_E2120_VP7                  .................................................A.............U.........A..U.......................C.
EEV1_Bryanston_VP7              .................................................G...................C...U....A.......A..U.......................C.
EEV3_Gamil_VP7                  .......A.......U..............A.......A...........U............A..U.......................G.....C....C.
EEV6_Potchefstroom_VP7          .........C..U.......A.............A......A.....U.......A................A..U..U..C........A.................C.
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                                   480        490        500        510        520        530        540        550        560        570        580        590        600        610        620
                                   ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7         CACUGAUUCCAACACUAUUCAGGUGAGCCUUACGCCGAACAGUGCUGCGCAAGUCAACGGCGCUAUGACGCCGCAAGGUGUGGGAGCUCGUGCAGUUAUUUUUUGUAUGGAGACCUUUUGCAAUGUACACAAGUCAGGCGGGAGCAGCCGU
4_2010_DVTD_E100058_01_VP7         ............................................................................................A......................................................
4_2008_DVTD_E080129_VP7 (7)        ...................................................................................................................................................
EEV5_Kyalami_VP7                   ........................C..................................................G.................A......................................................
EEV2_Cascara_VP7                   ......C..................C..............................A...................G...........U.A..........................................................U..
1_2009_DVTD_E090047_VP7 (5)        U......................U...............A...........U...................G............A.....................A.........................................
1_2009_DVTD_E090059_VP7            U......................U...............A...........U...................G............A.....................A.........................................
7_2008_DVTD_E080026_VP7            U.....G................U...............A...........C..................G............A.....................A........................................U..
6_2010_DVTD_E100020_VP7            U......................U...............A.............................G............A.....................A........................................U..
7_2008_DVTD_E080038_VP7 (4)        U......................................A............................G............A.........U.........................G....................U..
5_2009_DVTD_E090019_06_VP7 (3)     U......................................A............................G............A.........U.........................G....................U..
7_2008_DVTD_E080089_VP7            U......................................A............................G............A.........U.........................G....................U..
7_2008_DVTD_E080186_VP7 (2)        U......................................A............................G............A.........U.........................G....................U..
EEV4_Kaalplaas_VP7                 U......................................A............................G....................................................U..
2_2010_DVTD_E100043_VP7 (3)        ........G.....C........................A.......G....................G............A..................................................
5_2009_DVTD_E090010_19_VP7         ........G.....C........................A.......G....................G............A..................................................
EEV7_E2120_VP7                     U..............A......................A.......G....................G............A..................................................
EEV1_Bryanston_VP7                 U..C...........C......................A............................G........A....A......C...............................G........
EEV3_Gamil_VP7                     U.....C........C.....A.....C..........A.....G......................G........A....A..................................................
EEV6_Potchefstroom_VP7             U..............C.....A...............A............................G........A..U..A..A......................G..........C........G........

                                   630        640        650        660        670        680        690        700        710        720        730        740        750        760        770
                                   ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7         AGCUCAACCUCCCGGCGUGACUUUGUCGUUGGGGUGGAGUUAAUAUGCCAGAGGGUAGAAUUCUGAUAUGGGACGGUGUUGCUCCAGUUGUUGUACAAAAUCCAGGGAACGCGCCAGCUAUGGCUCAAAUUGAAGUCGUGAGAUUCACACA
4_2010_DVTD_E100058_01_VP7         ...................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)        ...................................................................................................................................................
EEV5_Kyalami_VP7                   ...................................................................................................................................................
EEV2_Cascara_VP7                   ......................A..............................................................U..............................................................
1_2009_DVTD_E090047_VP7 (5)        ........A........G...........C......................................................................................................................
1_2009_DVTD_E090059_VP7            ........A........G...........C......................................................................................................................
7_2008_DVTD_E080026_VP7            .................A...........C................................................................................................G.....................
6_2010_DVTD_E100020_VP7            .................A..A........C...............................................................................................G.....G...............
7_2008_DVTD_E080038_VP7 (4)        ......G..C.......A...........C................................................................................................G.....................
5_2009_DVTD_E090019_06_VP7 (3)     ......G..C.......A...........C................................................................................................G.....................
7_2008_DVTD_E080089_VP7            ......G..C.......A...........C................................................................................................G.....................
7_2008_DVTD_E080186_VP7 (2)        ......G..C.......A...........C................................................................................................G.....................
EEV4_Kaalplaas_VP7                 .................A...........C................................................................................................G.....................
2_2010_DVTD_E100043_VP7 (3)        .................A...........C.....................................................................C................................................
5_2009_DVTD_E090010_19_VP7         .................A...........C.....................................................................C.................U............................
EEV7_E2120_VP7                     .................A...........C......................................................................................................................
EEV1_Bryanston_VP7                 ......G..........G........................................U...............................................................U..A.....U.............
EEV3_Gamil_VP7                     ......G..........U.......................................C.......G....C.......C........................................A.............
EEV6_Potchefstroom_VP7             ................A..A.....A...............................G..C..................C...........G....U........................U..A.............

                                   780        790        800        810        820        830        840        850        860        870        880        890        900        910        920
                                   ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7         GCUCCUACACUCAUAUGAGACGGUUCCAGAAACCCGUGAGAAUAUGGCUAGAUGCUACUCAUUCCUUAGUGUUUACGUGGCACACCCUCCGAUCAGCUAUUCUGAGGUCCUUAGGAAUGCUUCCAGUGCACCAGUCCGCCUAUCCACCGAC
4_2010_DVTD_E100058_01_VP7         ...................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)        ...................................................................................................................................................
EEV5_Kyalami_VP7                   ..................................................................................U....................A.....................A.....U..C..........
EEV2_Cascara_VP7                   .......................G..........................................................U....................A.....................U..C..........
1_2009_DVTD_E090047_VP7 (5)        ....................U.....G......................................................U.................A...G...............U..C.......A..
1_2009_DVTD_E090059_VP7            ....................U.....G......................................................U.................A...C.G...............U..C.......A..
7_2008_DVTD_E080026_VP7            ..........................G......................................................U.................A...G...............U..C.......A..
6_2010_DVTD_E100020_VP7            ...............C..........G......................................................U.................A...G..G...............U..C.......A..
7_2008_DVTD_E080038_VP7 (4)        ..........................G......................................................U....C....A...G...............U..U..C..
5_2009_DVTD_E090019_06_VP7 (3)     ..........................G...............................A.....................U....C....A...G...............U..U..C..
7_2008_DVTD_E080089_VP7            ..........................G......................................................U....C....A...G...............U..U..C..
7_2008_DVTD_E080186_VP7 (2)        ..........................G......................................................U....C....A...G...............U..U..C..
EEV4_Kaalplaas_VP7                 ..........................G......................................................U....C....A...G...............U..U..C..
2_2010_DVTD_E100043_VP7 (3)        ...............G.....A.....G.....C..A............................................U..............A..U..G...............U..C..........
5_2009_DVTD_E090010_19_VP7         ...............G.....A.....G.....C..A............................................U..............A..U..G...............U..C..........
EEV7_E2120_VP7                     ...............G.....A.....G.....C..A............................................U..............A..U..G...............U..C..........
EEV1_Bryanston_VP7                 ..........................U......................................................U..............A...C.......C...........U..C..........
EEV3_Gamil_VP7                     ...............G..........U...............................U......................U..............A...C...................U..C..........
EEV6_Potchefstroom_VP7             ...............C....A........G.......A...............C..................G............A..C..............A.........U..C..........
```
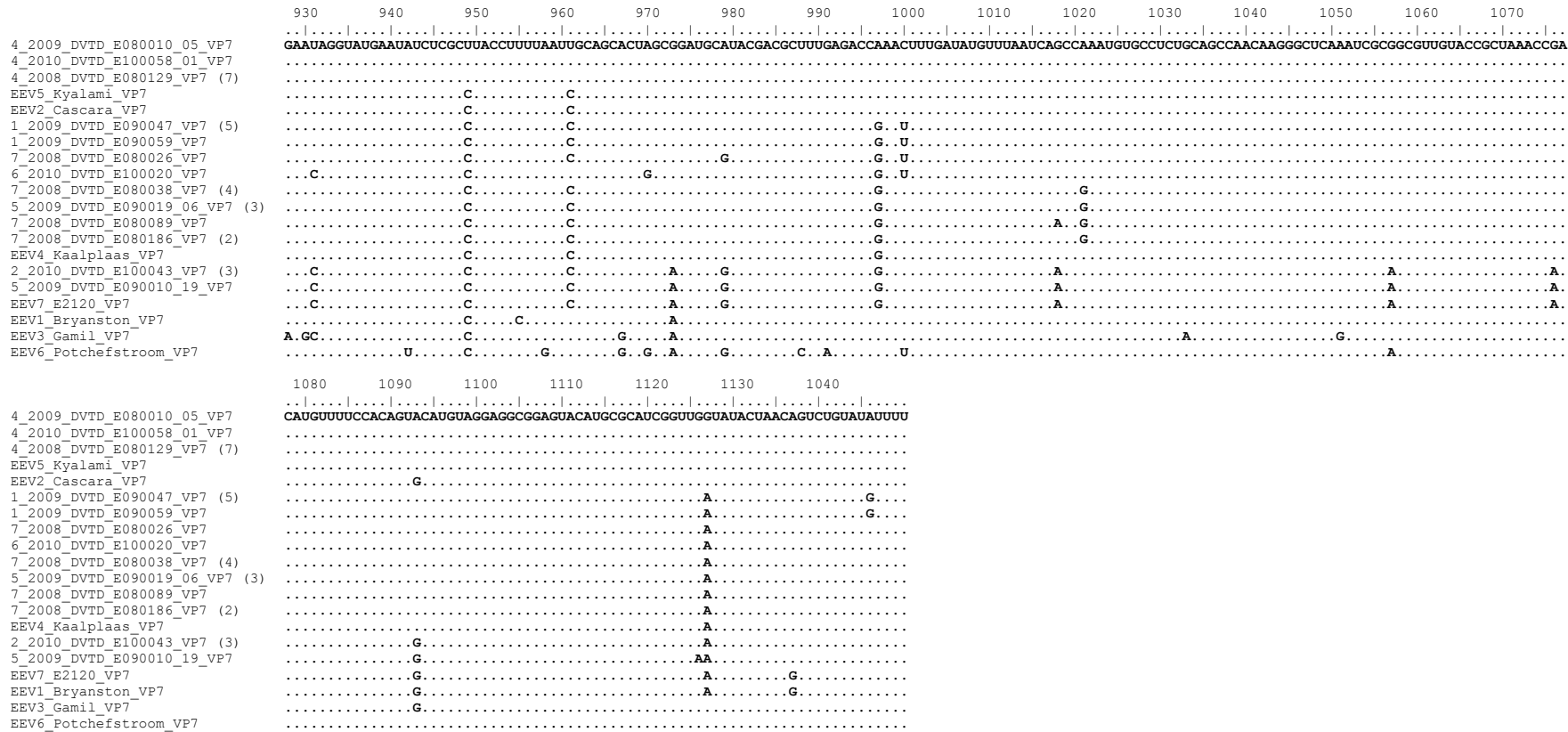
```
                    930       940       950       960       970       980       990       1000      1010      1020      1030      1040      1050      1060      1070
                    ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7    GAAUAGGUAUGAAUAUCUCGCUUACCUUUUAAUUGCAGCACUAGCGGAUGCAUACGACGCUUUGAGACCAAACUUUGAUAUGUUUAAUCAGCCAAAUGUGCCUCUGCAGCCAACAAGGGCUCAAAUCGCGGCGUUGUACCGCUAAACCGA
4_2010_DVTD_E100058_01_VP7    ...............................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)   ...............................................................................................................................................
EEV5_Kyalami_VP7              ..................C.........C..................................................................................................................
EEV2_Cascara_VP7              ..................C.........C..................................................................................................................
1_2009_DVTD_E090047_VP7 (5)   ..................C.........C................................G..U................................................................................
1_2009_DVTD_E090059_VP7       ..................C.........C................................G..U................................................................................
7_2008_DVTD_E080026_VP7       ..................C.........C...............G................G..U................................................................................
6_2010_DVTD_E100020_VP7       ...C..............C.........C...........G....................G..U................................................................................
7_2008_DVTD_E080038_VP7 (4)   ..................C.........C................................G................................G..................................................
5_2009_DVTD_E090019_06_VP7 (3)..................C.........C................................G................................G..................................................
7_2008_DVTD_E080089_VP7       ..................C.........C................................G...................A..G............................................................
7_2008_DVTD_E080186_VP7 (2)   ..................C.........C................................G................................G..................................................
EEV4_Kaalplaas_VP7            ..................C.........C..................................................................................................................
2_2010_DVTD_E100043_VP7 (3)   ...C..............C.........C......A....G....................G...................A........................................A.................A.
5_2009_DVTD_E090010_19_VP7    ...C..............C.........C......A....G....................G...................A........................................A.................A.
EEV7_E2120_VP7                ...C..............C.........C......A....G....................G...................A........................................A.................A.
EEV1_Bryanston_VP7            ..................C.....C..........A...........................................................................................................
EEV3_Gamil_VP7                A.GC..............C..............G....A......................................A...............G.................................................
EEV6_Potchefstroom_VP7        ..............U.....C........G........G.G.A.....G.........C.A.......U.....................................................A.....................

                    1080      1090      1100      1110      1120      1130      1040
                    ..|....|....|....|....|....|....|....|....|....|....|....|....|....|.....
4_2009_DVTD_E080010_05_VP7    CAUGUUUUCCACAGUACAUGUAGGAGGCGGAGUACAUGCGCAUCGGUUGGUAUACUAACAGUCUGUAUAUUUU
4_2010_DVTD_E100058_01_VP7    .....................................................................
4_2008_DVTD_E080129_VP7 (7)   .....................................................................
EEV5_Kyalami_VP7              .....................................................................
EEV2_Cascara_VP7              ..............G......................................................
1_2009_DVTD_E090047_VP7 (5)   ...........................................A................G....
1_2009_DVTD_E090059_VP7       ...........................................A................G....
7_2008_DVTD_E080026_VP7       ...........................................A....................
6_2010_DVTD_E100020_VP7       ...........................................A....................
7_2008_DVTD_E080038_VP7 (4)   ...........................................A....................
5_2009_DVTD_E090019_06_VP7 (3)...........................................A....................
7_2008_DVTD_E080089_VP7       ...........................................A....................
7_2008_DVTD_E080186_VP7 (2)   ...........................................A....................
EEV4_Kaalplaas_VP7            ...........................................A....................
2_2010_DVTD_E100043_VP7 (3)   ..............G............................A....................
5_2009_DVTD_E090010_19_VP7    ..............G............................AA...................
EEV7_E2120_VP7                ..............G............................A........G..........
EEV1_Bryanston_VP7            ..............G............................A........G..........
EEV3_Gamil_VP7                ..............G................................................
EEV6_Potchefstroom_VP7        .....................................................................
```

Figure 4.3. EEV S7 (VP7) gene sequences. Dots indicate identity and letters differences with the first sequence in the group. Identical sequences are represented only once in the figure and the number of identical sequences are indicated in brackets after the name of a random sequence in the group. The location of rtRT-PCR assay primers and probe are indicated by grey shaded boxes.

4.5. Real time reverse transcription polymerase chain reaction (rtRT-PCR)

The EEV rtRT-PCR assay was specific and did not detect AHSV nor BTV viruses (Figure 4.4 and Table 4.3).
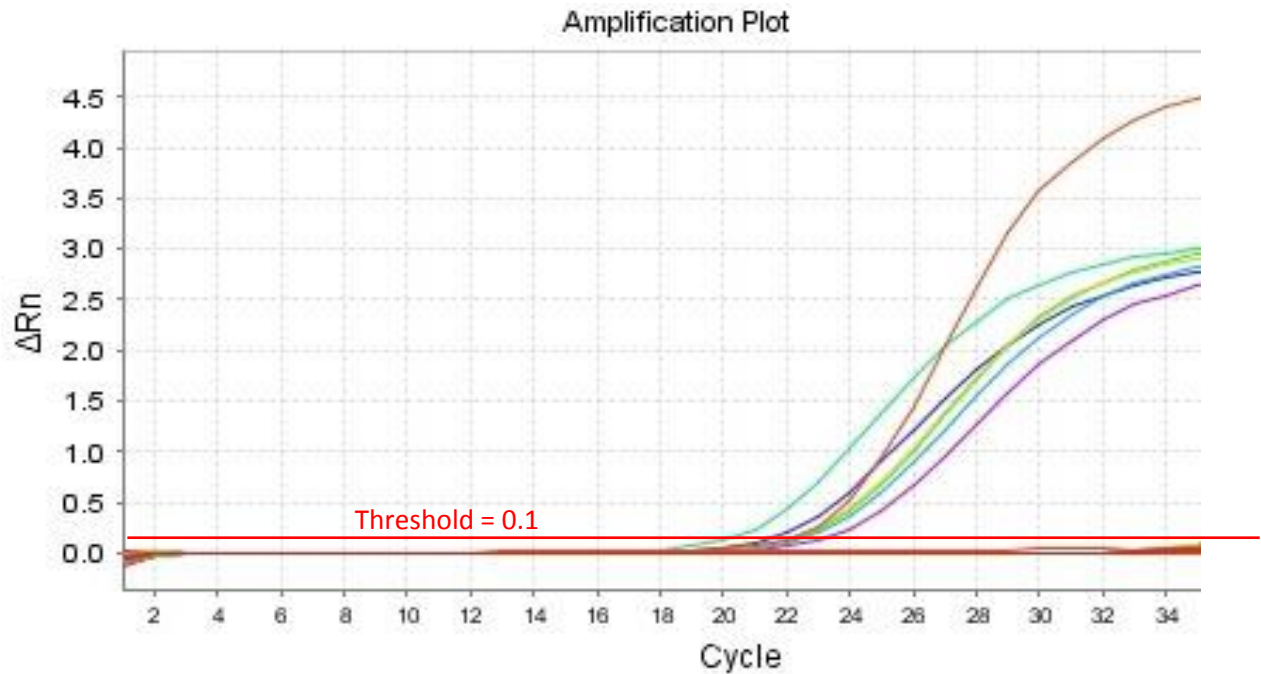

Figure 4.4. Amplification plots from a TaqMan EEV S7 rtRT-PCR assay showing detection of the seven serotypes of EEV (sigmoidal curves) and no detection of BTV or AHSV (baselines).

Table 4.3. Specificity of the EEV S7 (VP7) real-time RT-PCR assay. Samples were classified as negative if no fluoresence above the threshold (0.1) was detected within 35 cycles. AHSV - African horse sickness virus, BHK – baby hamster kidney, BTV - bluetongue virus, C_T - cycle threshold, EEV - equine encephalosis virus, dash indicates unknown.

Virus	Serotype	C_T value	Passage no.	Cell line	Date
AHSV	1	Negative	4	BHK	2002/10
AHSV	2	Negative	4	BHK	2002/10
AHSV	3	Negative	5	BHK	2002/10
AHSV	4	Negative	4	BHK	2002/09
AHSV	5	Negative	4	BHK	2002/10
AHSV	6	Negative	3	BHK	2002/08
AHSV	7	Negative	5	BHK	2003/03/14
AHSV	8	Negative	5	BHK	2002/10
AHSV	9	Negative	5	BHK	2002/05/23
BTV	1	Negative	-	-	1998/01/28
BTV	2	Negative	-	-	1998/01/28
BTV	3	Negative	-	-	1998/01/28
BTV	4	Negative	-	-	1998/03/13
BTV	5	Negative	-	-	1998/01/26

BTV	6	Negative	-	-	1998/01/26
BTV	7	Negative	-	-	1998/01/26
BTV	8	Negative	-	-	1998/07/09
BTV	9	Negative	-	-	1997/11/21
BTV	10	Negative	-	-	1997/11/21
BTV	11	Negative	-	-	1998/07/09
BTV	12	Negative	-	-	1998/07/09
BTV	13	Negative	-	-	1998/07/20
BTV	14	Negative	-	-	1998/07/20
BTV	15	Negative	-	-	1998/07/20
BTV	16	Negative	-	-	1998/02/09
BTV	17	Negative	-	-	1998/02/09
BTV	18	Negative	-	-	1998/02/10
BTV	19	Negative	-	-	1998/02/10
BTV	20	Negative	-	-	1998/02/16
BTV	21	Negative	-	-	1998/02/16
BTV	22	Negative	-	-	1998/10/08
BTV	23	Negative	-	-	1998/02/17
BTV	24	Negative	-	-	1998/03/13
EEV	1	21.57	1	BHK	2004/11/26
EEV	2	21.54	3	BHK	2002/09/09
EEV	3	21.07	-	-	1998/08/06
EEV	4	19.64	-	-	1998/08/06
EEV	5	21.28	2	BHK	2002/01/28
EEV	6	20.79	-	-	2002/02/25
EEV	7	22.59	6	BHK	2003/05/23

CHAPTER

5. DISCUSSION AND CONCLUSIONS

The aim of this study was to design and develop a specific, rapid and robust rtRT-PCR assay for the detection of EEV. The S7 gene was selected as it is known to be conserved in other related orbiviruses. Primers were designed to amplify and sequence 38 EEV isolates, and included all seven serotypes of EEV, as well as all the reference strains of EEV

Sequences generated contain slightly conserved sites of nucleotides within the region of VP7 gene, terminal regions was observed to be more conserved and constitute the highest score of similar nucleotides compared to the interior region, and this were the regions used to design one step RT-PCR primers to amplify full length of VP7 gene. Similarly, the 5' and 3' end terminal nucleotides of the EEV-1 sequences contain conserved regions and are also gene specific (Potgieter *et al.,* 2002). In AHSV VP7 and NS2, the terminal regions of those genes were more conserved compared to the interior region (Quan *et al.,* 2010) similar to the EEV VP7 gene. The first 115 nucleotides on the 5' end terminal sequence of VP7 gene are highly conserved compared to the other 3' end terminal sequence, and the assay targeting VP7 gene using TaqMan®MGB™hydrolysis probe was designed within same region. There was no more than 55% variation of nucleotides throughout the region of VP7 sequences. It is because only two epitopes of VP7 gene are expressed on the surface of the virus (Anthony *et al.*, 2007), and the immune pressure caused by the host cell suppresses the viral VP7 gene to slightly change the arrangement of sequence nucleotides thus leading to mutation. Epitopes expressed might not be enough to elicit high immune pressure that can cause nucleotide variation. In contrast, it leads to low exposure of the VP7 gene, thus leading to other regions still conserved.

The TaqMan®MGB™ RT-PCR assay developed for detection of EEV nucleic acid was specific and quick to perform, as it does not require visualization of the PCR products by electrophoresis. The elimination of this step saves laboratory equipment and reagents, but require a more advanced and expensive real-time thermocycler which is not available in every diagnostic laboratory. The real-time reagents are much more expensive than those used in conventional PCR, but the assay provides a convenient way of obtaining reproducible results within a short time.

EE itself is a mild disease and not regarded as an important disease of horses. Where EE is important is that it can be confused easily with mild cases of AHS, as both viruses present with similar clinical signs, are in the same genus (*Orbivirus*) and occur at the same time, as

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they are both transmitted by *Culicoides* midges. AHS is a high impact disease and is a listed disease by the World Organisation for Animal Health (OIE). Where cases present as possible AHS/EE cases, it is imperative to distinguish between the two viruses, as the control measures and consequences differ vastly, depending on the causative virus. In South Africa AHS is a controlled disease, and EE is not. A real-time RT-PCR assay has been developed for AHSV to rapidly detect cases of AHS and implement control measures as soon as possible. A real-time RT-PCR assay for EEV is needed to complement the assay for AHSV and distinguish between the two viruses.

The rtRT-PCR described here provides a valuable diagnostic assay method that has been evaluated for all EEV serotypes. The segment 7 assay can be used to rapidly and reliably detect any of the EEV strains that were tested in cell or tissue culture. The assay can provide a differential diagnosis between EEV and AHSV rapidly. It will be useful in endemic countries where both viruses are circulating to ensure that correct control strategies are used.

The results described in this study contribute to the improvement of the available techniques for the detection of EEV antigen and/or antibodies against EEV. So far, the only group-specific EEV assay is the indirect sandwich ELISA for the detection of EEV antigen (Crafford *et al.*, 2003). The assay takes a day to obtain results. To date, there is no published molecular assay for EEV and this is the first study to describe an rtRT-PCR assay for the detection of EEV.

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6. APPENDIX

Customized protocol for the MagMax^{TM} Express Particle Processor (Lifetech) for RNA extraction.

[PROTOCOL PROPERTIES]

Name = AM1830_Mod3
Protocol template version = 2.6.0
Instrument type = KingFisher
Creator = mquan
Created = 30/9/2009 9:31:36
Description = RNA isolation from whole blood.
Kit = MagMAX-96 viral RNA Isolation Kit , AM1836
Plate layouts = Default

[PLATE LAYOUTS]

Default

Plate type = KingFisher plate 200 ul
Plate change message = Change Default

A:
- volume = 20, name = Bead Mix (10 ul RNA binding beads, 10 ulLysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ulLysis/Binding Solution, 1 ul Carrier RNA, 2 ulXeno RNA, 65 ul Isopropanol
B:
- volume = 150, name = Wash Solution 1
C:
- volume = 150, name = Wash Solution 1
D:
- volume = 150, name = Wash Solution 2
E:
- volume = 150, name = Wash Solution 2
F:
- volume = 50, name = Elution Buffer
G:
- EMPTY
H:
- EMPTY

[STEPS]

BIND
Step parameters
- Name = Lysis Binding 5 min
- Well = A, Default

Beginning of step:

- No Action = Yes

Bind parameters:
- Bind time = 5min 0s, speed = Fast dual mix

End of step:
- Collect beads = Yes, count = 5

WASH
Step parameters
- Name = 1st Wash I 1 min
- Well = B, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = 2nd Wash I 1 min
- Well = C, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = 1st Wash II 1 min
- Well = D, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 2

WASH
Step parameters
- Name = 2nd Wash II 1 min
- Well = E, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:

- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 2

DRY
Step parameters
- Name = Dry 1 min
- Well = E, Default
- Dry time = 1min 0s
- Tip position = Outside well

ELUTION
Step parameters
- Name = Elution 3 min
- Well = F, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Elution parameters:
- Elution time = 3min 0s, speed = Bottom medium

Pause parameters:
- Pause for manual handling = No

Remove beads:
  - Remove beads = Yes, collect count = 5, disposal well = B