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………………………………..
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Postgraduate students (2009 to 2011) of the Department of Veterinary Tropical Disease (DVTD), you have made my studies and laboratory work worthwhile and easy. I appreciate all the help from the DVTD.
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 ethylenediaminetetraacetic acid
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
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RSA</td>
<td>Republic of South Africa</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>rtRT-PCR</td>
<td>real-time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>second/s</td>
</tr>
<tr>
<td>sELISA</td>
<td>sandwich enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VIB</td>
<td>virus inclusion bodies</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein</td>
</tr>
</tbody>
</table>
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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™ Express Particle Processor and MagMax™-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™ 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.
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*.**

<table>
<thead>
<tr>
<th>Family</th>
<th>Reoviridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfamily</td>
<td>Sedoreovirinae</td>
</tr>
<tr>
<td>Genus</td>
<td>Orbivirus</td>
</tr>
<tr>
<td>Species</td>
<td>African horse sickness virus</td>
</tr>
<tr>
<td></td>
<td>Changuinola virus</td>
</tr>
<tr>
<td></td>
<td>Bluetongue virus</td>
</tr>
<tr>
<td></td>
<td>Chenuda virus</td>
</tr>
<tr>
<td></td>
<td>Chobor Gorge virus</td>
</tr>
<tr>
<td></td>
<td>Corriparta virus</td>
</tr>
<tr>
<td></td>
<td>Epizootic hemorrhagic disease virus</td>
</tr>
<tr>
<td></td>
<td>Equine encephalosis virus</td>
</tr>
<tr>
<td></td>
<td>Eubenangee virus</td>
</tr>
<tr>
<td></td>
<td>Great island virus</td>
</tr>
<tr>
<td></td>
<td>Ieri virus</td>
</tr>
<tr>
<td></td>
<td>Lebombo virus</td>
</tr>
<tr>
<td></td>
<td>Orungo virus</td>
</tr>
<tr>
<td></td>
<td>Palyam virus</td>
</tr>
<tr>
<td></td>
<td>Peruvian horse sickness virus</td>
</tr>
<tr>
<td></td>
<td>St Croix River virus</td>
</tr>
<tr>
<td></td>
<td>Umatilla virus</td>
</tr>
<tr>
<td></td>
<td>Wad Medani virus</td>
</tr>
<tr>
<td></td>
<td>Wallal virus</td>
</tr>
<tr>
<td></td>
<td>Warrego virus</td>
</tr>
<tr>
<td></td>
<td>Wongorr virus</td>
</tr>
<tr>
<td></td>
<td>Yunnan orbivirus</td>
</tr>
</tbody>
</table>

*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.*, 2005).*
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).

<table>
<thead>
<tr>
<th>Name of virus</th>
<th>Numerical serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bryanston</td>
<td>EEV-1</td>
</tr>
<tr>
<td>Cascara</td>
<td>EEV-2</td>
</tr>
<tr>
<td>Gamil</td>
<td>EEV-3</td>
</tr>
<tr>
<td>Kaalplaas</td>
<td>EEV-4</td>
</tr>
<tr>
<td>Kyalami</td>
<td>EEV-5</td>
</tr>
<tr>
<td>Potchefstroom</td>
<td>EEV-6</td>
</tr>
<tr>
<td>E21/20</td>
<td>EEV-7</td>
</tr>
</tbody>
</table>
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’GUUWAD.......HSUUAC3’ (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.

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

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.
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 Culicoides 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 °C) for one or two days, or a higher fever (40 °C to
41 °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) (Kalender 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Serotype</th>
<th>Passage number</th>
<th>Isolation date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference strain</td>
<td>1 (Bryanston)</td>
<td>1</td>
<td>2004/11/26</td>
</tr>
<tr>
<td>E080099</td>
<td>1 (Bryanston)</td>
<td>6</td>
<td>2008/07/03</td>
</tr>
<tr>
<td>E080243</td>
<td>1 (Bryanston)</td>
<td>6</td>
<td>2008/08/14</td>
</tr>
<tr>
<td>E080340_1</td>
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<td>3</td>
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<td>E080341_2</td>
<td>1 (Bryanston)</td>
<td>3</td>
<td>2009/02/19</td>
</tr>
<tr>
<td>E080342</td>
<td>1 (Bryanston)</td>
<td>3</td>
<td>2009/02/12</td>
</tr>
<tr>
<td>E090039</td>
<td>1 (Bryanston)</td>
<td>4</td>
<td>2009/04/03</td>
</tr>
<tr>
<td>E090047</td>
<td>1 (Bryanston)</td>
<td>3</td>
<td>2009/04/03</td>
</tr>
<tr>
<td>E090059</td>
<td>1 (Bryanston)</td>
<td>1</td>
<td>2009/04/09</td>
</tr>
<tr>
<td>Reference strain</td>
<td>2 (Cascara)</td>
<td>3</td>
<td>2002/09/09</td>
</tr>
<tr>
<td>E100043</td>
<td>2 (Cascara)</td>
<td>-</td>
<td>2010/05/06</td>
</tr>
<tr>
<td>Reference strain</td>
<td>3 (Gamil)</td>
<td>-</td>
<td>1998/08/06</td>
</tr>
<tr>
<td>Reference strain</td>
<td>4 (Kaaplaas)</td>
<td>-</td>
<td>1998/08/06</td>
</tr>
<tr>
<td>E080010_5</td>
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<td>2</td>
<td>2008/01/31</td>
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<tr>
<td>E080013_2</td>
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<td>2008/02/07</td>
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<td>2008/03/20</td>
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<td>E080129</td>
<td>4 (Kaaplaas)</td>
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<td>2008/04/10</td>
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<tr>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>3</td>
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<td>Reference strain</td>
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<td>2002/01/28</td>
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<td>E090010_6</td>
<td>5 (Kyalami)</td>
<td>5</td>
<td>2009/04/09</td>
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<tr>
<td>E090010_15</td>
<td>5 (Kyalami)</td>
<td>3</td>
<td>2009/03/05</td>
</tr>
</tbody>
</table>
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) (Quantet 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 MgCl₂, 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 Multiscribete™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 °C for 12 min and then heated at 94 °C for 10 min, using a GeneAmp PCR system 9700 (Lifetech). This was followed by 40 amplification cycles consisting of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 60 s, before a terminal extension step at 72 °C for 7 min and a hold cycle at 4 °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 °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 2500g for 30 min using a Sorvall® RC6 centrifuge (Kendro Laboratory Products) and then inverted immediately and spun gently at 1000g for 1 min to remove the supernatant; 35 μl 70% ethanol were added to each reaction and the plate centrifuged at 1650g for 15 min and then inverted immediately and spun gently at 1000g 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™) 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™ system (Lifetech), and then cooled quickly on ice. VetMAX™-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™ system (Lifetech) and a standard protocol run: 48 °C for 10 min, 95°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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Orientation</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEV_VP7_F0007_0027</td>
<td>Forward</td>
<td>TTTGGCCAACAAGATGGATGC</td>
</tr>
<tr>
<td>EEV_VP7_F0495_0516</td>
<td>Forward</td>
<td>TTCAGGTGAGCCTACGCGGA</td>
</tr>
<tr>
<td>EEV_VP7_R0588_0609</td>
<td>Reverse</td>
<td>CTCGTGTACATTGCAAAAGG</td>
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<tr>
<td>EEV_VP7_R1151_1175</td>
<td>Reverse</td>
<td>GTAACACGTTTGGCCTACAGCCTTT</td>
</tr>
</tbody>
</table>

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](image)

**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).
4.4. **Primers and TaqMan® 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.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
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<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
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<td>28-48</td>
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<tr>
<td>EEV_VP7_P0054_0072</td>
<td>MGB™ probe</td>
<td>54-72</td>
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<td>EEV_VP7_R0085_0106</td>
<td>Reverse primer</td>
<td>85-106</td>
<td>AACTTGAGGAGCCATR*GTAGCT</td>
</tr>
<tr>
<td>Sequence Name</td>
<td>Accession</td>
<td>Description</td>
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</tr>
<tr>
<td>-------------------------------------</td>
<td>-----------</td>
<td>-------------</td>
<td></td>
</tr>
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<td></td>
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<td>EEV2_Cascara_VP7</td>
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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).

![Amplification Plot](image)

**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 fluorescence 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.

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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
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.
REFERENCES


Customized protocol for the MagMax™ 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 ul Lysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ul Lysis/Binding Solution, 1 ul Carrier RNA, 2 ul Xeno 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

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### 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

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### 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

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### 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

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**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