Characterization and sequence variation of the virulence-associated proteins of different tissue culture isolates of African Horsesickness Virus serotype 4

By

Jeanne Nicola Korsman

Submitted in partial fulfilment of the requirements for the degree
Magister Scientiae

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Department of Genetics
University of Pretoria
Pretoria

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in loving memory of my Grandmother

Agnes Elizabeth Hobbs

10·03·1913 – 16·06·2006
Declaration:

I declare that the dissertation that I hereby submit for the degree MSc Genetics at the University of Pretoria has not been previously submitted by me for degree purposes at any other university.

Signature: ______________________

Date: ________________
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Summary:

Characterization and sequence variation of the virulence-associated proteins of different tissue culture isolates of African Horsesickness Virus serotype 4

By
Jeanne Nicola Korsman

Supervisor: Prof. H. Huismans
Department of Genetics, University of Pretoria

Co-supervisor: Dr. W. Fick
Department of Genetics, University of Pretoria

For the degree MSc

African horsesickness, a disease of equines caused by African horsesickness virus (AHSV), is often fatal, although the pathogenic effect in different animals is variable. Current AHSV vaccines are live attenuated viruses generated by serial passage in cell culture. This process affects virus plaque size, which has been considered an indicator of AHSV virulence (Erasmus, 1966; Coetzer and Guthrie, 2004). The most likely AHSV proteins to be involved in viral virulence and attenuation are the outer capsid proteins, VP2 and VP5, due to their role in attachment of viral particles to cells and early stages of viral replication. Nonstructural protein NS3 may play an equally important role due to its function in release of viral particles from cells.

Two viruses were obtained for this study, AHSV-4(1) and AHSV-4(13). The thirteenth passage virus, AHSV-4(13), originated from the primary isolate AHSV-4(1). The three most variable AHSV proteins are VP2, VP5 and NS3. The question of sequence variation of these proteins between AHSV-4(1) and AHSV-4(13) arising during the attenuation process was addressed. The subject of plaque size variation between these viruses was also investigated.

Some of the sequence variation observed in NS3, VP2 and VP5, between AHSV-4(1) and AHSV-4(13), occurred in protein regions that may be involved in virus entry into and exit from cells. The sequence information also indicated that AHSV-4(1) and AHSV-4(13) consist of genetically heterogeneous viral pools. The plaque size of AHSV-4(1) was
variable, with small to relatively large plaques, whereas the plaques of AHSV-4(13) were mostly large. During serial plaque purification of AHSV-4(1) plaque size increased and became homogenous in size. No sequence variation in NS3 or VP5 of any of the plaque variants could be linked to variation or change in plaque size.

NS3 and VP5 have a possible role in the AHSV virulence phenotype, and exhibit cytotoxic properties in bacterial and insect cells. As these proteins have not been studied in mammalian cells, an aim of this study was to express them in Vero cells and investigate their cytotoxic and membrane permeabilization properties within these cells.

The NS3 and VP5 genes of AHSV-4(1) and AHSV-4(13) were successfully inserted into a mammalian expression vector and transiently expressed in Vero cells. The transfection procedure was optimized using eGFP, but expression levels were still low. When NS3 and VP5 were expressed, no obvious signs of cytotoxicity were observed. Cell viability and membrane integrity assays were performed and expression of NS3 and VP5 in Vero cells had no detectable effect on cell viability or membrane integrity. Low expression levels may have resulted in protein levels too low to cause membrane damage or affect cell viability. As Vero cells support AHSV replication, low levels of NS3 and VP5 may not be cytotoxic in these cells. NS3 was further investigated by expressing an NS3-eGFP fusion protein in Vero cells. Putative localization with membranous components and possible perinuclear localization of the fusion protein was observed. These observations may be confirmed with more sensitive microscopic techniques for a better assessment of the localization.
List of Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>AHS</td>
<td>African horsesickness</td>
</tr>
<tr>
<td>AHHSV</td>
<td>African horsesickness virus</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cells</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BTV</td>
<td>Bluetongue virus</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cm</td>
<td>centimetres</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl terminal</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled H₂O</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled H₂O</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>e.g.</td>
<td>for example</td>
</tr>
<tr>
<td>et al</td>
<td><em>et alia</em> (and others)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescence protein</td>
</tr>
<tr>
<td>EHDV</td>
<td>Epizootic haemorrhagic disease virus</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<td>Fig.</td>
<td>Figure</td>
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<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>×g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>HBS</td>
<td>Hepes-buffered saline</td>
</tr>
<tr>
<td>i.e.</td>
<td>that is</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>g/l</td>
<td>grams per liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<td>mm</td>
<td>millimeter</td>
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<tr>
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</tr>
<tr>
<td>MMOH</td>
<td>methyl mercuric hydroxide</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>ng</td>
<td>nanograms</td>
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<tr>
<td>nm</td>
<td>nanometers</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
</tr>
<tr>
<td>PSB</td>
<td>protein solvent buffer</td>
</tr>
<tr>
<td>R.F.U.</td>
<td>relative fluorescent units</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sf9</td>
<td>&lt;i&gt;Spodoptera frugiperda&lt;/i&gt;</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>µg</td>
<td>micrograms</td>
</tr>
<tr>
<td>µl</td>
<td>microliters</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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Literature Review

1.1 Introduction

Viruses are generally associated with disease, but many viruses are fairly benign and cause very little, if any, damage to their host. It is the more virulent viruses that are often thought to be more interesting, and most likely to be noticed. Indeed, their existence is more likely to be detected than a virus that causes no harm (Weiss, 2002). This can be illustrated by poliovirus, which is known for the paralysis it causes. However infection of the central nervous system with resulting paralysis occurs in only 1-2% of infections. Most polio infections result in symptoms such as sore throat and fever and can easily go undiagnosed (Racaniello, 2006). With such variation in virulence it can be asked why certain viruses cause disease, and how they give rise to the particular disease phenotype.

African horsesickness virus (AHSV) is an intracellular parasite of equine animals and Culicoides midges. African horsesickness (AHS), the disease AHSV causes in horses, is often fatal, although the pathogenic effect in different animals is highly variable. The Culicoides midges are the arthropod vectors that transmit AHSV (Roy et al., 1994; Coetzer and Guthrie, 2004), yet no obvious pathogenic effects have been noted in infected insects in contrast to vertebrates. The causes of AHSV virulence and the reasons for the variation in the virulence phenotype are still mostly unknown.

There are various ideas on how to define virulence, but most take into account the damage caused to the host. Virulence is a complex trait with multiple genes involved. The evolution of virulence is complex with many influencing factors, such as the mode of transmission of the specific parasite as well as host factors, e.g. susceptibility. Attenuation can be considered a form of directed virulence evolution, but much is still unknown about the process of attenuation and its success is varied. An attenuated phenotype can often be obtained by serial passage in cell culture or serial plaque-to-plaque transfers (Bull, 1994; Ebert, 1998).

Currently, live attenuated viruses serve as vaccines for AHSV (Coetzer and Guthrie, 2004). Due to the nature of the vaccines there is a possibility of reversion to virulence. Serotype 5 has been removed from the polyvalent vaccine due to reported deaths in vaccinated animals (Mellor and Hamblin, 2004). An in-depth study of virulence and attenuation
mechanisms may make it possible to engineer new vaccine candidates or create effective subunit vaccines.

Virus virulence can be studied fairly easily due to viruses’ short generation time. One way of studying virulence is through attenuation, or the loss of virulence. AHSV can be attenuated fairly rapidly by serial passage in cell culture. This process may have an effect on virus plaque size in cell culture, which has been considered an indicator of AHSV virulence (Erasmus, 1966; Coetzer and Guthrie, 2004).

Certain viral genes can play a significant role in virulence characteristics, such as genes influencing the rate of virus replication or virus transmission. Proteins found on the virus surface and proteins involved in the release of viruses from cells often play a role in virulence (Zhang et al., 1998; Goto et al., 2003; Kobasa et al., 2004). In studies on virus attenuation, point mutations and deletions have been observed in regions of viral genomes that may affect virulence (Mandl et al., 1998; Zhang et al., 1998). This study looks at the process of attenuation of AHSV on the molecular level by studying sequence variation between certain proteins of a virus with lowered virulence and the virulent virus from which it was derived. Sequence variation pertaining to viral plaque size is also considered.

In general, all components of the virus life cycle are involved in the virulence phenotype (Schneider-Schaulies, 2000). Viral entry into a host cell, viral replication within the host cell and viral exit from the host cell by extrusion, cell lysis or budding each have an effect on virulence. Host factors, such as the immune response and cell receptors, also play a role in virulence (Schneider-Schaulies, 2000; Weiss, 2002). This makes viral virulence a complex phenomenon as these virulence-determining factors may act singularly or in concert to produce the final phenotype.

From the virulence perspective of the AHSV replication cycle, specific focus is placed on VP2 and VP5 due to their role in virus entry into cells and NS3 with its role in virus exit from cells. These proteins, thought to play a role in the virulence characteristics of AHSV, and which exhibit cytotoxic properties in other cells, are investigated by expression in mammalian cells.

1.2 Virulence
An early view of virulence was that it was a property of the pathogen. Later, host factors, such as susceptibility or resistance, were also taken into account and the role of the host response to the pathogen was acknowledged (Casadevall and Pirofski, 1999).
Virulence is the ability of a pathogen to multiply and cause harm to its host. In ecology and evolutionary biology the harm caused to the host is more important than parasite fitness (Poulin and Combes, 1999). Pathogen virulence, viewed in this way, can be described as the reduction of host fitness, which is often measured by host morbidity or mortality due to infection, as fitness can be difficult to quantify (Ganusov, 2003; Lipsitch and Moxon, 1997; Bull, 1994; Ebert, 1999). The terms virulence and pathogenicity have been used interchangeably in the literature (Lipsitch and Moxon, 1997). The distinction between these terms has caused some debate (Casadevall and Pirofski, 1999; Poulin and Combes, 1999; Shapiro-Ilan et al., 2005). There is no universally accepted definition for virulence (Bull, 1994), but most definitions focus on the pathogenic effect of the pathogen on the host (Poulin and Combes, 1999). The consensus in medical fields such as pathology is that pathogenicity is a qualitative term, i.e. an organism is either pathogenic or not; virulence is quantitative or variable, i.e. one pathogen may have a higher virulence than another (Shapiro-Ilan et al., 2005).

Virulence is a complex trait with multiple genes involved. These genes may play a role in such factors as tissue specificity, generation time and cytotoxicity (Lipsitch and Moxon, 1997). The level of virulence depends on the host species as well as the individual host within the species (Poulin and Combes, 1999), with host-pathogen interactions influencing virulence (Casadevall and Pirofski, 1999; Ebert and Hamilton, 1996).

The relationship between virulence and pathogen fitness is complex. Selection for high replication is associated with an increase in virulence, suggesting a link between pathogen fitness and virulence (Lipsitch and Moxon, 1997). Serial passage experiments support the idea that virulence and pathogen fitness are genetically correlated as the increase in fitness is accompanied by an increase in virulence in the new host (Ebert, 1998).

1.2.1 Virulence and transmission

Lipsitch and Moxon (1997) state two views on the relationship of virulence and pathogen transmission. The first is that selection favours reduced virulence because living and mobile hosts transmit pathogens more efficiently. The second view is that selection can favour higher virulence if it accompanies an advantage that overcomes the decline in transmission opportunities. This could be a higher transmission rate early in infection, or the ability to out-compete less virulent strains. A trade-off between an extended time of disease transmission and rapid reproduction and transmission from a host is evident from the second view.
Virulence may benefit the pathogen by enhancing transmission through symptoms that promote pathogen spread, e.g. coughing enhances the spread of respiratory pathogens and diarrhoea enhances the spread of enteric pathogens (Bull, 1994; Lipsitch and Moxon, 1997; Ebert, 1999; Weiss, 2002). Such characteristics may also be advantageous to the host in terms of expelling the pathogen (Ebert, 1999). If higher virulence is connected to a high replication rate it may also be associated with a low clearance rate by the host’s immune system (Lipsitch and Moxon, 1997). Certain symptoms of disease may be due to the immune response and not virus levels or replication; these will not aid in the transmission of viruses (Weiss, 2002).

Virulence and transmissibility are positively correlated for a wide range of pathogens, but there are a number of exceptions. Virulence and transmission may not be linked in some instances, e.g. if symptoms are due to the host immune response and not pathogen replication (Lipsitch and Moxon, 1997). Virulence in a novel or unusual host that does not spread the disease (Bull, 1994; Lipsitch and Moxon, 1997; Ebert, 1999), or virulence occurring after transmission, as with HIV and oncogenic viruses, will also have a neutral effect on transmission (Bull, 1994; Ebert, 1999).

Virulence may lower pathogen fitness by increasing the host death rate, but this may be necessary for the production of a high pathogen concentration or viral load which increases early pathogen transmission (Bull, 1994; Ebert, 1999). Transmission events per day may be higher due to virulence, but transmission events per infection may be lower (Ebert, 1999). Certain deadly diseases of humans result from infections by viruses such as Ebola and Hantavirus, whose natural hosts are other mammals. In these cases the virulence in humans exceeds that in the natural host and is merely a by-product of the virus’s evolution in another host (Bull, 1994; Ebert, 1999). In most cases of infections of novel hosts, the infections are probably avirulent, but these usually go unnoticed and only the chance virulent infections receive much attention (Ebert, 1999). This high virulence in novel hosts is sometimes not associated with high reproduction levels or transmission. If the parasite is to establish itself as a pathogen of the novel host, it will evolve a suitable level of virulence (Ebert, 1999).

Selection of pathogen genotypes competing within a host is not well understood, but higher virulence and higher pathogen growth rates usually correlate (Bull, 1994). An increase in multiple infections of one host seems to lead to an increase in virulence (Ebert, 1998). Within-host evolution induces an increase in within-host growth rate and virulence; a strain with a higher growth rate will out-compete any slower growing strains within the host.
Host density may influence virulence levels of pathogens. When there are less contact opportunities between infected and uninfected hosts there will be lower transmission and the pathogen will require more time before host death for transmission to occur. When there are more contact events between hosts there will be more transmission events. If pathogen fitness is measured as the rate at which the pathogen spreads in the host population, low host density should be correlated with low virulence and high host density should be correlated with high virulence (Bull, 1994).

Diseases which can survive outside the host in a vector or in a resistant form in the environment, e.g. in the form of a spore, often have higher levels of virulence than diseases spread by bodily fluids (Myers and Rothman, 1995). This may be due to efficient transmission by a vector allowing greater virulence, because the movement of the host for transmission, e.g. by contact between individuals, is not necessary, as the vector moves the pathogen between hosts. An inactive host will also be more susceptible to a vector taking a blood meal from it, thus promoting transmission of the parasite (Ewald, 1994).

Pathogens of low virulence can be transmitted vertically, i.e. between generations, in host populations, whereas more virulent pathogens are usually transmitted horizontally, between individuals, as infected hosts are likely to die before reproduction (Myers and Rothman, 1995; Lipsitch and Moxon, 1997). In vertically transmitted pathogens, virulence would have a fitness reducing effect on the pathogens themselves, as their fitness is directly linked to that of the host (Ebert, 1999).

1.2.2 Attenuation

The study of virulence evolution may lead to knowledge useful in designing attenuated viruses. To date, some of the most successful vaccines have been attenuated viruses, e.g. smallpox, rubella, measles and mumps (Bull, 1994), yet the success of the attenuation process is mixed and it is unknown which conditions will work well for a new virus. The possibility exists for a live attenuated virus to revert back to a virulent phenotype (Bull, 1994; Ebert, 1998).

The process of attenuation is based upon artificial selection of a virus or pathogen with reduced virulence, possibly from a population of pathogens consisting of virulence variants or by mutations or deletions. The virulent virus or pathogen is usually grown in novel
conditions such as a new host or at a different temperature. The enhanced growth under new conditions is accompanied by reduced virulence and growth rate in the original host (Bull, 1994; Ebert, 1998; Ebert, 1999). The process is usually fastest in RNA viruses, slower for DNA viruses, followed by bacteria, and slowest for eukaryotes (Ebert, 1998).

The new host is usually clonal or inbred, reducing the amount of host genetic diversity (Ebert, 1998); normal host diversity would prevent a pathogen from adapting to a particular genotype and would enable evolution of host resistance (Ebert and Hamilton, 1996). In addition, hosts with short generation times evolve resistance to disease more rapidly than hosts with long generation times (Myers and Rothman, 1995).

Serial passage experiments transfer the pathogen from one host to the next, simulating growth within a host without real transmission events, so there is no cost of virulence (Ebert, 1998; Ebert, 1999). These experiments select for strains with high infectivity, a fast growth rate, and often an increase in virulence in the host in which the pathogen is passaged (Lipsitch and Moxon, 1997; Ebert, 1999). This increased virulence in the novel host is usually accompanied by a reduction in virulence in hosts other than the one in which they are passaged, i.e. the pathogen is attenuated for these hosts (Ebert, 1999).

Many serial passage experiments consist of a large number of individuals being transferred during each passage. This rules out genetic drift as the main cause of attenuation (Ebert, 1998). If the population size during each passage is much lower than usual, the probability of genetic drift is much higher, and repeated bottlenecks occur increasing fixation of deleterious mutations and decreasing fixation of advantageous mutations (Ebert, 1998). Viruses with high mutation rates can have their fitness reduced by passing them through repeated bottlenecks, such as serial plaque-to-plaque transfers. The reduced fitness is usually accompanied by reduced virulence (Bull, 1994).

1.2.3 Virulence genes and virulence factors
A virulence factor can be described as a component of a pathogen that causes damage to the host (Casadevall and Pirofski, 1999). Virulence genes are genes encoding these virulence factors.

Virulence genes of pathogenic bacteria have been found on DNA segments termed pathogenicity islands (Hacker et al., 1997). These pathogenicity islands are found in genomes of pathogenic bacterial strains, but are usually absent in non-pathogenic strains. They may contain one or more virulence genes, and are able to undergo horizontal gene
transfer between bacteria, thus enabling rapid development of a virulence phenotype in a bacterial strain. These virulence genes can be selected for when the reduction of host fitness provides an advantage to the pathogen (Poulin and Combes, 1999).

There are fewer and fewer options available for treating diseases due to the increase of antibiotic resistance. A new possibility of targeting virulence factors, such as surface proteins or toxins, is emerging (Alekshun and Levy, 2004). It would be a shift from direct growth inhibition to targeting virulence factors using proteins or small molecules. Such a strategy is still in its infancy with no such drugs in use, although it should hold potential for the treatment of bacterial as well as viral infections.

1.2.4 Viral virulence

Pathogens such as viruses and bacteria have short generation times, so the evolution of their virulence phenotypes can be observed and studied in experimental systems (Bull, 1994).

Certain virus-receptor interactions play a role in virus tropism and pathogenesis (Schneider-Schaulies, 2000; Forrest and Dermody, 2003). Proteins found on the virus surface, be they outer capsid proteins or envelope proteins, are under immune selection and often variable, but interaction between these viral proteins and cell receptors can limit the variation. Mutations in these proteins may change virus tropism and virulence (Schneider-Schaulies, 2000). Entry of the virus into the cell is initiated by the virus-receptor interaction. This can affect virulence by influencing the rate of virus replication, virus transmission between cells and organs (Schneider-Schaulies, 2000), and the immune response mounted by the host, which is partially due to receptor initiation of signal transduction pathways which induce cytokine and interferon secretion (Schneider-Schaulies, 2000; Forrest and Dermody, 2003). Release of viruses from cells also influences virulence (Schneider-Schaulies, 2000).

There have been many studies on viral genes and proteins that contribute to virulence. Many of these studies have identified specific genes that have been associated with a change in the virulence phenotype. For example, the viral haemagglutinin of a virulent influenza A virus was found to confer virulence on previously avirulent viruses when transferred to the previously avirulent recombinant influenza strains (Kobasa et al., 2004). The matrix protein of an influenza B virus was also found to confer a virulent phenotype when a single mutation was introduced (McCullers et al., 2005). The attenuation of poliovirus neurovirulence, in types 1, 2 and 3, has been attributed to a mutation in the 5'
noncoding region and to mutations in capsid proteins with other mutations possibly also influencing the virulence phenotype (Omata et al., 1986; Moss et al., 1989; Westrop et al., 1989).

In the family *Flaviviridae*, attenuating mutations have been found in the 5’ noncoding region and nonstructural proteins (Butrapet et al., 2000), and in the 3’ noncoding region (Blaney et al., 2006) of dengue virus. In tick-borne encephalitis virus a neuroinvasiveness attenuating mutation was observed in the E protein, possibly affecting receptor binding and thus cell tropism (Goto et al., 2003). Additional mutations in a nonstructural protein and in the 3’ noncoding region were observed, but these were less important in the attenuation phenotype. Similarly, Mandl et al. (1998) determined that deletions in the 3’ noncoding region of tick-borne encephalitis virus led to attenuation.

A similar diversity of virulence mechanisms has been observed in the *Reoviridae* family. Reovirus protein σ1, the viral attachment protein, is responsible for varying pathogenic phenotypes between two virus strains infecting newborn mice. One of the strains infects ependymal cells of the central nervous system and causes hydrocephalus, while the other infects neurons causing lethal encephalitis. This suggests that σ1 determines which cell type the virus will infect by binding to receptors expressed by the cell type for which it is specific (Forrest and Dermody, 2003; O'Donnell et al., 2003). In addition, σ1 binds to certain receptors in apoptotic signalling pathways, and may influence reovirus virulence through its role in the tissue damage caused by apoptosis (O'Donnell et al., 2003). Entry of the virus into cells is also needed for apoptosis and μ1 is involved in virus entry into cytoplasm from the endosome (Forrest and Dermody, 2003; O'Donnell et al., 2003). These factors, along with host cell properties, influence tissue damage and consequently virus virulence. Reovirus nonstructural protein, σ1s, also contributes to virus pathogenesis and virulence through its influence on the amount of apoptosis and the resultant tissue damage (Hoyt et al., 2005).

In a study on virulent and attenuated porcine rotavirus strains, Zhang et al. (1998) showed that mutations in NSP4, an enterotoxin, are associated with the protein’s capability to cause diarrhoea, and therefore proposed that NSP4 mutations were involved in altered virus virulence. Hoshino et al. (1995) also identified NSP4, as well as VP3, an inner capsid protein, VP4 and VP7, the outer capsid proteins, as being involved in the virulence phenotype in a genome reassortment study. All four proteins derived from the virulent strain were required to induce diarrhoea, and the inclusion of one protein from the avirulent strain in the normally virulent virus resulted in an attenuated virus. In another genome
reassortment study of virulent and avirulent strains of an avian rotavirus, Mori et al. (2003) demonstrated that both the outer capsid proteins, VP4 and VP7, are involved in the virulence phenotype. However, not all studies are in agreement with regard to the association of the outer capsid proteins and NSP4 with virulence. Broome et al. (1993) found no linkage between the outer capsid proteins and the virulence phenotype in a mouse model and Ward et al. (1997) indicated that the attenuation of a human rotavirus was not related to mutations in NSP4. This shows the complexity of viral virulence, the molecular basis of which is still poorly understood.

Little is known about the molecular mechanisms behind orbivirus virulence. However, in bluetongue virus (BTV), a genome reassortment study of two strains with different neurovirulence properties, the outer capsid protein VP5 segregated with the neurovirulent phenotype observed in neonatal mice (Carr et al., 1994). While Bernard et al. (1994) found that the antigenicity of the outer capsid protein VP2 and the electrophoretic mobility of the gene encoding the inner core protein VP3 differed between virulent and avirulent BTV isolates. Some of the amino acid differences between the VP2 proteins of these BTV isolates were found in three clusters, one of which coincided with a neutralization epitope. The VP3 proteins were more conserved and less likely to be determinants of virulence compared to VP2 (Bernard et al. 1997). Hybridization studies by Huismans and Howell (1973) also suggest that the proteins involved in serotype determination, VP2 and VP5, are involved in virulence determination. The ability of BTV to trigger apoptosis in mammalian cells, in a similar manner to reovirus, may play a role in the virulence phenotype. Mortola et al. (2004) found that virus uncoating, or the addition of both VP2 and VP5, triggered apoptosis in mammalian cells.

1.3 African horsesickness virus
African horsesickness is a highly infectious, non-contagious disease of equids. It is endemic to sub-Saharan Africa and is transmitted by biting midges of the *Culicoides* genus (Roy et al., 1994; Coetzer and Guthrie, 2004). AHSV, which causes AHS, is one of 19 serogroups in the *Orbivirus* genus, which falls within the *Reoviridae* family. AHSV includes 9 serotypes that are distinguished between by means of neutralization assays (Roy, 2001; Coetzer and Guthrie, 2004). BTV, which infects ruminants, is the prototype virus of the orbiviruses, and has certain properties that are similar to AHSV (Roy et al., 1994).
1.3.1 Pathogenesis and Disease

The mortality rate in AHSV infected horses is as high as 95% (Coetzer and Guthrie, 2004; Mellor and Hamblin 2004). Serotypes 1-8, which cause 90-95% mortality, are more virulent than serotype 9, which causes approximately 70% mortality (Coetzer and Erasmus, 1994).

The symptoms of AHS, such as oedema, effusion and haemorrhage, develop as a result of damage to the circulatory and respiratory systems (Mellor and Hamblin 2004). The four clinical forms of AHS are reviewed by Roy (2001), Coetzer and Guthrie (2004) and Mellor and Hamblin (2004). The pulmonary form has a high mortality rate, above 95%, and the onset of symptoms can be rapid and death follows quickly. The cardiac form has a lower mortality rate of about 50%. Symptoms are drawn out, but milder than those of the pulmonary form. The mixed form is the most common, and is a combination of the pulmonary and cardiac forms; the death rate is approximately 70%. Horsesickness fever is a very mild form of the disease with no mortality. It usually occurs in animals with some immunity against the virus, or is due to infection with a less virulent strain. This form may be observed in infected donkeys and zebras (Coetzer and Guthrie, 2004). According to Laegreid et al. (1993) the clinical form of AHSV that manifests in naïve horses is primarily due to the virulence phenotype of the virus with which the horse is infected. Experimental infection with an AHSV-4 field isolate, an AHSV-9 and an AHSV-4 variant isolated from mouse brain demonstrated that the virulent AHSV-4 variant resulted in the cardiac form with detectable viraemia occurring at three days post inoculation. The AHSV-9 variant resulted in the pulmonary form with detectable viraemia at seven to ten days post inoculation. The avirulent AHSV-4 variant resulted in the fever form with no detectable viraemia. Laegreid et al. (1993) suggest that the difference in time of viraemia onset between the virulence variants is due to the primary replication rate of the virus variant or the secondary spread from the site of primary replication. These factors are involved in the different pathologies observed. Additionally, Skowronek et al. (1995) suggest that AHSV pathogenesis involves endothelial cell damage, resulting in loss of the endothelial cell barrier function, which increases vascular permeability and contributes to the oedema, effusion and haemorrhage observed in AHSV infection.

*Culicoides* species are the main vectors of AHSV and BTV. These insects that transmit the viruses between vertebrate hosts. The initial virus replication occurs in the host’s lymph nodes giving rise to the primary viraemia. Subsequent infection is in the lungs, spleen and other lymphoid tissues and produces a secondary viraemia (Roy, 2001; Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004). Not all *Culicoides* midges are susceptible to BTV infection, but those that are become infected with BTV by taking in a viraemic blood
meal from an infected vertebrate host. The virus replicates in the insect’s mid-gut from where progeny viruses are released into the haemacoel, from where secondary target organs such as the salivary glands are targeted. The virus can be transmitted to a new vertebrate host 10-14 days post infection. The virus does not cause obvious damage to the insect cells, as it does to vertebrate cells, so it is possible that virus replication persists in susceptible cells until death or a certain physiological age (reviewed by Mellor, 1990).

At times when the pathogen’s vector is scarce, a virulent pathogen can disappear from the system (Myers and Rothman, 1995). Thus, it is necessary for the virus to survive from one “vector season” (i.e. when environmental conditions support adult vector survival) to the next (Coetzer and Guthrie, 2004). This is called overwintering.

One possible mechanism of overwintering is in the *Culicoides* vector. White et al. (2005) demonstrated the feasibility of this possibility for BTV. They detected RNA from BTV segment seven in *Culicoides sonorensis* larvae as well as in adult midges reared from larvae, indicating vertical transmission of the virus. Furthermore, they detected BTV segment seven in *Culicoides* cell lines, and Wechsler et al. (1989) found that BTV can persistently infect *Culicoides* cell lines with no obvious cytopathic effects.

Alternatively, orbiviruses may overwinter in a vertebrate host. Takamatsu et al. (2003) found that BTV persistently infected ovine γδ T-cells and that certain receptors could convert the infection to a lytic one. It was hypothesised that vector feeding could induce skin inflammation and bring γδ T-cells to the site of vector feeding where they would be triggered to release infectious virus. A similar mechanism may be present in a reservoir host of AHHSV. Zebra are susceptible to AHHSV infection and can be viraemic for up to 6 weeks. They are the most likely reservoir of the virus (reviewed by Barnard, 1998). Donkeys are also susceptible to AHHSV infection. They may also act as virus reservoirs, but are unlikely to be long-term reservoirs (Hamblin et al., 1998). Alexander et al. (1995) have found that a number of carnivores (e.g. lion, spotted hyena and African wild dog) can be infected with AHHSV and produce neutralizing antibodies. This infection is possibly due to ingestion of infected prey, such as zebra (Alexander et al., 1995).

### 1.3.2 AHHSV Attenuation

Current vaccines against AHHSV are polyvalent, live, attenuated viruses (Coetzer and Guthrie, 2004). Two vaccines are available. One contains serotypes 1, 3 and 4 and the other contains serotypes 2, 6, 7 and 8. Serotype 9 is rare and protection is provided by cross-reaction with serotype 6 (Coetzer and Guthrie, 2004). Serotype 5 has been removed...
due to reported deaths in vaccinated animals (Mellor and Hamblin, 2004) and protection is provided by cross-reaction with serotype 8 (Coetzer and Guthrie, 2004).

AHSV can be attenuated by serial passage in mice, embryonated chicken eggs, or cell culture. Passage in cell culture attenuates the virus very rapidly. It takes 5 to 20 passages, compared to approximately 100 passages in mouse brain to achieve adequate attenuation (Erasmus, 1966; Coetzer and Guthrie, 2004). The cytopathic effect of AHSV in cell culture appears 3 to 7 days after the first inoculation and appears more rapidly after a few passages (Coetzer and Guthrie, 2004). Virus plaque size in cell culture is considered a marker of virulence of AHSV, with large plaque variants usually less virulent than small plaque variants, making them more attractive candidates for vaccine production (Coetzer and Guthrie, 2004).

In studies on attenuation of other viruses, researchers have found point mutations and deletions in conserved regions, which could conceivably have an effect on virulence. For example, Puri et al. (1997) found that the degree of dengue virus attenuation increased with passage level, indicating that the contribution of the 25 nucleotide mutations, which resulted in 11 amino acid changes, to attenuation were cumulative. Five of these amino acid changes were found in the E protein, the major surface antigen, and six amino acid changes were found in nonstructural proteins. Sequence comparisons between virulent and cell culture attenuated rotaviruses (Zhang et al., 1998), showed that amino acid changes between position 131 and 140 in NSP4 are important in the virulence phenotype. Experimental mutations and deletions in this area confirmed their association with attenuation. Butrapet et al. (2000) found that a virulent parental dengue virus and a candidate attenuated vaccine virus differed by nine nucleotides. A mutation in the 5' noncoding region and an amino acid change in the NS1 protein were determined to be the main attenuation determinants. Attenuation may also affect plaque size, which may be associated with viral release.

1.3.3 Orbivirus structure and molecular biology

The double stranded RNA (dsRNA) viruses have similarities in the structure of the inner capsid layer and the enzymes it houses. Cognate proteins can even be identified in distantly related dsRNA viruses (Mertens, 2004). BTV is the best-studied member of the orbiviruses. Due to the similarity in the structure and molecular biology of different orbiviruses, BTV is taken as the example, except where stated.
The dsRNA genome of orbiviruses is encased in the viral capsid that consists of two layers: the outer capsid and the core, as shown in Fig. 1.1 (reviewed by Roy et al., 1994).

1.3.3.1 Core proteins
VP3 and VP7 are the two major core proteins. VP3 forms the inner scaffold of the core and interacts with the inner core proteins and the genomic dsRNA. VP7 forms the surface layer of the core. The three minor proteins, VP1, VP4 and VP6, as well as the dsRNA genome make up the inner part of the core as can be seen in Fig. 1.1 (Roy, 2001).

1.3.3.2 Outer capsid proteins
The outer capsid of the viral particle has an icosahedral structure. It consists of two proteins, VP5 and VP2 (Fig. 1.1), the two least conserved proteins of BTV. VP5 occupies the space formed by six-membered rings of VP7 trimers. VP2 is positioned above the VP7 trimers and protrudes past VP5 (reviewed by Roy, 2001). BTV VP2 is the serotype-determining antigen (Huismans and Erasmus, 1981; Mertens et al., 1989) and plays a role in virus attachment to cells (Hassan and Roy, 1999). BTV VP5 plays a role in virus penetration of the endosomal membrane and releasing the virus core into the cytoplasm.
(Hassan et al., 2001). AHSV VP5 may also contain neutralization epitopes (Martinez-Torrecuadrada et al., 1999).

1.3.3.3 Nonstructural Proteins
NS1 and NS2 are expressed at high levels while NS3 and NS3A are expressed at very low levels in host cells (Van Dijk and Huismans, 1988). NS1 forms tubules and may play a role in the release of virus from cells by budding rather than lytic release (Owens et al., 2004). NS2 forms multimeric inclusion bodies and binds single stranded RNA (ssRNA) (Huismans et al., 1987; Fillmore et al., 2002; Lymeropoulos et al., 2003; Butan et al., 2004). AHSV NS2 binds ssRNA less efficiently than BTV NS2 (Uitenweerde et al., 1995). NS3/NS3A facilitates virus release from cells (Hyatt et al., 1993) and has been found to be associated with the plasma membrane and intracellular vesicles (Hyatt et al., 1991).

1.3.3.4 Virus Genome
The AHSV genome consists of 10 dsRNA segments, which are situated inside the virus core (Fig. 1.1). The genome segments have been designated L1-L3, M4-M6, and S7-S10 according to their electrophoretic mobility (reviewed by Roy et al. 1994). Each genome segment codes for one protein, except S10, which codes for two proteins, NS3 and NS3A (Roy et al., 1994; Roy, 2001). The coding assignments are shown in Table 1.1.

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Nucleotide length</th>
<th>Protein</th>
<th>Amino acid length</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>3965</td>
<td>VP1</td>
<td>1305</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>L2</td>
<td>3221</td>
<td>VP2</td>
<td>1057</td>
<td>Serotype determining antigen; virus attachment to cells and virus entry into cells; haemagglutination</td>
</tr>
<tr>
<td>L3</td>
<td>2792</td>
<td>VP3</td>
<td>905</td>
<td>Forms inner scaffold of the core</td>
</tr>
<tr>
<td>M4</td>
<td>1978</td>
<td>VP4</td>
<td>642</td>
<td>mRNA capping and methylation</td>
</tr>
<tr>
<td>M5</td>
<td>1748</td>
<td>NS1</td>
<td>548</td>
<td>Forms tubules</td>
</tr>
<tr>
<td>M6</td>
<td>1566</td>
<td>VP5</td>
<td>505</td>
<td>Release of the viral core into the cytoplasm</td>
</tr>
<tr>
<td>S7</td>
<td>1167</td>
<td>VP7</td>
<td>349</td>
<td>Forms surface layer of the core</td>
</tr>
<tr>
<td>S8</td>
<td>1166</td>
<td>NS2</td>
<td>365</td>
<td>Forms cytoplasmic inclusion bodies; binds ssRNA</td>
</tr>
<tr>
<td>S9</td>
<td>1169</td>
<td>VP6</td>
<td>369</td>
<td>Helicase; binds ssRNA</td>
</tr>
<tr>
<td>S10</td>
<td>756</td>
<td>NS3/NS3A</td>
<td>217/206</td>
<td>Virus exit from cells</td>
</tr>
</tbody>
</table>
1.3.3.5 Viral Replication

Orbiviruses replicate in both their vertebrate hosts and in their arthropod vectors (reviewed by Roy, 2001). There are a few known differences in modes of virus entry into, and exit from the mammalian and insect cells. Due to the similarity in the structure of different orbiviruses, it can be assumed that their replication cycles are similar. The orbivirus replication cycle is illustrated in Fig. 1.2.

Adsorption to cells is due to VP2 (Hassan and Roy, 1999), which has haemagglutination properties (Mertens et al., 1987). It seems that the cell receptors to which BTV binds are glycophorins (Eaton and Crameri, 1989). BTV core particles with VP7 exposed bind to invertebrate cells better than whole viruses do, but not as well to mammalian cells (Xu et al., 1997). VP2 is responsible for virus entry into mammalian cells via receptor-mediated endocytosis (Hassan and Roy, 1999). An endocytic vesicle containing the virus is formed by the membrane invaginating and separating from the cell membrane (Eaton et al., 1990). VP2 is degraded and VP5 probably assists in virus entry into the cytoplasm by destabilizing the endosomal membrane (Hassan et al., 2001). VP2 and VP5 are then removed from the
virus leaving the core particle (Huismans et al., 1987). The virus core particles can then transcribe the viral RNA (Van Dijk and Huismans, 1980).

VP6 has an RNA binding and helicase function and catalyses the unwinding of the dsRNA segments (Stäuber et al., 1997; Kar and Roy, 2003; De Waal and Huismans, 2005). VP1 is the mRNA producing RNA-dependant RNA polymerase (Urakawa et al., 1989) and has been shown to synthesise dsRNA from the positive strand viral RNA (Boyce et al., 2004). The positive-strand viral mRNA is capped by VP4, which also has a methylation function (Ramadevi et al., 1998). The processed mRNA is released into the cytoplasm through pores in the five-fold axis of the virus core (Diprose et al., 2001; Mertens and Diprose, 2004). Viral proteins are synthesised in infected cells soon after infection until cell death. Inclusion bodies in infected cells may be sites of virus core assembly. These inclusion bodies are known to contain mRNA, NS2, VP3 and VP7. NS1 forms tubules in the cytoplasm of infected cells. It is also found in the inclusion bodies and may have a role in virus assembly. VP3 and VP7 form the virus core. VP1, VP4 and VP6 are enclosed in the virus core and interact with VP3. The outer capsid proteins attach to the core by interactions with VP7 (reviewed by Roy, 2001). Newly formed virions are then released from the cells.

Virus particles may bud through the cell membrane, obtaining a temporary envelope in the process, or they may exit the cells via disrupted cellular membranes (Hyatt et al., 1989; Stoltz et al., 1996). The nonstructural protein, NS3, has been implicated in the process of virus exit from cells (Hyatt et al., 1991; Hyatt et al., 1993; Stoltz et al., 1996). Beaton et al. (2002) indicated a mechanism for virus egress from cells. They have shown that the N-terminal region of BTV NS3 interacts with p11, a cellular protein that forms part of the calpactin complex, which is involved in exocytosis pathways. They have also shown that the C-terminal region of BTV NS3 interacts with VP2, suggesting that NS3 mediates the interaction between the virus, through VP2, and the pathway through which the virus particles exit the cell. Owens et al. (2004) have also linked NS1 to virus release from cells and cellular pathogenesis. They found that reduced NS1 tubule formation resulted in virus release by budding and a reduction in cell lysis. Furthermore, they propose that the ratio of NS1 and NS3 in cells may affect the mechanism of virus release. The differences in mechanisms of virus release can be observed in insect and mammalian cells, where virus is released from insect cells mainly by budding, and from mammalian cells by cell permeabilization or lysis resulting in cell death (Owens et al., 2004). Wirblich et al. (2006) have recently demonstrated that the late domain motifs PTAP and PPXY are present in orbivirus NS3 and are functional, although weakly so. The PTAP motif in NS3 is needed to
bind Tsg101, which is involved in the budding mechanism of release resulting in transiently enveloped virus particles. NS3 has been shown to have viroporin-like activity (Han and Harty, 2004), which may be involved in virus extrusion through permeabilized cell membranes. The exact mechanisms of virus exit from cells are not yet well understood.

### 1.3.4 Virulence associated proteins of AHSV

O’Hara et al. (1998) have indicated that VP2, VP5 and NS3 are associated with the virulence phenotype of AHSV in a study involving genome segment reassortment between virulent and avirulent AHSV strains in a mouse model. All the virulent reassortant viruses contained genome segments encoding VP2, VP5 and NS3. VP2 from the virulent parent was always present in the virulent reassortant viruses. The absence of VP2 from the virulent parent was associated with either an intermediate or an avirulent phenotype depending on which other segments from the virulent parent were present. VP5 and NS3 from the virulent parent were always present in the virulent reassortants, but were also present in the other phenotypes together with the VP2 from the avirulent parent. NS3 was found to confer intermediate virulence in the absence of VP2. VP5 may also play a role in the intermediate phenotype.

These three proteins are all involved in either viral cell entry or exit. NS3 (Van Staden et al., 1995) as well as VP5 (Hassan et al., 2001) are known to have cytotoxic properties. The role of VP2 in cell attachment (Hassan and Roy, 1999) indicates it may influence tissue tropism. While NS3 and VP2 have been implicated in a possible mechanism of virus release from cells (Beaton et al., 2002). VP2, VP5 and NS3 are also the most variable proteins between the different AHSV serotypes (Van Niekerk et al., 2001b).

The interaction between the virus and cell receptor initiates the viral entry process, and mutations in outer capsid proteins or envelope proteins as well as proteins involved in viral exit from cells can influence virus virulence (Schneider-Schaulies, 2000). A variety of studies on other viruses have identified viral genes connected with virulence. Viral genes encoding structural proteins that are likely to be involved in virus attachment and entry into cells and possibly also cell tropism, have been found to play a role in virulence in a number of viruses (Omata et al., 1986; Moss et al., 1989; Westrop et al., 1989; Hoshino et al., 1995; Forrest and Dermody, 2003; Goto et al., 2003; Mori et al., 2003; Kobasa et al., 2004). Certain nonstructural proteins have also been linked to virulence (Hoshino et al., 1995; Zhang et al., 1998; Butrapet et al., 2000; Hoyt et al., 2005), and mutations in noncoding regions of viral genomes have also been correlated with virulence changes (Omata et al., 1986; Moss et al., 1989; Westrop et al., 1989; Mandl et al., 1998; Butrapet et
al., 2000; Blaney et al., 2006). Studies in BTV have also pointed to the outer capsid proteins being involved in differences in the virulence phenotype (Huismans and Howell, 1973; Bernard et al., 1994; Carr et al., 1994).

1.3.4.1 NS3

The minor nonstructural proteins, NS3 and NS3A, are expressed at low levels in infected cells. The smallest genome segment, S10, encodes both these proteins, which are translated from two conserved in-phase initiation codons (Van Staden and Huismans, 1991). NS3 is 217 amino acid residues in length. NS3A differs in that its initiation codon is the 11th or 12th codon of NS3, depending on the serotype, thus, making NS3A approximately 207 amino acid residues in length. NS3 is the second most variable AHSV protein. It has certain conserved regions, some of which play a role in membrane association properties of NS3, which may contribute to AHSV virulence characteristics (Van Niekerk et al., 2001a; Van Niekerk et al., 2001b).

1.3.4.1.1 NS3 sequence variation

Sequence analysis of the NS3 encoding genome segment of different AHSV serotypes shows that AHSV NS3 is not as conserved as BTV NS3 (Sailleau et al., 1997; Martin et al., 1998). There is 30% divergence in the AHSV NS3 gene’s nucleotide sequence of the two most divergent AHSV serotypes. And there is approximately 36% amino acid variation between NS3 proteins of different serotypes, and as much as 27% within serotypes (Van Niekerk et al., 2001b). NS3 membrane association may expose it to immune selection, especially the region between the two hydrophobic domains (Van Niekerk et al., 2001b).

There are two conserved hydrophobic domains from residues 116-137 and residues 154-170, which may assist in membrane association (Van Staden et al., 1995). The second in-phase start codon for the initiation of NS3A is conserved at residue 11 or 12. A group of five prolines between residues 22-34 are conserved. There is another conserved region of about 50 amino acids between residues 43 and 92 (Van Staden and Huismans, 1991). Within this conserved region, there is a possible myristylation region from residue 60-65 that is also conserved in other orbivirus NS3 proteins (Van Niekerk et al., 2001b).

NS3 variation between virulent field isolates and non-virulent vaccine isolates, is between 2.3% and 9.7% depending on the serotype (Van Niekerk et al., 2001b). Sailleau et al. (1997) compared NS3 gene sequences from a vaccine strain, two virulent strains and a strain of unknown virulence and found the vaccine strain’s nucleotide sequence differed
from the other strains by 5.4% - 7.6%, with the other strains being more closely related. It was suggested the divergence could be due to mutations induced by a high number of passages in mouse brain. None of the NS3 sequences from vaccine strains have been found to be identical to the field isolates from the same serotype, although no correlations have yet been made between mutations and attenuation (Van Niekerk et al., 2001b).

Variation in NS3 protein sequences of a virulent and an attenuated strain has been shown to influence virulence characteristics (O'Hara et al., 1998). Martin et al. (1998) linked different patterns of viral release from Culicoides cells to NS3 using the same virulent and attenuated strains of AHSV as O'Hara et al. (1998). The virulent and avirulent parental viruses were released from cells at different times after infection, and the reassortant virus had the same pattern of virus release as the parent virus from which the NS3 encoding genome segment originated.

1.3.4.1.2 NS3 membrane association and virus release

There is evidence that NS3 plays a role in cell membrane damage and viral release from infected cells. It has been found to be cytotoxic in Spodoptera frugiperda (Sf9) cells. NS3 was expressed in a baculovirus expression system and was present at lower levels than expected, possibly due to membrane association and damage, causing membrane permeability and loss of osmotic control, which may explain the cytotoxicity (Van Staden et al., 1995; Van Staden et al., 1998). There was also an indication of membrane association from an investigation using immunofluorescence (Van Staden et al., 1995). Han and Harty (2004) have shown that NS3 has viroporin-like properties, which result in membrane permeability in mammalian cells.

BTV NS3 has also been found to be responsible for viral release from insect cells (Hyatt et al., 1993). This viral release can take place by either budding or extrusion through a disrupted cell membrane. It is likely that AHSV NS3 enables viral release from cells in much the same way as BTV NS3, thus contributing to virulence in AHSV (Van Niekerk et al., 2001b). NS1 has also been implicated in the viral release process. Reduction in NS1 tubule formation led to an increase in viral release by budding and less cytopathic effect, indicating the possibility that the ratio of NS1 to NS3 affects the mechanism of virus release (Owens et al., 2004).

The hydrophobic domains of AHSV NS3 have been implicated in membrane association as mutations in these domains prevent the cytotoxicity of NS3 in insect cells (Van Staden et al., 1998; Van Niekerk et al., 2001a). Virus-like particles, synthesized in a baculovirus
system expressing BTV proteins in Sf9 cells were released in the presence of NS3, but not in its absence (Hyatt et al., 1991). Stoltz et al. (1996) demonstrated that AHSV NS3 is associated with virus release from infected Vero cells, and that NS3 is present in the membrane components at the sites of virus release in Vero cells. Beaton et al. (2002) have shown that BTV NS3 interacts with the p11 component of the calpactin complex through its N-terminal residues, as well as with VP2 through its C-terminal residues, indicating a possible mechanism of virus egress via calpactin. Wirblich et al. (2006) indicate another possible mechanism of viral release. NS3 of BTV and AHSV have weak, but functional late-domains that play a role in virus budding through the cell membrane.

1.3.4.2 VP2

VP2 is the main outer capsid protein, and is encoded by the L2 genome segment that is 3229 nucleotides in length. AHSV VP2 has an observed size of 115kDa (Martinez-Torrecuadrada et al., 1994), while the calculated size is 124kDa (Iwata et al., 1992). VP2 has the most variable sequence of the AHSV proteins (Potgieter et al., 2003). Due to VP2 forming part of the outer capsid, it is subject to immune selection, which explains its high variability.

VP2 has been found to be the major serotype specific antigen on which most of the neutralizing epitopes are found (Huismans and Erasmus, 1981; Mertens et al., 1989; Burrage et al., 1993; Martinez-Torrecuadrada et al., 1994; Vreede and Huismans, 1994). The region of the protein from amino acids 200 to 413 (nucleotides 606-1251) has been found to be a major antigenic domain, with the N and C-terminal regions, to either side of this domain, having low immunogenicity. This study was not performed on conformational epitopes but on linear epitopes, which may not be exposed on the virus surface (Martinez-Torrecuadrada and Casal, 1995).

The outer capsid is involved in attachment to the cell and cell entry (Hassan and Roy, 1999; Hassan et al., 2001), after which VP2 and VP5 are removed to reveal the core particle, which is transcriptionally active (Van Dijk and Huismans, 1988). BTV VP2 has been shown to bind and enter cells, indicating that VP2 plays a role in BTV entry into cells (Hassan and Roy, 1999). BTV VP2 may also play a role in virus egress from cells through its interaction with NS3 and the cellular protein calpactin (Beaton et al., 2002). O’Hara et al. (1998) identified VP2 as playing a role in AHSV virulence. The role of VP2 in cell attachment and penetration may account for this role in virulence. Due to its role in cell attachment (Hassan and Roy, 1999), VP2 may influence tissue tropism, which plays a role in virulence (Schneider-Schaulies, 2000).
1.3.4.3 VP5

VP5 is the second of the two outer capsid proteins, and is encoded by the M6 genome segment of AHSV that is 1566 nucleotides in length. VP5 has an observed size of 56kDa (Martinez-Torrecuadrada et al., 1994); the calculated size is 57kDa (Iwata et al., 1992). A smaller protein of 50kDa, known as truncated VP5, has also been observed when the VP5 encoding gene is expressed in the baculovirus expression system (Grubman and Lewis, 1992; Martinez-Torrecuadrada et al., 1994). The M6 sequence of AHSV is comparable with the M5 genes of BTV and EHDV (epizootic haemorrhagic disease virus) (as reviewed by Roy et al., 1994).

VP5 is the third most variable AHSV protein (Van Niekerk et al., 2001b). It is probably less exposed than VP2 on the virus surface, and therefore undergoes less immune selection than VP2, making it less variable, though still more so than most of the other proteins (Roy et al., 1994).

AHSV VP5 has been expressed at low levels in the baculovirus expression system; the low levels of expression may be due to cytotoxicity of the protein (Martinez-Torrecuadrada et al., 1994; Du Plessis and Nel, 1997; Filter, 2000). AHSV-4 VP5, expressed in the baculovirus expression system, has been found to be less soluble than VP2 with much of the protein remaining associated with the cellular debris (Martinez-Torrecuadrada et al., 1994). They suggest that the low solubility may be due to the hydrophobic regions of VP5, which may be involved in membrane association. In BTV, the amino end of VP5 has been correlated with a low expression level in the baculovirus system. This indicates that the N-terminus, a model of which has two amphipathic helices supporting membrane association, plays a role in membrane destabilization. Both amphipathic helices were shown to permeabilize cell membranes experimentally (Hassan et al., 2001).

Purified BTV VP5 has been shown to be cytotoxic and to permeabilize both mammalian and insect cells (Hassan et al., 2001). BTV VP5 was also shown to bind to mammalian cell membranes, but was not internalised as VP2 was (Hassan et al., 2001; Hassan and Roy, 1999). It was suggested that BTV VP5 plays a role in membrane destabilization, enabling the virus core to enter the cytoplasm from the endocytic vesicle (Hassan et al., 2001; Forzan et al., 2004; Roy, 2005). This role in viral entry into cells and the results of O’Hara et al. (1998) indicate that VP5 may also play a role in the virulence phenotype of AHSV.
1.4 Aims

Much is still unknown about the molecular basis of attenuation of AHSV. The sequence differences that may arise in the proteins of AHSV during passage in cell culture, and to what degree such variation could contribute to attenuation and other phenotypic characteristics such as plaque morphology, are not known. Although AHSV NS3 and VP5, have been associated with virus virulence and have known cytotoxic properties, their effect on mammalian cells has not been well characterized.

The long-term aims of this project were to study the contribution of some of the AHSV proteins, such as the outer capsid proteins VP2 and VP5 and nonstructural protein NS3, to the phenotypic characteristics of the virus, especially virulence-related characteristics.

The following short-term questions were addressed in the project:
1. What sequence differences are observed in the VP2, VP5 and NS3 genes between a low passage AHSV isolate and a virus produced by passage of the low passage virus isolate in cell culture?
2. What sequence differences in the VP5 and NS3 genes and changes in plaque morphology are observed after repeated plaque purifications of a low passage AHSV isolate?
3. Are VP5 and NS3 cytotoxic in mammalian cells and do they cause membrane permeabilization?
4. Is it possible to associate differences in the cytotoxicity of VP5 and NS3, with changes in AHSV plaque morphology?
Chapter 2:
Variation of the nonstructural protein, NS3, and the outer capsid proteins, VP2 and VP5, after a process of attenuation by passage in cell culture

2.1 Introduction
VP2 is the most variable of the AHSV proteins with amino acid variation as high as 71% between serotypes (Potgieter et al., 2003). There is up to 36% amino acid variation in NS3, making it the second most variable AHSV protein, and VP5 is the third most variable protein with 19% amino acid variation between different serotypes (Van Niekerk et al., 2001b). VP2 is the major serotype-specific antigen (Huismans and Erasmus, 1981) and both VP2 and VP5 are immuno-reactive in virus-infected animals (Martinez-Torrecuadrada et al., 1994). Both proteins are subject to immune selection, but VP5 to a lesser extent. This partially explains their high variability. Hydrophobicity profiles of VP2 between AHSV serotypes (Vreede and Huismans, 1994; Potgieter et al., 2003) as well as between orbivirus serogroups (Williams et al., 1998) are similar, suggesting structural similarity despite the high sequence variation. Similarly, VP5 hydrophobicity profiles are comparable between serogroups (Du Plessis and Nel, 1997).

O’Hara et al. (1998) recognized VP2, VP5 and NS3 as having a function in the AHSV virulence phenotype. Due to its role in cell attachment and entry (Hassan and Roy, 1999), VP2 may influence tissue tropism and virus fitness. VP5 is involved in the permeabilization of the endosome membrane and in the release of the viral core into the cytoplasm (Hassan et al., 2001; Forzan et al., 2004; Roy, 2005). Stoltz et al. (1996) showed that NS3 is associated with virus exit from Vero cells, thus influencing cell-to-cell spread. VP2 may also be involved in virus exit from cells through an interaction with NS3 and a cellular exocytosis pathway (Beaton et al., 2002).

How attenuation is achieved when an original virus has a virulent phenotype is of interest in this project. It could occur either due to mutation of the original virulent virus, or due to selection of a less virulent phenotype from a pool of viruses present in the original virus isolate. The selection of a virus with reduced virulence or an attenuated virus can be brought about by passage of the virus in cell culture. Certain AHSV strains can be attenuated in this way, taking only 5 to 20 passages to produce an avirulent virus (Erasmus, 1966; Coetzer and Guthrie, 2004). In South Africa, the current AHSV vaccines
are based on live attenuated viruses. A range of small and large plaques can be observed for AHSV (Mirchamsy and Taslimi, 1966). Virus plaque size has been used as an indicator of suitable candidates for vaccine production, with large plaque variants usually chosen for the attenuation procedure (Coetzer and Guthrie, 2004). Viruses that produce larger plaques may have a higher fitness in the cell type used for attenuation and have a faster growth rate, spreading from cell to cell faster than a small plaque virus variant.

The quasispecies structure of viruses, or viral populations or pools, may play a role in the attenuation process. A specific viral genotype can be selected from a viral pool during artificial selection such as passage in cell culture, especially if the virus is passed through one or more genetic bottlenecks. Furthermore, a new idea has emerged where a change in the level of heterogeneity in a viral population, and not the selection of a specific mutation, may be correlated to a change in virulence (Sauder et al., 2006; Vignuzzi et al., 2006).

Relatively little is known about the molecular basis of attenuation. It is difficult to determine which changes in a virus are responsible for a change in the virulence phenotype during the attenuation process. There are, however, numerous examples in the literature where certain mutations are linked to changes in virulence. Rotavirus NSP4 sequence comparisons between virulent and cell culture attenuated viruses showed certain mutations in NSP4 to be virulence associated. The association was confirmed by site directed mutagenesis (Zhang et al., 1998). Butrapet et al. (2000) found nine nucleotide changes between an attenuated dengue type 2 virus, a flavivirus, and the virulent parental virus. Experimentation with recombinant viruses determined that a mutation in the 5' noncoding region, and an amino acid change in the NS1 protein were the main determinants of attenuation. Certain deletions in the 3' noncoding region of tick-borne encephalitis virus, another flavivirus, resulted in attenuation of the virus (Mandl et al., 1998). Multiple determinants of attenuation were found in the poliovirus type 1 vaccine strain, a picornavirus, including mutations in the 5' noncoding region and in the capsid proteins (Omata et al., 1986). Moss et al. (1989) found attenuation-determining mutations in the 5' noncoding region as well as in a genomic region encoding structural and nonstructural proteins of poliovirus type 2. Westrop et al. (1989) found two out of ten point mutations to be determinants of attenuation between a parental virulent type 3 poliovirus and its avirulent vaccine derivative. One mutation was in the 5' noncoding region, and one resulted in an amino acid change in a structural protein. Research by Cohen et al. (1987) indicated that the 5' noncoding region and the capsid region may be important for attenuation in hepatitis A virus, also a picornavirus.
This chapter focuses on the process of attenuation of AHSV on a molecular level. The selection of attenuated AHSV is generally carried out by repeated passage in cell culture. During earlier studies there was no means of determining whether attenuation occurred by random mutation, or by selection of a less virulent virus variant from a pool of viruses with differing levels of virulence. The original virus stocks are no longer available, but it may be of some value to recreate such an experiment to investigate the attenuation process. Such an opportunity arose from an experiment carried out at the Equine Research Centre at Onderstepoort Faculty of Veterinary Science, where an AHSV-4 strain was isolated from a horse, passaged 13 times in an undefined experiment, possibly resulting in lowered virulence, although the virulence phenotype has not been confirmed. The original material was available for further study. This enabled the detection of sequence differences between the virulence-associated proteins, VP2, VP5 and NS3, of the different passage level isolates. Additional information could be gathered on the plaque size of the isolates and on any sequence variation that may be associated with plaque size variation.

2.2 Materials and Methods

2.2.1 Cells

Vero (African green monkey) and BHK (baby hamster kidney) cells were grown as monolayers on 75cm² flasks at 37°C in a 5% CO₂ environment. The cells were grown in Minimum Essential Medium (MEM) or Dulbecco’s Modified Eagle’s Medium (DMEM) (Highveld Biological) supplemented with 2.5% to 5% foetal calf serum (FCS) (Highveld Biological), Penicillin and Streptomycin at a final concentration of 0.12mg/ml and Fungizone at a final concentration of 0.3mg/ml (Highveld Biological).

2.2.2 Virus propagation and passaging

AHSV serotype 4 seed stock (AHSV-4(1)) and a thirteenth passage virus derived from this isolate (AHSV-4(13)) were provided by Prof. A. Guthrie from the Equine Research Centre at Onderstepoort Faculty of Veterinary Science. Plaque titrations were carried out in a manner similar to that described by Dulbecco (1952). A serial dilution of AHSV-4(1) was infected on 80-90% confluent Vero cells seeded on 6 well plates. The virus was allowed to adsorb to the cells for 90 minutes at 37°C. The virus-containing medium was replaced with a 0.5% agarose overlayer containing 3 parts DMEM and 1 part Earle’s medium (11mM Glucose, 1.8mM CaCl₂, 5mM KCl, 0.8mM MgSO₄, 116mM NaCl, 26mM NaHCO₃, 1mM NaH₂PO₄). The 6 well plates were incubated at 37°C until plaques were visible; usually about five to six days post infection, as previously described by
Oellermann (1970). Plaques were then stained using 0.05% Neutral red and counted. Single unstained plaques were picked, resuspended in DMEM, and stored at 4°C. Virus isolated from single plaques was continually re-infected on Vero cells and the next generation of viral plaques were obtained.

Virus was grown on 75cm² flasks for RNA isolations. The virus was infected at a multiplicity of infection of approximately 0.1 plaque-forming units per cell, on 80-90% confluent Vero cells in a small volume of medium without FCS and antibiotics. Medium containing FCS and antibiotics was added after one hour. The cells were harvested when there was a 60-80% cytopathic effect, at approximately 3 to 4 days post infection.

2.2.3 RNA isolations

2.2.3.1 RNA extraction
Total RNA was isolated from infected Vero or BHK cells using TRIZOL (Gibco BRL), according to the manufacturer's instructions. The RNA pellet was air-dried in a nuclease free environment and resuspended in diethylpyrocarbonate (DEPC) treated ddH₂O.

2.2.3.2 dsRNA precipitation
Double stranded RNA was isolated from total RNA by lithium chloride precipitation. A final concentration of 2mM lithium chloride was added to the RNA and left at 4°C overnight. The dsRNA was isolated from the supernatant by centrifugation at 15000g at 4°C for 30 minutes. A final concentration of 0.2M sodium chloride was added to the dsRNA, which was then precipitated by the addition of 2.5 volumes of 96% ethanol and collected by centrifugation at 15000g at 4°C for 15 minutes. The dsRNA pellet was washed with 70% ethanol, air-dried and again resuspended in DEPC-treated ddH₂O and stored at -20°C until further use.

2.2.4 RT-PCR
AHSV NS3, VP5 and VP2 cDNA was synthesized using the method described by Wade-Evans et al. (1990). Approximately 200ng of dsRNA, in a volume of 5µl, was denatured using an equal volume of 10mM methylmercuric hydroxide (MMOH). The MMOH was then reduced by 0.7M β-mercaptoethanol and any RNases present were inhibited by an RNase inhibitor (Roche). The denatured RNA was used for cDNA production by incubation at 42°C for 90 minutes with 5U AMV reverse transcriptase (Promega) and 5× AMV buffer, 2.5mM dNTPs, and 100pmol of each forward and reverse gene-specific primer (Table 2.1). The cDNA was stored at -20°C until further use.

The cDNA was amplified by the polymerase chain reaction (PCR) in a 50µl volume containing 2.5U Taq polymerase (Promega) or Ex Taq (Takara), 10× Taq buffer, 25mM MgCl₂, 1mM dNTPs, and 100pmol of each primer (the same primers as used for cDNA synthesis). To amplify the NS3 gene, cDNA was denatured for 2 minutes at 95°C, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing of primers at 55°C for 30 seconds, and elongation at 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes.
To amplify the VP5 gene, cDNA was denatured for 5 minutes at 95°C, followed by 35 cycles of
denaturation at 94°C for 45 seconds, annealing of primers at 58°C for 30 seconds, and elongation at
72°C for 2 minutes, followed by a final elongation step of 72°C for 10 minutes.
To amplify the VP2 gene, cDNA was denatured for 5 minutes at 95°C, followed by 30 cycles of
denaturation at 94°C for 1 minute, annealing of primers at 60°C for 30 seconds, and elongation at
72°C for 4 minutes, followed by a final 10 minute elongation step at 72°C.
The PCR products were analysed by agarose gel electrophoresis.

2.2.5 Agarose gel electrophoresis
A 1% agarose gel was stained with a final concentration of 0.5µg/ml Ethidium Bromide and
electrophoresed in 1× TAE buffer (0.04M Tris-acetate, 1mM EDTA, pH 8.5) at 80V for 30 to 60
minutes and visualized under UV light.

2.2.6 Insertion of VP5 and VP2 PCR products into pCR-XL-TOPO
The VP5 and VP2 PCR products were purified from a 0.8% agarose gel and inserted into pCR-XL-
TOPO using the TOPO XL PCR Cloning Kit (Invitrogen) according to the manufacturer’s
instructions. The ligation reaction was set up according to the manufacturer’s instructions and
incubated at room temperature for 5 minutes, followed by transformation into One Shot TOP10
competent cells (Invitrogen). The cells were then plated out on agar plates with selective media, i.e.
50µg/ml Kanamycin. Plasmid DNA was isolated for analysis as described in paragraph 3.2.1.7 and
restriction enzyme digestion reactions were carried out as described in paragraph 3.2.1.1.

2.2.7 DNA sequencing and sequence analysis
2.2.7.1 DNA purification
PCR products and plasmid constructs were purified for sequencing using a High Pure PCR Product
Purification Kit (Roche) and a High Pure Plasmid Purification Kit (Roche) respectively.

2.2.7.2 Cycle sequencing and automated sequencing
The cycle sequencing reaction was performed using 50-200ng of PCR product or approximately
200ng plasmid DNA, 3.2pmol of the appropriate primer (Table 2.1), and 4µl ABI prism BigDye
Terminator ready reaction mix (Applied Biosystems). The cycle sequencing product was precipitated
using the ethanol sodium acetate precipitation or the ethanol precipitation method according to the
manufacturer’s instructions (Applied Biosystems), and sequenced using an ABI 377 or ABI 3100
automated sequencer (Applied Biosystems).

2.2.7.3 Sequence analysis
The sequences were translated using Sequence Navigator v.1.0.1 (Applied Biosystems). Nucleotide
and predicted protein sequences were aligned using Clustal X v.1.83 (Thompson et al., 1997).
Table 2.1: Primers used for NS3, VP5 and VP2 gene segment amplification and sequencing.

<table>
<thead>
<tr>
<th>Gene/plasmid</th>
<th>Primer name</th>
<th>Purpose</th>
<th>Forward (F) / Reverse (R)</th>
<th>Binding site on gene or plasmid</th>
<th>Primer sequence (5’ to 3’)</th>
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<tr>
<td>NS3</td>
<td>NS3 I</td>
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<td>GTT TAA ATT ATC CCT TGT CAT G</td>
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<td>NS3 II</td>
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<td>3’ noncoding region</td>
<td>GTA AGT CGT TAT CCC GGC TCC</td>
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<td>NS3pBam</td>
<td>cDNA synthesis, PCR &amp; Sequencing</td>
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<td>CGG GAT CCG TTT AOA TTA TCC CTT G</td>
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<tr>
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<td>NS3pEco</td>
<td>cDNA synthesis, PCR &amp; Sequencing</td>
<td>R</td>
<td>3’ noncoding region</td>
<td>CGG AAT TCG TAA GTC GAT ATC CCG G</td>
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<tr>
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<tr>
<td></td>
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<td>cDNA synthesis, PCR &amp; Sequencing</td>
<td>R</td>
<td>3’ noncoding region</td>
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<td></td>
<td>HS4L2SEQ-Z</td>
<td>Sequencing</td>
<td>R</td>
<td>Bases 2590-2611</td>
<td>CGA TAA CCC TTA ACC CTT TGG</td>
</tr>
<tr>
<td></td>
<td>pCR-XL-TOPO</td>
<td>M13 Forward</td>
<td>Sequencing</td>
<td>Bases 205-2210 on pCR-XL-TOPO</td>
<td>GTA AAA CGA CCG CCA G</td>
</tr>
<tr>
<td></td>
<td>pCMV Script</td>
<td>M13 Reverse</td>
<td>Sequencing</td>
<td>Bases 433-448 on pCR-XL-TOPO</td>
<td>CAG GAA ACA GCT ATG A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3</td>
<td>Sequencing</td>
<td>Bases 620-639 on pCMV Script (12 bases upstream of multiple cloning site)</td>
<td>AAT TAA CCC TCA CTA AAG GG</td>
</tr>
</tbody>
</table>
2.3 Results

At the start of this investigation two AHSV-4 isolates were provided by Prof. A. Guthrie from the Equine Research Centre. The first of these, AHSV-4(1), was the primary isolate from a diseased horse that had been passaged in tissue culture only once. Such primary isolates are usually still virulent. The second virus isolate, AHSV-4(13), was the same strain, but after it had been passaged thirteen times in tissue culture (Fig. 2.1). AHSV-4(13) may have lost its virulence characteristics. It was not known if this attenuation in cells was associated with a change in plaque morphology or genome sequence.

This provided an opportunity to determine whether any differences could be detected between AHSV-4(1) and AHSV-4(13) on a molecular and phenotypic level. On the phenotypic level this was done by investigating plaque morphology, and on the molecular level by sequencing the VP2, VP5 and NS3 genes. This was followed up with a closer look at plaque size by passaging small and large plaque variants of AHSV-4(1) by plaque-to-plaque transfer and subsequently investigating them on a molecular level.

2.3.1 Variation in virus plaque size

In order to investigate differences in plaque morphology and plaque size of AHSV-4(1) and AHSV-4(13) the viruses were plaque titrated (paragraph 2.2.2).

Vero cells in a 6-well plate were infected with serial dilutions (1 × 10⁻¹ to 1 × 10⁻⁶) of virus stock and an agarose overlayer was placed over the infected cells. Plaques were visible from three to four days post infection. The cell layer was stained with neutral red, and the unstained plaques counted and photographed six days post infection.

The AHSV-4(13) plaques were generally larger than those of AHSV-4(1). The majority of the AHSV-4(13) plaques were approximately 3-3.5mm in diameter at six days post infection with some smaller plaques present. The AHSV-4(1) plaques varied in size, but were relatively small compared to AHSV-4(13) plaques, ranging from approximately 0.4-1.7mm in diameter, with a few larger plaques present, at six days post infection (Fig. 2.2, compare B and C).

Variation was therefore found between the viruses with regard to plaque size.
Figure 2.1  Schematic diagram showing the viruses used in this study. The AHSV-4(1) isolate being from a presumed pool of viruses and the AHSV-4(13) isolate being selected from that presumed pool. The attenuation procedure carried out at the Equine Research Centre is illustrated.

Figure 2.2  Photos of plaques from titrations of AHSV-4(1) and AHSV-4(13) in Vero cells stained with neutral red at six days post infection.
A: Uninfected Vero cells in a well of a six well plate.
B: AHSV-4(1) infected Vero cells in a well of a six well plate.
C: AHSV-4(13) infected Vero cells in a well of a six well plate.
2.3.2 VP2, VP5 and NS3 sequence variation between AHSV-4(1) and AHSV-4(13)

In order to determine if sequence differences could be detected between the AHSV-4(1) and AHSV-4(13) VP2, VP5 and NS3 genes, they were sequenced and compared.

2.3.2.1 VP2 sequence variation

The AHSV-4(1) and AHSV-4(13) isolates viral dsRNA was extracted from virus infected Vero cells, reverse transcribed, PCR amplified and sequenced (paragraphs 2.2.3, 2.2.4, and 2.2.7). Overlapping nucleotide sequences were aligned and the amino acid sequences were deduced. The nucleotide and amino acid sequences of VP2 of the AHSV-4(1) and AHSV-4(13) isolates were compared in order to observe any variation between the viruses’ VP2 sequences. The nucleotide comparison of VP2 is shown in Appendix A and the sequence comparison is summarized in Table 2.2. The deduced amino acid sequences are aligned in Fig. 2.3.

There are five variable nucleotide sites, resulting in four variable amino acid sites between the VP2-PCR amplicons of AHSV-4(1) and AHSV-4(13). Two of these variable amino acids occur in known antigenic regions of VP2. Three of these variable sites are polymorphic in the sequence of the VP2 PCR product, i.e. the sequence is ambiguous, or both bases are present in one position in the sequencing electropherogram. This shows that there are sequence differences between the VP2 proteins of AHSV-4(1) and AHSV-4(13).

The occurrence of ambiguous VP2 sequences indicates a mixed virus population. It was therefore decided to analyse the sequences further to determine whether AHSV-4(1) and AHSV-4(13) did indeed consist of heterogeneous viral populations. This was done by cloning and sequencing the VP2 genes of the respective viruses.

Nucleotide sequences from PCR amplicons of VP2 and from the VP2 genes inserted into pCR-XL-TOPO (Invitrogen), as described in paragraph 2.2.6, were compared (Appendix A, Table 2.2, Fig 2.3). The deduced amino acid sequence of the cloned VP2 gene and the PCR product from AHSV-4(13) were the same, with the exception of the ambiguous sites in the VP2 PCR amplicon sequence. The nucleotides at those ambiguous sites in the cloned VP2 gene of AHSV-4(13) were the same as those nucleotides of the VP2 PCR amplicon sequence of AHSV-4(13) that differed from the AHSV-4(1) sequence. In addition, eight variable nucleotide sites, resulting in 6 variable amino acids, were observed between the cloned VP2 sequence and the sequenced VP2-PCR amplicon of AHSV-4(1).
These sequence differences indicate that AHSV-4(1) and AHSV-4(13) from which the dsRNA was cloned, are viral pools or quasispecies. Most of these differences and the variation between AHSV-4(1) and AHSV-4(13) occurred in the first antigenic region of AHSV-4 VP2 identified by Martinez-Torrecuadrada et al. (2001).

Table 2.2: Variation in VP2 nucleotide and amino acid sequences.

<table>
<thead>
<tr>
<th>sequence compared to AHSV-4(1) PCR amplicon</th>
<th>nucleotide difference (length = 3183bp) position</th>
<th>amino acid difference (length = 1060aa) position</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-4(13) PCR amplicon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U → A</td>
<td>52</td>
<td>Leu → Ile</td>
</tr>
<tr>
<td>U → C *</td>
<td>1037</td>
<td>Met → Thr *</td>
</tr>
<tr>
<td>A → G *</td>
<td>1063</td>
<td>Thr → Ala *</td>
</tr>
<tr>
<td>A → G</td>
<td>1920</td>
<td>silent</td>
</tr>
<tr>
<td>A → G *</td>
<td>2465</td>
<td>Gln → Arg *</td>
</tr>
<tr>
<td>AHSV-4(13) clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U → A</td>
<td>52</td>
<td>Leu → Ile</td>
</tr>
<tr>
<td>U → C</td>
<td>1037</td>
<td>Met → Thr</td>
</tr>
<tr>
<td>A → G</td>
<td>1063</td>
<td>Thr → Ala</td>
</tr>
<tr>
<td>A → G</td>
<td>1920</td>
<td>silent</td>
</tr>
<tr>
<td>A → G</td>
<td>2465</td>
<td>Gln → Arg</td>
</tr>
<tr>
<td>AHSV-4(1) clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C → U</td>
<td>7</td>
<td>Pro → Ser</td>
</tr>
<tr>
<td>A → C</td>
<td>674</td>
<td>His → Pro</td>
</tr>
<tr>
<td>C → U</td>
<td>761</td>
<td>Ala → Val</td>
</tr>
<tr>
<td>G → U</td>
<td>932</td>
<td>Cys → Phe</td>
</tr>
<tr>
<td>U → C</td>
<td>1093</td>
<td>Phe → Leu</td>
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<tr>
<td>A → U</td>
<td>1202</td>
<td>Gln → Leu</td>
</tr>
<tr>
<td>A → G</td>
<td>1548</td>
<td>silent</td>
</tr>
<tr>
<td>G → A</td>
<td>3177</td>
<td>silent</td>
</tr>
</tbody>
</table>

* polymorphic site in virus pool, indicated by both bases present in one position in the sequencing electropherogram

2.3.2.2 VP5 sequence variation
The AHSV-4(1) and AHSV-4(13) isolates were investigated on the VP5 gene and protein sequence levels to identify sequence variation between the virus isolates.

AHSV-4(1) and AHSV-4(13) infected Vero cells were used for dsRNA isolation. The RNA was then reverse transcribed and amplified. Nucleotide sequences of the VP5 encoding genes of each virus were determined and translated to amino acid sequences in order to identify any differences between the AHSV-4(1) and AHSV-4(13) isolates. The nucleotide comparison of VP5 is shown in Appendix B and is summarized in Table 2.3. The deduced amino acid sequence comparison is shown in Fig. 2.4. One change was found between the
VP5 sequences of AHSV-4(1) and AHSV-4(13), an adenine (A) to a guanine (G), resulting in an amino acid change from a glutamine to an arginine within an antigenic region of VP5.

The sequences were also investigated for indications of AHSV-4(1) and AHSV-4(13) consisting of a variable viral pool. VP5 encoding segments from AHSV-4(1) and AHSV-4(13) were inserted into pCR-XL-TOPO (Invitrogen) (paragraph 2.2.6), sequenced and compared to the sequences obtained from the PCR products (Appendix B, Table 2.3, Fig. 2.4). The A to G nucleotide change, resulting in the glutamine to arginine amino acid change mentioned above, was also observed in the sequence of the cloned VP5 gene of AHSV-4(13). An additional silent nucleotide mutation and three more amino acid differences were observed within the cloned VP5 genes from AHSV-4(1) and AHSV-4(13).

The differences between the cloned genes and the PCR amplicons are indicative of heterogeneous viral pools. The majority of the variation observed occurred in the antigenic regions of AHSV-4 VP5 identified by Martinez-Torrecuadrada et al. (1999), including the glutamine to arginine change which was consistent between the PCR derived sequences as well as the cloned gene sequences of AHSV-4(1) and AHSV-4(13). There was no variation present in the amphipathic helices, which are likely to play a role in possible cytotoxic properties of AHSV VP5, as was shown for BTV VP5 (Hassan, et al., 2001).

Table 2.3: Variation in VP5 nucleotide and amino acid sequences.

<table>
<thead>
<tr>
<th>sequence compared to AHSV-4(1) PCR amplicon</th>
<th>nucleotide (length = 1518bp) difference</th>
<th>amino acid (length = 505aa) difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-4(13) PCR amplicon</td>
<td>A → G 278 Gln → Arg 93</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13) clone</td>
<td>A → G 278 Gln → Arg 93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A → G 562 Thr → Ala 188</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G → A 700 Glu → Lys 234</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(1) clone</td>
<td>G → A 588 silent 292</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A → G 995 Lys → Arg 332</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2.3 NS3 sequence variation

The NS3 sequences of the AHSV-4(1) and AHSV-4(13) isolates were investigated in order to identify any sequence variation in NS3 between the viruses.

Viral dsRNA was extracted from Vero cells infected with each virus. RT-PCR was performed and the PCR products were purified and sequenced. The NS3 gene sequences from the AHSV-4(1) and AHSV-4(13) isolates were compared. The full nucleotide comparison for NS3 is shown in Appendix C. The results are summarized in Table 2.4. Deduced amino acid sequences were also aligned and compared (Fig. 2.5). A transition
from a uracil (U) to a cytosine (C) in the second nucleotide position of codon number 208 was found in the NS3 encoding gene of AHSV-4(13). It resulted in an amino acid change from leucine to serine in the C-terminal region of NS3. The same amino acid difference was identified between the AHSV-8 field strains (HS2/98; accession number AF276692, and HS7/98; accession number AF276691) and the AHSV-8 vaccine strain (S8 Vaccine; accession number AF276690) (Van Niekerk, 2001).

In order to determine if the difference observed between the NS3 genes of AHSV-4(1) and AHSV-4(13) was due to the selection of a specific genotype from a heterogeneous viral pool during viral passaging, NS3 genes from AHSV-4(1) and AHSV-4(13) were inserted into pCMV Script (Stratagene) (paragraph 3.3.2). Two clones from both AHSV-4(1) and AHSV-4(13) were sequenced and compared to the AHSV-4(1) and AHSV-4(13) NS3 sequences obtained from the PCR products (Appendix C, Table 2.4, Fig. 2.5). The transition from U to C, resulting in the leucine to serine amino acid change, was found in both cloned NS3 genes of AHSV-4(13) and not found in either of the AHSV-4(1) cloned NS3 genes. Three more non-synonymous and two synonymous mutations were identified in the cloned NS3 genes; these differences were not evident in the PCR product sequences, which represent the viral pool consensus sequence as the PCR amplicons were obtained from the viral pools.

The leucine to serine change at residue 208 in NS3 is the only difference that was consistent between AHSV-4(1) and AHSV-4(13) in the PCR derived sequences as well as the cloned gene sequences. Additional differences between the AHSV-4(1) clones and PCR sequence, as well as between the AHSV-4(13) clones and PCR sequence are indicative of a heterogeneous viral pool.

Table 2.4: Variation in NS3 nucleotide and amino acid sequences.

<table>
<thead>
<tr>
<th>sequence compared to AHSV-4(1) PCR amplicon</th>
<th>nucleotide (length = 654bp) difference position</th>
<th>amino acid (length = 217aa) difference position</th>
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<tbody>
<tr>
<td>AHSV-4(13) PCR amplicon</td>
<td>U → C 623</td>
<td>Leu → Ser 208</td>
</tr>
<tr>
<td>AHSV-4(13) clone1</td>
<td>U → C 623</td>
<td>Leu → Ser 208</td>
</tr>
<tr>
<td>AHSV-4(13) clone2</td>
<td>A → G 121</td>
<td>Ser → Gly 41</td>
</tr>
<tr>
<td></td>
<td>A → G 230</td>
<td>Glu → Gly 77</td>
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<td></td>
<td>U → C 623</td>
<td>Leu → Ser 208</td>
</tr>
<tr>
<td>AHSV-4(1) clone2</td>
<td>A → G 15</td>
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<td></td>
<td>G → A 57</td>
<td>silent</td>
</tr>
<tr>
<td></td>
<td>U → C 62</td>
<td>Val → Ala 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>790</td>
<td>800</td>
<td>810</td>
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<tr>
<td>AHSV-4(1)</td>
<td>LRFFPTYRHYYLETQRVFNDERRLEVFYDFMYQYDVTQREQLNTFTDFHRCVESELLLPITKLNFLLWIVFEMENVEVNAAYKRHPPLISBNTEAKLRLTVIGVDFNGLS1SMGWIYPYVERMCAESKVQ</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(1)-clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)-clone</td>
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<tr>
<td>920</td>
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<td>940</td>
</tr>
<tr>
<td>AHSV-4(1)</td>
<td>TKLTADELKLRWFISYYTTLKLDRRAEPRMSFKFEGLSTWIGSCNGVQVYIVLQMPKTEAMVYARDSRIEWMIEAEELSQWLGMLTGLLGFLILVHDSGIINKSVLRARTKLYNYRGSMDTLILIS</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(1)-clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)-clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1050</td>
<td>1060</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(1)</td>
<td>SGVYTFGNKFLSSLKLAKTE</td>
<td></td>
</tr>
<tr>
<td>AHSV-4(1)-clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV-4(13)-clone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.3**  Amino acid sequence alignment of VP2 from AHSV-4(1) and AHSV-4(13). Dashes indicate identity to AHSV-4(1) VP2 sequence and letters indicate amino acid changes with respect to the AHSV-4(1) VP2 sequence. The antigenic regions are shaded turquoise. Two neutralizing epitopes are shaded red. Polymorphic sites in a virus pool, which had two bases at a single position in the sequencing electropherogram, are highlighted in yellow.
Figure 2.4  Amino acid sequence alignment of VP5 from AHSV-4(1) and AHSV-4(13). Dashes indicate identity to AHSV-4(1) VP5 sequence and letters indicate amino acid changes with respect to the AHSV-4(1) VP5 sequence. The major antigenic region is shaded grey. Two more specific antigenic regions are shaded turquoise. The amphipathic helices are shaded yellow.
Figure 2.5  Amino acid sequence alignment of NS3 from AHSV-4(1) and AHSV-4(13). Dashes indicate identity to AHSV-4(1) NS3 sequence and letters indicate amino acid changes with respect to the AHSV-4(1) NS3 sequence. The conserved methionine coded for by the NS3A start codon is shaded grey. The proline-rich region is shaded green and the L-domains within this region are indicated by lines above them. The conserved region is shaded turquoise with the myristilation motif within this region in yellow. The hydrophobic domains are shaded red.
2.3.3 Variation in virus plaque size and NS3 and VP5 sequences between AHSV-4(1) and a derived plaque purified line

In the previous sections (2.3.1 and 2.3.2) variation in plaque morphology and sequence variation were observed between the AHSV-4(1) and AHSV-4(13) isolates. The sequence variation was indicative of a quasispecies structure. As the serial passage experiment from which AHSV-4(1) and AHSV-4(13) were obtained was relatively undefined, it was decided that a new experiment was to be carried out, where AHSV-4(1) was subjected to a certain number of controlled serial plaque-to-plaque transfers (Fig. 2.6). This made it possible to monitor the changes in plaque morphology and to identify how much sequence variation is introduced in NS3 and VP5 by a number of passages. This process also enabled the identification of the same or similar outcomes to those observed in the comparison between the AHSV-4(1) and AHSV-4(13) isolates.

Figure 2.6  Schematic diagram showing the viruses used in this study. The AHSV-4(1) isolate being from a presumed pool of viruses. The serial plaque purifications carried out to investigate the effect on plaque morphology and sequence variability are illustrated.

2.3.3.1 Virus plaque size

A small plaque and a relatively large plaque of AHSV-4(1) were selected and individually passaged by serial plaque purification for a total of eight passages (Fig. 2.6) to observe changes in plaque morphology.

Vero cells were infected with serial dilutions of the virus and covered with a nutrient and agarose overlayer. Plaques became visible at three to four days post infection. Small and large plaque variants of the AHSV-4(1) isolate were picked for further passage at six days post infection, at which stage plaques were easily detectable. Plaques 8a and 8b were both derived, as separately passaged lines, from an original small plaque. The original small plaque was isolated from the first plaque titration of the AHSV-4(1) isolate, after which two progeny plaques were isolated and passaged in parallel by serial plaque isolation. Plaques
8c and 8d were derived, also as separately passaged lines, from an original large plaque of AHSV-4(1). Plaque size can be influenced by the contents of the overlay (Mirchamsy and Taslimi, 1966) as well as by the cell type on which the virus is propagated (Oellermann, 1970). Therefore the overlay and cell type, i.e. Vero cells, were kept constant throughout the experiment.

After the fourth passage, an overall increase in plaque size was observed with both the small and large plaque variants increasing in size. No more small plaques could be found in the original small plaque line, and it was no longer possible to distinguish between the small and large plaque variants on the basis of plaque size. By the eighth passage the average plaque size had decreased slightly.

Thus, passaging the virus by serial plaque purification resulted in a change in plaque size.

2.3.3.2 Sequence variation

Sequences of only the NS3 and VP5 encoding genes of the eighth plaque purified passage viruses originating from small (8a and 8b) and large plaque (8c and 8d) variants of AHSV-4(1) were investigated. These sequences were used to identify any differences which may have arisen during passage by serial plaque isolation, or which may have been present between small and large plaque variants. Any sequence variation providing evidence of a quasispecies structure was also noted.

The NS3 and VP5 nucleotide sequences from the serially plaque purified AHSV-4 viruses were aligned and compared to the sequences of the AHSV-4(1) isolate. Deduced amino acid sequences were also aligned and compared. No variation was found in the amino acid sequence of VP5. However, a silent mutation of a G to an A at nucleotide 894, was observed in plaque 8d (see Appendix B). In NS3, the same change from a leucine to a serine at amino acid position 208 was found in plaque 8d, as was observed in AHSV-4(13) (Fig. 2.7).

The sequences of the NS3 encoding genes of the third passage were determined to establish whether the change from leucine to serine was present early on during passaging or whether it appeared later. However, the NS3 sequences of the third passage were found to be identical to that of the original AHSV-4(1) isolate (results not shown).

No sequence changes in NS3 or VP5 were found to be consistent with the variation in plaque size.
Figure 2.7  Amino acid sequence alignment of NS3 from the 8th passages of the small (8a and 8b) and large plaque variants (8c and 8d) derived from AHSV-4(1), compared to the original AHSV-4(1) and AHSV-4(13) isolates. Dashes indicate identity to the AHSV-4(1) NS3 sequence. Letters indicate amino acid changes with respect to the AHSV-4(1) NS3 sequence. The conserved methionine coded for by the NS3A start codon is shaded grey. The proline-rich region is shaded green and the L-domains within this region are indicated by lines above them. The conserved region is shaded turquoise with the myristilation motif within this region in yellow. The hydrophobic domains are shaded red.
2.4 Discussion

The purpose of these experiments was firstly to examine any differences between the NS3, VP5 and VP2 sequences of the AHSV-4(1) isolate and the AHSV-4(13) isolate obtained by serial passage at the Equine Research Centre, and to ascertain whether the differences are located in certain domains within the proteins, such as the hydrophobic domains in NS3, the antigenic regions in VP5 or VP2, or the amphipathic helices of VP5, and to establish whether the differences may be correlated with plaque size. Secondly, it was investigated whether plaque size may be related to differences in protein sequences. This was done by passaging purified plaque size variants and sequencing the NS3 and VP5 genes of these virus variants. These proteins have been associated with virus virulence in a genomic reassortment study (O’Hara et al., 1998), and virus entry and exit from cells (Stoltz et al., 1996; Hassan and Roy, 1999; Hassan et al., 2001). NS3 plays a role in virus exit from cells via the cell membrane (Stoltz et al., 1996), and VP5 plays a role in virus release from the endosome into the cytoplasm (Hassan et al., 2001). The genes encoding VP2, NS3 and VP5 are also the most variable in the AHSV genome (Van Niekerk et al., 2001b). This made them good candidates for the study of sequence variation between virulence and plaque size variants.

There was variation in the plaque size of AHSV-4(1) with a range of sizes from small to relatively large plaques. The plaques of AHSV-4(13) were larger than those of AHSV-4(1), and their size was more uniform, indicating a more homogeneous virus population with regards to plaque phenotype. Plaque size may be determined by the viral replication rate and the spread of the virus between cells. Therefore, the replication rate of AHSV-4(13) in the cells in which the viruses were grown may be faster than that of AHSV-4(1), which had smaller plaques compared to AHSV-4(13), and possibly a lesser ability to spread from cell to cell. The passaging process may have selected a virus adapted to replication in the cells. The variation in the plaque size of AHSV-4(1) also indicates a mixed population of viruses with different replication abilities in the cells.

The AHSV-4(1) isolate was independently passaged by serial plaque purification, and plaque size was observed for any change throughout the process. The increase in plaque size after the fourth passage may have been due to adaptation of the virus to the Vero cells. Since it is easier to pick large plaques there may be a bias towards picking the larger, more visible plaques, although a conscious attempt was made to
pick the smallest plaques. The increase in plaque size occurred at the same time for both the small and large plaque variants which both increased in size to a fairly uniform size larger than the original large plaques of AHSV-4(1). Thereafter, the variants could no longer be distinguished from each other on the basis of their plaque size. No difference between the plaque size variants could be detected based on their apparent ability to adapt to cell culture. There was a subsequent decrease in plaque size by the eighth passage. This may have been caused by a reduction of the fitness components of the virus due to the repetitive genetic bottlenecks resulting in an accumulation of deleterious mutations in accordance with Muller’s ratchet (Chao, 1990; Duarte et al., 1992). None of the sequence variation found in either NS3 or VP5 of any of the plaque variants could be linked to the variation or the change in plaque size.

The genes encoding VP2, VP5 and NS3 of AHSV-4(1) and AHSV-4(13) were sequenced to determine if there were differences between the viruses. The gene sequences obtained for AHSV-4(1) and AHSV-4(13) showed signs of ambiguity, indicating that both virus isolates are composed of mixed populations of virus variants. This supports the phenotypic evidence for mixed virus populations observed in the variable plaque size. The ambiguities in the gene sequences are supported by differences between the sequences obtained from PCR amplified genes and cloned genes of the same virus isolate. This variation is unlikely to be caused by polymerase error because the changes in the outer capsid proteins tend to be clustered in the antigenic regions and are not entirely random as they would be if due to polymerase error.

In the NS3 sequences, evidence was found of a single amino acid change occurring in two independently passaged lines of AHSV-4(1). The leucine to serine amino acid change in NS3 was observed between the AHSV-4(1) and AHSV-4(13) isolates, as well as between the AHSV-4(1) and eighth passage plaque, 8d. A virus variant with a serine at NS3 amino acid position 208 is likely to have been selected from a virus pool by the passaging process in both instances, rather than the same mutation arising twice. The leucine being the amino acid present at position 208 in the NS3 sequence of the third passage plaque precursor of 8d may indicate that the process of picking plaques does not necessarily produce a single virus genotype. A number of viruses may be associated with a piece of cell debris and therefore may form a viral pool within a single plaque. It is remarkable that the leucine/serine amino acid difference was also present between the serotype 8 field and vaccine strains.
Niekerk, 2001), and that it was one of the independently passaged large plaque variants of AHSV-4(1) in which this difference was found. In the past, large plaque variants were used for the manufacture of AHSV vaccine strains and thus avirulent viruses.

There was no variation in the hydrophobic domains that play a role in the cytotoxic properties of NS3 (Van Staden et al., 1998; Van Niekerk et al., 2001a). Also, no variation was present in the amphipathic helices of VP5, which may play a role in cytotoxic properties of AHSV VP5 as has been illustrated for BTV VP5 (Hassan et al., 2001). These are areas of the proteins that are likely to contribute to the cytotoxic characteristics of the proteins when expressed in the absence of other viral proteins.

No variation could be observed in the noncoding regions of the genes, as the DNA primers used for gene amplification bind to these regions concealing any variation that may have arisen in these sections. Other protein domains, which may be more important in protein-protein interaction, may be significant in the virulence characteristics of the virus.

The leucine to serine amino acid change between the NS3 proteins of AHSV-4(1) and AHSV-4(13) was found in the C-terminal region of NS3, which, in BTV, has been shown to interact with VP2 in a possible mechanism of virus release (Beaton et al., 2002). The majority of the variation observed in VP2 occurred in the first antigenic region of the protein. This first major antigenic region in AHSV-4 VP2 identified by Martinez-Torrecuadrada et al. (2001) corresponds to the first half of a general antigenic region identified in AHSV-3 VP2 from residue 224 to 543 (Bentley et al., 2000). A neutralization domain in AHSV-4 VP2 (Martinez-Torrecuadrada et al., 2001) within the first major antigenic region corresponds to the linear epitope identified in AHSV-9 VP2 between residues 369 and 403 (Venter et al., 2000). The antigenic regions of VP2 are the most likely regions to be exposed on the outside of the virus particles; this is likely to be where NS3-VP2 binding would take place. These antigenic regions are also likely to be involved in cell attachment, which VP2 is involved in (Hassan and Roy, 1999). Therefore the variation in these regions may also affect cell or tissue tropism. In addition to the variation in antigenic regions of VP2, most of the variation observed in VP5 also occurred in the antigenic regions of AHSV-4 VP5 identified by Martinez-Torrecuadrada et al. (1999). A study of hepatitis A virus passaged in cell culture showed that much of the amino acid variation within the two capsid proteins studied was located in or near antigenic domains (Sanchez et al., 2003). Antigenic regions of viral proteins are usually highly variable. This is often
attributed to immune selection, which is absent in cell culture. Therefore the variation arising in the antigenic regions during passage in cell culture may be due to less stringent structural constraints and interaction between viral proteins found in antigenic regions (Domingo et al., 1993).

To summarize, sequence differences were observed in NS3, VP2 and VP5 between AHSV-4(1) and AHSV-4(13) and some of these differences occur in areas that may be involved in virus entry into and exit from cells. Whether any of the mutations are attenuation determinants would have to be investigated experimentally. Viral plaque size was found to increase after a few passages in cell culture. No genetic variation in NS3 or VP5 could be correlated to variation or change in plaque size. The VP2, VP5 and NS3 sequences were studied as these were thought to be the most likely candidates to acquire mutations during the attenuation process, but there may also be variation in other genome segments worth investigating.
Chapter 3:  
Cytotoxic effect of AHSV-4 VP5 and NS3 on mammalian cells

3.1 Introduction  
AHSV VP5 and NS3 have cytotoxic properties (Martinez-Torrecuadrada et al., 1994; Van Staden et al., 1995) and both are involved in the virulence phenotype of the virus as shown by exchange of the genome segments encoding VP5 and NS3 in a genomic reassortment study (O’Hara et al., 1998).

Martinez-Torrecuadrada et al. (1994) have found that insect cells co-expressing AHSV-4 VP5 and VP2 showed earlier signs of cell death than cells infected with wild-type baculovirus. Furthermore, the expression levels were relatively low, indicating possible toxicity of the expressed proteins. Relatively low expression levels of AHSV-4 VP5, in E. coli cells were observed by Wall (2006), along with a decrease in cell growth rate, indicating a cytotoxic effect of AHSV VP5 in bacterial cells. Martinez-Torrecuadrada et al. (1999) also observed that AHSV VP5 expressed in E. coli is toxic to the cells, resulting in cell lysis. The toxic properties were specific to the N-terminal of VP5. All the VP5 fragments containing the N-terminal region exhibited toxicity to E. coli cells and those without the N-terminal were non-toxic and were expressed at high levels. This correlates with work on BTV VP5 that showed the predicted N-terminal amphipathic helices of BTV VP5 are necessary for cytotoxicity of the protein (Hassan et al., 2001). In addition, BTV VP5 applied exogenously to both mammalian and insect cells, was shown to permeabilize the cell membranes and to have cytotoxic properties. Furthermore, BTV VP5 expressed in Sf9 cells is cytotoxic and exhibits membrane fusion activity when expressed on the cell surface (Forzan et al., 2004). The conformation of BTV VP5 that allows it to interact with membranes is pH dependent, and VP5-membrane interaction is optimal at low pH. These findings are consistent with the hypothesis that VP5 is responsible for permeabilization of the endosome membrane in order to release the viral core into the cytoplasm after activation at low pH (Hassan et al., 2001; Forzan et al., 2004; Roy, 2005; Wall, 2006).

AHSV NS3 has been found to be cytotoxic in insect cells. It causes membrane permeabilization of the insect cell membranes and it localizes at both the cell membranes of Sf9 cells expressing NS3 (Van Staden et al., 1995) and at the cell membranes of AHSV infected Vero cells (Stoltz et al., 1996). Mammalian cell membranes were also found to be permeabilized by BTV NS3 (Han and Harty, 2004), and exogenous addition of AHSV NS3 to Vero cells resulted in permeabilization of the cell membrane (Meiring, 2001). The
hydrophobic domains of AHSV NS3 are involved in membrane localization and are necessary for NS3 cytotoxicity in Sf9 cells (Van Staden et al., 1998; Van Niekerk et al., 2001a). Furthermore, BTV and AHSV are released from cells by both budding and extrusion through disrupted membranes of mammalian cells with NS3 being present at sites of membrane disruption (Hyatt et al., 1989; Hyatt et al., 1991; Stoltz et al., 1996). Recently BTV and AHSV NS3 have been shown by Wirblich et al. (2006) to have functional, although fairly weak late-domains, involved in a virus release mechanism, which results in virus budding through the cell membrane. BTV NS3 has also been shown to interact with a protein in a cellular exocytosis pathway as well as with VP2, indicating a possible mechanism for virus release (Beaton et al., 2002). This, or the viroporin-like function of NS3 which results in cell membrane permeability (Han and Harty, 2004), may provide an explanation for the observed virus extrusion through the cell membrane.

The mechanisms of virus entry and release from infected cells, and the proteins involved in these mechanisms, impact on cell damage and pathogenicity. Information on the role of NS3 and VP5 in cytotoxicity is available from studies carried out on insect and bacterial cells. However, the effect of AHSV NS3 and VP5 has not yet been studied in mammalian cells, although the virus has a natural mammalian host in which the virus causes much damage. This chapter focuses on establishing a system to express these proteins in mammalian cells, including the optimization and confirmation of protein expression. Attention is also directed at establishing a system for measuring cytotoxicity and membrane permeabilization properties of viral proteins expressed within the mammalian cells. This may make it possible to correlate differences in cytotoxicity to observed sequence variation. If such correlations can be made, they may open up possibilities of studying protein function in more depth by site directed mutagenesis or deletion mutations.
3.2 Materials and Methods

3.2.1 Insertion of the NS3, VP5, NS1 and eGFP genes into pCMV-Script and NS3 and VP5 into pUEX3

3.2.1.1 Restriction enzyme digestion of DNA
Restriction enzyme digestion reactions were carried out in the appropriate buffer and at the optimal temperature recommended by the manufacturer (Roche). Restriction enzyme digestion of PCR products was carried out overnight to ensure complete digestion of the PCR products. In general, reactions containing plasmid DNA were incubated for 2 or 3 hours.

3.2.1.2 Dephosphorylation
Approximately one microgram of plasmid DNA was dephosphorylated with 0.5U alkaline phosphatase (Roche) in a final concentration of 1x dephosphorylation buffer in a volume of 40µl for 10 minutes at 37ºC.

3.2.1.3 Purification of DNA fragments
The digested DNA was purified either directly from restriction endonuclease reactions, or from excised agarose gel pieces after electrophoresis (2.2.5) using the High Pure PCR product purification kit (Roche) according to the manufacturer's instructions. Alternatively, DNA was purified from restriction endonuclease reactions by ethanol precipitation. Sodium acetate pH 4.8 was added to a final concentration of 1M, followed by the addition of 2 volumes of 96% ethanol, this was incubated at -20ºC for 1 hour. The DNA was collected by centrifugation at 15000g for 10 minutes, washed with 80% ethanol, air-dried and resuspended in ddH₂O.

3.2.1.4 DNA ligation
Ligation reactions were carried out at 16ºC overnight. The reactions contained approximately 300ng of plasmid DNA and up to 800ng of insert DNA and 1U of T4 DNA ligase (Roche) in a final concentration of 1x ligation buffer (Roche) in a volume of 10µl.

3.2.1.5 Preparation of competent E. coli cells
Competent cells were prepared using the CaCl₂ method described by Sambrook et al. (1989). Stationary phase culture (1ml) was used to inoculate 100ml of Luria-Bertani (LB) broth (1% NaCl (w/v), 1% Bacto-tryptone (w/v), 0.5% Yeast extract (w/v), pH 7.4). This was grown at 37°C until mid log phase. The cells were collected by low speed centrifugation and resuspended in 50ml ice-cold 50mM CaCl₂ and incubated on ice for 30 minutes. The cells were again collected by low speed centrifugation, resuspended in 5ml ice-cold 50mM CaCl₂ and incubated on ice for an hour. Cells were used immediately or frozen at -70°C in 15% glycerol for future use.
3.2.1.6 Transfection of competent cells with DNA

Ligation reactions (3.2.1.4) were transfected into chemically competent *E. coli* cells. The ligation reaction was mixed with 100µl of competent cells in a test tube, and incubated on ice for 30 minutes. The transformation mix was subjected to heat shock at 42°C for 90 seconds and then cooled on ice for 2 minutes. The cells were allowed to recover in 1ml LB broth at 37°C for 1 hour, with shaking, before being plated out on agar plates with selective media. Cells were incubated at 37°C or at 30°C if transformed with pUEX3 or a pUEX3 construct.

3.2.1.7 Plasmid DNA isolation

The alkaline lysis method (Birnboim and Doly, 1979) was used for plasmid DNA isolation. A colony of *E. coli* cells containing the plasmid contract was grown for 16 hours at 37°C or 30°C in 3 to 5ml LB broth supplemented with the appropriate antibiotics. *E. coli* cells were collected by centrifugation at 15000g for 1 minute and resuspended in 100µl ice-cold Solution I (10mM EDTA, 50mM glucose, 25mMTris-HCl, pH 8.0). The cell suspension was incubated at room temperature for 5 minutes, then briefly on ice. Two hundred microliters of fresh Solution II (0.2N NaOH, 1% SDS) was added, mixed and incubated on ice for 5 minutes. A volume of 150µl sodium acetate (3M NaAc, pH 4.8) was added, mixed and incubated on ice for 10 minutes. Plasmid DNA was isolated in the supernatant by centrifugation at 15000g for 10 minutes, then precipitated by the addition of two volumes of 96% ethanol and collected by centrifugation at 15000g for 10 minutes. The precipitated plasmid DNA was washed with 80% ethanol. The DNA was air-dried, and resuspended in ddH₂O. Plasmid DNA was analysed by agarose gel electrophoresis.

3.2.2 Production of β-galactosidase (β-gal)-NS3 and β-gal-VP5 antibodies

3.2.2.1 Induction of fusion protein expression

Overnight cultures of *E. coli* transformed with pUEX3 constructs were grown at 30°C in 100µg/ml ampicillin supplemented LB broth, for 16 to 20 hours. The cultures were diluted 1:10 in 100µg/ml ampicillin supplemented LB broth and grown at 37°C for two hours followed by induction at 42°C for two hours. *E. coli* cells were pelleted by low speed centrifugation and resuspended in 1× Phosphate buffered saline (PBS) (1mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄·2H₂O, 1.4mM KH₂PO₄, pH 7.3).

3.2.2.2 SDS-polyacrylamide gel electrophoresis (PAGE)

An 8-15% polyacrylamide separating gel (8-15% acrylamide, 0.2-0.4% bisacrylamide, 0.375M Tris pH 8.8, 0.1% SDS, 0.008% TEMED, 0.08% ammonium persulphate) was cast between two 8cm × 9cm or 16cm × 18cm glass plates and allowed to set at room temperature. A 5% stacking gel (5% acrylamide, 0.13% bisacrylamide, 0.125M Tris pH 6.8, 0.1% SDS, 0.008% TEMED, 0.08% ammonium persulphate) was poured on top of the separating gel and allowed to set. Once set, the gels were assembled in Mighty Small™ II SE 250 units or Sturdier SE400 vertical slab gel units (Hoefer Scientific instruments). Protein samples, in 2× protein solvent buffer (2× PSB) (0.125M Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), were heated at 95°C for 5 minutes before
loading. The samples were then electrophoresed in 1× TGS (25mM Tris, 190mM glycine, 0.1% SDS) at 120V for 2 to 4 hours or at 100V for 16 hours, depending on gel size and protein size.

Polyacrylamide gels used directly for protein analysis were stained with 0.125% Coomassie blue, 50% methanol, 10% acetic acid for 20 to 30 minutes, then destained with 5% methanol, 5% acetic acid until the background was transparent. Polyacrylamide gels used for protein purification were reverse stained (3.2.2.3.1).

3.2.2.3 Purification of protein from SDS-polyacrylamide gels
3.2.2.3.1 Reverse staining of SDS-polyacrylamide gels

The 8% SDS-polyacrylamide gels used to separate proteins for purification were reverse stained as described by Fernandez-Patron et al. (1995). The gels were rinsed in dH₂O for 60 seconds and then soaked in 100ml 0.2M imidazole, 0.1% SDS for 15 min. Subsequently, the gel was submerged in 0.2M zinc sulphate for 15 to 60 seconds, until the gel background became white with clear protein bands. Rinsing the gel 3 times in dH₂O for approximately 5 seconds each time stopped the staining reaction. A clear band corresponding to the protein of interest was excised from the gel and soaked in 25mM Tris-HCl, 192mM glycine, pH 8.3 for 5 to 10 minutes, until the gel background became totally transparent, in order to mobilise the proteins (Fernandez-Patron et al., 1995).

3.2.2.3.2 Elution of protein from SDS-polyacrylamide gels

Proteins were eluted from 8% reverse stained SDS-polyacrylamide gels. The gel pieces were placed in elution buffer (50mM Tris pH 9.0, 1% Triton X-100, 2% SDS) as described by Szewczyk and Summers (1988). The gel pieces were homogenised in 0.5ml elution buffer per cm² of gel, using an Ultra-Turrax homogeniser. Proteins were eluted from the crushed gel pieces at 37ºC with shaking for 2 hours.

3.2.2.3.3 Acetone precipitation

Proteins were precipitated by the addition of 4 volumes of pre-cooled acetone and incubated at -70ºC for 1 hour before collection by centrifugation at 25000g at 4ºC for 30 minutes. The protein pellet was air dried in a sterile environment, resuspended in filter sterilized 1× PBS and stored at -20ºC.

3.2.2.4 Immunization of hens

Immunization of hens for the production of antibodies took place at Onderstepoort Veterinary Institute of the Agricultural Research Council (ARC) and was carried out by Dr. Marco Romito. For the primary inoculation, 100µg of purified β-gal-VP5 or β-gal-NS3 in sterile 1× PBS mixed with an equal volume of ISA 70 Seppic oil adjuvant was injected into the pectoral muscles of Leghorn hens. The second inoculation, using a similar amount of antigen, took place four weeks later. Further inoculations only apply to the hen inoculated with β-gal-NS3, as the hen inoculated with β-gal-VP5
died. The third inoculation took place a month after the second, followed by the fourth after a further six weeks. Eggs from each hen were collected for IgY extraction and use.

3.2.2.5 IgY purification from chicken eggs

3.2.2.5.1 Chloroform/PEG 6000 method

Chicken egg yolk antibodies (IgY) were extracted from eggs according to the method described by Tini et al. (2002). One hundred millimolar sodium phosphate buffer, pH7, was added to the egg yolk of a single egg to make the volume up to 25ml. The solution was mixed before the addition of 20ml chloroform. The egg yolk-chloroform mixture was shaken until a semi-solid phase was obtained and then centrifuged at 1200g for 30 minutes. The supernatant was collected and solid polyethylene glycol 6000 (PEG 6000) was added to a final concentration of 10 to 12%. Antibodies were pelleted by centrifugation at 15800g for 10 minutes. The IgY containing pellet was resuspended in 1× PBS.

3.2.2.5.2 Ammonium sulphate precipitation

An alternative method for the extraction of chicken egg yolk antibodies was modified from Cook et al. (2001) and Hansen et al. (1998). The egg yolk was diluted 1:5 in dH2O and the pH set to 5 with 10% Acetic Acid. Lipids were allowed to settle out overnight at 4ºC. The supernatant of the settled material was clarified by centrifugation at 4600g for 30 min. Ammonium sulphate was added to a final concentration of 200g/l and incubated at room temperature with agitation for 30 minutes. Antibodies were pelleted by centrifugation at 13000g for 30 minutes and the IgY-containing pellet was resuspended in 1× PBS.

3.2.2.6 Western blot analysis

Protein samples were separated by SDS-PAGE and transferred to a Hybond-C Extra nitrocellulose membrane (Amersham) using a submerged blotter (EC 140 Mini Blot Module) in transfer buffer (25mM Tris, 190mM glycine, pH 8.3). After transfer, the membrane was removed from the blotting apparatus and washed in 1× PBS for 5 minutes followed by blocking against non-specific binding by incubation in 1% blocking solution (1% milk powder in 1× PBS) for 30 minutes to 1 hour. The membrane was transferred to a primary antibody solution in 1× PBS, the dilution of which depended on the antibody used, and incubated with gentle agitation overnight. After antibody binding, the membrane was washed in wash buffer (0.05% Tween in 1× PBS) 3 times for 5 minutes each time to remove unbound antibody. The membrane was then incubated in secondary antibody in 1× PBS with gentle agitation for an hour. A 1:500 dilution of Protein A peroxidase conjugate was used for serum obtained from rabbit and a dilution of 1:4000 of Anti-Chicken IgY (IgG) Peroxidase Conjugate developed in rabbit (Sigma) was used for IgY obtained from egg yolk. After removal from the secondary antibody solution, the membrane was washed in wash buffer again, 3 times for 5 minutes each time, after which it was rinsed in 1× PBS for 5 minutes. Antibody-bound bands were detected by the addition of a freshly prepared enzyme substrate solution, 60mg 4-chloro-1-naphthol in 20ml ice cold methanol was added to 60µl hydrogen peroxide in 100ml 1× PBS immediately before soaking the membrane in the solution while protected from light. When bands became visible the
membrane was rinsed with dH₂O to stop the reaction. The membrane was dried for storage and scanned to preserve the image.

3.2.3 Plasmid isolation for transfection
Plasmid DNA of pCMV-Script constructs to be used for transfections into mammalian cells was isolated from overnight cultures grown in LB broth supplemented with 50µg/ml Kanamycin and 12.5µg/ml Tetracyclin, using the GenElute Plasmid Miniprep Kit (Sigma) according to the manufacturer’s instructions.

3.2.4 DNA concentration determination
The concentration of DNA was determined spectrophotometrically using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc).

3.2.5 Transfection of DNA into Vero cells
Plasmid DNA was transfected into Vero cells using DOSPER Liposomal Transfection Reagent (Roche). Vero cells were seeded on 6, 24, or 96 well plates (Nunc) the day before transfection and transfected when approximately 80% confluent. DNA was diluted to the appropriate concentration in 1× Heps-buffered saline (HBS), pH7.4, and added to the appropriate concentration of DOSPER in 1× HBS, pH7.4, in the appropriate volume for the size well. A volume of 100µl DNA-DOSPER mix containing 10µg DOSPER was used to transfect a well of a 6 well plate; 25µl was used per well of a 24 well plate and 7µl per well of a 96 well plate. The DNA-DOSPER mix was gently agitated for 15 minutes at room temperature to allow DNA-DOSPER complexes to form. The culture medium was washed and then replaced with serum and antibiotic-free medium. The DNA-DOSPER complex was added to the medium drop by drop while rocking the plate back and forth to disperse the complex evenly. The plate was incubated at 37°C in a 5% CO₂ environment for 6 hours, after which the transfection medium was replaced with medium containing antibiotics and 2.5% FCS and kept at 37°C in a 5% CO₂ environment until analysis.

3.2.6 Cytotoxicity assays
3.2.6.1 CellTiter-Blue assay
The CellTiter-Blue Cell Viability Assay (Promega) was carried out according to the manufacturer’s instructions. Briefly, cells seeded on 96 well plates were transfected with the construct to be assayed in 100µl of medium and incubated at 37°C for the required time. Twenty microliter CellTiter-Blue Reagent was added to each well and the plate was shaken for 10 seconds. The cells were incubated at 37°C again for up to 4 hours. The plate was shaken for 10 seconds and fluorescence (544/590nm) was measured for each well using a Fluoroskan Ascent FL (Thermo Labsystems). Background fluorescence was determined by measuring the fluorescence of triplicate wells containing medium, but no cells, treated with CellTiter-Blue Reagent. The average background fluorescence was subtracted from all fluorescence readings.
3.2.6.2 CytoTox-ONE assay

The CytoTox-ONE Homogenous Membrane Integrity Assay (Promega) was carried out according to the manufacturer's instructions. Briefly, cells in a 96 well plate were transfected with the construct to be assayed in 100µl of medium and incubated at 37°C for the required time. Two microliters of Lysis Solution was added to the Maximum LDH release control to disrupt all cell membranes. The plate was equilibrated to 22°C and 100µl of CytoTox-ONE Reagent was added to each well and the plate shaken for 30 seconds. The cells were incubated at 22°C for 10 minutes after which 50µl of Stop Solution was added to each well. The plate was shaken for 10 seconds and fluorescence (544/590nm) was measured for each well using a Fluoroskan Ascent FL (Thermo Labsystems). Background fluorescence was determined by measuring the fluorescence of triplicate wells containing medium, but no cells, subjected to the assay. The average background fluorescence was subtracted from all readings.
3.3 Results

3.3.1 Production of polyclonal antibodies against AHSV-4 NS3 and VP5

Preliminary results using the pCMV-Script expression vector for protein expression in mammalian cells indicated that expression levels were too low to be detected by means of Coomassie blue staining. In order to detect such low levels of AHSV-4 NS3 and VP5 by means of Western blot analysis, it was necessary to produce polyclonal antibodies. A series of experiments were initiated to raise antibodies against β-gal-NS3 and β-gal-VP5 fusion proteins in Leghorn hens. Chicken egg antibodies (IgY) were extracted from the egg yolks of each hen's eggs and used for protein detection. As the source of NS3 and VP5 antigen, the proteins were expressed in *E. coli* by means of the pUEX expression system.

3.3.1.1 Insertion of genes encoding AHSV-4 NS3 and VP5 into pUEX3

The NS3 and VP5 genes of AHSV-4 were inserted into the pUEX3 expression vector in frame with the *LacZ* gene (Fig. 3.1 A; B) to produce β-gal fusion proteins to be used as antigens in anti-β-gal-NS3 and anti-β-gal-VP5 IgY production.

Both NS3 and VP5 genes were excised from the pCMV-Script constructs (the cloning of which is described in paragraph 3.3.2) using the restriction enzymes *Bam*HI and *Sal*I. The NS3(13)-pCMV-Script and VP5(1)-pCMV-Script constructs, as well as the pUEX3 expression vector, were fully digested with *Sal*I, ethanol precipitated and resuspended in ddH₂O. Plasmids NS3(13)-pCMV-Script and pUEX3 were then fully digested with *Bam*HI. However VP5(1)-pCMV-Script was partially digested with *Bam*HI because the VP5 gene contains an internal *Bam*HI site at nucleotide position 1038. The partial digest contained four DNA fragments, of which a fragment of approximately 1600 nucleotides corresponded to the full-length VP5 gene. The fragments corresponding to the expected size of NS3 and full-length VP5 were excised from an agarose gel and purified (3.2.1.3).

The NS3 and VP5 genes were ligated to the *Bam*HI and *Sal*I sites of the pUEX3 plasmid DNA and transfected into chemically competent DH5α *E. coli* cells. The cells were plated out on selective medium containing ampicillin as well as IPTG and X-gal. Colonies were selected based on blue/white colour selection, cultured and screened for the presence of the applicable insert by restriction endonuclease analysis. The NS3 gene was excised from the vector using *Bam*HI and *Eco*RI. The VP5 gene was excised from the vector using *Eco*RI as the *Eco*RI sites were present in the segments subcloned from the pCMV-Script constructs (Fig. 3.1 B). NS3-pUEX containing the NS3 gene, and VP5-pUEX containing the
VP5 gene, were shown to contain inserts of the correct sizes (Fig. 3.1 C). These clones were then used for the production of β-gal-NS3 and β-gal-VP5 fusion proteins.

3.3.1.2 Expression and purification of β-gal fusion proteins

The NS3-pUEX and VP5-pUEX constructs were used for the production of β-gal-NS3 and β-gal-VP5 fusion proteins that were subsequently used as antigens for IgY production.

The expression of fusion proteins was induced as described in paragraph 3.2.2.1 and analysed by SDS PAGE to confirm the expression of the correct sized proteins (Fig. 3.2 A). The size of the β-gal-NS3 fusion protein was estimated as 140 kDa and that of β-gal-VP5 was estimated as 173 kDa (Fig. 3.1 B). Thereafter, fusion proteins were purified from preparative 8% SDS-polyacrylamide gels. The gels were reverse stained and the clear protein bands corresponding to either β-gal-NS3 or β-gal-VP5 excised from the gels. The gel pieces were destained and the proteins eluted and precipitated (paragraph 3.2.2.3), thereby isolating the fusion proteins in a relatively pure form suitable for antibody production (Fig. 3.2 B lanes 3 and 6). The proteins were resuspended in sterile 1× PBS. The concentration of purified protein was determined by comparison with a concentration gradient of proteins of known concentration on Coomassie blue stained SDS-polyacrylamide gels using the VersaDoc Imaging System with the Quantity One v. 4.4.1 software (BioRad).
Figure 3.1 pUEX3 plasmid map (A) showing the multiple cloning site, the lacZ gene, and the Ampicillin resistance gene. Schematic diagram (B) illustrating the insertion of the NS3 and VP5 genes into pUEX3 in frame with the LacZ gene. Restriction enzyme sites incorporated due to the cloning procedure are shown, as well as estimated fusion protein sizes. Restriction endonuclease analysis (C), by agarose gel electrophoresis of recombinant pUEX3 plasmids containing the VP5 gene and the NS3 gene. Molecular Weight Marker III (Roche) was used for size determination (lane 1). Undigested (lane 2) and partially digested pUEX3 (lane 3) showing the linear fragment of pUEX3, are shown. Undigested VP5-pUEX (lane 4) and VP5-pUEX digested with EcoRI (lane 5) confirming the presence of the VP5 gene insert (arrowhead), and undigested NS3-pUEX (lane 6) and NS3-pUEX digested with BamHI and EcoRI (lane 7) confirming the presence of the NS3 gene insert (arrowhead) are shown.
Figure 3.2 SDS-PAGE of β-gal fusion proteins expressed in *E. coli* cells (A) and purified β-gal fusion proteins (B). Protein molecular weight marker (lane 1, A and B) indicates protein sizes. Uninduced *E. coli* cell lysates (lane 2A, lane 4B) are compared to lysates of induced cells expressing β-gal (lane 3A), β-gal-NS3 (lane 4A, lane 2B) and β-gal-VP5 (lane 5A, lane 5B). Purified β-gal-NS3 (lane 3B) and β-gal-VP5 (lane 6B) are shown. Arrowheads indicate expressed proteins.
3.3.1.3 IgY production, purification and determination of antigen specificity

The β-gal-NS3 and β-gal-VP5 fusion proteins were used to elicit an immune response in Leghorn hens, after which IgY were isolated from hen’s egg yolk and tested for reactivity with AHSV-4 proteins.

Purified β-gal-NS3 and β-gal-VP5 proteins were injected into Leghorn hens to elicit a primary immune response, and a booster injection was given a month later. This procedure was carried out by a qualified veterinarian at Onderstepoort Veterinary Institute. Pre-inoculation and post-inoculation eggs were collected and the IgY isolated from the egg yolks as described in paragraph 3.2.2.5. Antibody specificity was tested by Western blot analysis. The pre-inoculation IgY showed no specificity to the viral proteins, with very little background reaction with Vero cell proteins (results not shown). The post-inoculation IgY from the hen inoculated with β-gal-NS3 and the hen inoculated with β-gal-VP5 were found to bind with proteins from AHSV-4 infected Vero cells that corresponded in size to NS3 (Fig. 3.3 A, lane 1) and VP5 (Fig. 3.3 B, lane 1) respectively. In addition, smaller proteins were detected in Fig. 3.3 B lane 1; these smaller proteins are probably truncated versions of VP5 as the IgY were specific for β-gal-VP5.

The antisera used for the determination of antigen specificity were obtained from eggs collected early in the inoculation procedure. Different eggs are not immunogenically identical, and independent extractions can also introduce variation. Therefore, there is some variation between the reactivity of the antibodies obtained from different eggs. However, enough reactive antibodies were available for the detection of NS3 and VP5 expressed in mammalian cells.
Figure 3.3 Western blot analysis showing anti-β-gal-NS3 IgY reactivity with AHSV-4 NS3 (A) and anti-β-gal-VP5 IgY reactivity with AHSV-4 VP5 (B). In A, AHSV-4 infected Vero cells show anti-β-gal-NS3 IgY specificity for NS3 (lane 1); the arrowhead indicates NS3. Uninfected Vero cells (lane 2). Partially purified β-gal-NS3 (lane 3, arrowhead) and uninduced *E. coli* cells (lane 4). In B, AHSV-4 infected Vero cells show anti-β-gal-VP5 IgY specificity for VP5 (lane 1); the arrowhead indicates full length VP5. Uninfected Vero cells (lane 2). Unpurified β-gal-VP5 expressed in *E. coli* cells (lane 3, arrowhead) and uninduced *E. coli* cells (lane 4).
3.3.2 Construction of recombinant pCMV-Script plasmids for mammalian expression of AHSV-4 NS3 and VP5 proteins

In order to determine whether AHSV-4 NS3 and VP5 are cytotoxic or permeabilize the cell membrane when expressed in mammalian cells, the VP5 and NS3 genes from AHSV-4(1) and AHSV-4(13) were expressed in Vero cells using the mammalian expression vector, pCMV-Script (Fig. 3.4 A). The genes from both AHSV-4(1) and AHSV-4(13) were included in order to establish whether there are any detectable differences between the proteins of viruses with different plaque morphologies, and possibly different virulence characteristics, with regard to their cytotoxic or the membrane permeabilization properties, when expressed in mammalian cells.

The eGFP protein is not cytotoxic and is fluorescent allowing in situ visualization of the expressed eGFP. It therefore served as an excellent tool for optimization of the transfection procedure and as a non-cytotoxic control. NS1 was also included as a non-cytotoxic control. An NS3-eGFP fusion gene was included to reveal the localization properties of NS3 due to the fluorescent properties of the attached eGFP, allowing in situ visualization of the fusion protein.

In order to compare the cytotoxic and membrane permeabilization properties of the NS3 proteins of two serotype 2 strains with each other as well as with those of the serotype 4 viruses, when expressed in Vero cells, the NS3 genes from two serotype 2 strains, the AHSV-2 reference strain 82/61 and the AHSV-2 vaccine strain originally derived from the AHSV-2 reference strain (Van Niekerk, 2001), were incorporated in the study. Their membrane permeabilization properties have previously been studied in insect cells. NS3 from both serotype 2 strains were found to permeabilize Sf9 cell membranes, although no significant difference between their permeabilization abilities was observed. However, permeabilization of Vero cells during infection with the serotype 2 viruses was significantly greater than during infection with a serotype 4 virus (Van Niekerk, 2001). Although other viral proteins may also play a role in membrane permeabilization during infection, the serotype 2 NS3 is likely to play an important role in virus release and membrane permeabilization. Therefore, the inclusion of the serotype 2 NS3 genes could be used to look into possible differences between the serotype 2 and serotype 4 NS3 in Vero cell permeabilization. See Table 3.1 for a summary of the pCMV-Script constructs.

The VP5 encoding genes from the AHSV-4(1) and AHSV-4(13) viruses were excised from pCR-XL-TOPO with EcoRI (the cloning of VP5 into pCR-XL-TOPO is described in
The pCR-XL-TOPO plasmid DNA was then digested with XhoI to ensure that the VP5 insert DNA did not religate with the pCR-XL-TOPO DNA. pCMV-Script vector DNA was prepared by digestion with EcoRI and dephosphorylation. The digested insert and vector DNA were purified directly from restriction endonuclease digestions (3.2.1.3) and then ligated (3.2.1.4). The correct size pCMV-Script and VP5 DNA fragments are shown in restriction endonuclease digestions in Fig. 3.4 B, lanes 5 and 6.

The NS3 genes encoding NS3 from both AHSV-4 isolates were PCR amplified with primers NS3pBam and NS3pEco (Table 2.1). The PCR amplicons as well as pCMV-Script vector DNA were digested with BamHI and EcoRI and purified. The NS3 inserts were then ligated to pCMV-Script. NS3 from the AHSV-2 reference strain 82/61 (accession number AF276694) and AHSV-2 vaccine strain (accession number AF276693) were inserted into pCMV-Script by Dr. Michelle van Niekerk (University of Pretoria), also using BamHI and EcoRI, and used in this study. The pCMV-Script and NS3 insert DNA are shown in Fig. 3.4 B, lanes 8 to 11 for each construct.

The eGFP gene was excised from the eGFP-pGEM-T Easy construct, provided by Dr. Vida van Staden (University of Pretoria), with HindIII and XhoI. ScaI was used to digest pGEM-T Easy to ensure the eGFP gene did not religate with pGEM-T Easy. The pCMV-Script vector was prepared with HindIII and XhoI. The insert and vector DNA were purified directly from restriction endonuclease digestions and ligated. The eGFP gene is excised from pCMV-Script in Fig. 3.4 B, lane 13 to confirm insertion. The NS3 gene originating from AHSV-3 was fused to eGFP in a pFastBac construct made by Tracey-Leigh Hatherell (University of Pretoria). This NS3-eGFP fusion gene was excised from pFastBac and inserted into pCMV-Script with BamHI and HindIII by Dr. Vida van Staden. The NS3-eGFP fusion gene is excised from pCMV-Script in Fig. 3.4 B, lane 15. The AHSV-6 NS1 encoding gene was excised from the pET vector and inserted into pCMV-Script using BamHI and EcoRI. The enzyme EcoRV was used to digest the pET vector to stop the NS1 gene from religating into pET before purifying the DNA. The pCMV-Script and NS1 gene are shown in Fig. 3.4 B, lane 17, confirming insertion.

All ligation reactions were transformed into chemically competent XL1-Blue MRF' E. coli cells, prepared by the CaCl₂ method (3.2.1.5) and plated out on LB agar plates containing kanamycin and tetracyclin. The plates were incubated at 37°C for 16 to 20 hours after which colonies were selected and grown in LB broth overnight to be screened for the presence of the cloned insert by restriction endonuclease analysis. Confirmation of pCMV-Script containing each of the cloned inserts is shown in Fig. 3.4 B. The AHSV-4 VP5 and
NS3 genes were sequenced using a T3 primer (Table 2.1) which binds upstream of the pCMV-Script multiple cloning site as an additional confirmation of the presence of the inserts. In the case of the VP5 genes the correct orientation was also confirmed. These clones were then used for transfection into Vero cells, and production and analysis of the proteins of interest.

Table 3.1: pCMV-Script constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Purpose</th>
<th>RE sites</th>
<th>Serotype of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP-pCMV-Script</td>
<td>transfection optimization non-cytotoxic control</td>
<td>HindIII - Xhol</td>
<td>-</td>
</tr>
<tr>
<td>NS3(1)-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>BamHI - EcoRI</td>
<td>AHSV-4 AHSV-4(1)</td>
</tr>
<tr>
<td>NS3(13)-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>BamHI - EcoRI</td>
<td>AHSV-4 AHSV-4(13)</td>
</tr>
<tr>
<td>S2-82/61-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>BamHI - EcoRI</td>
<td>AHSV-2</td>
</tr>
<tr>
<td>(cloned by Dr. M. van Niekerk)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2-vac-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>BamHI - EcoRI</td>
<td>AHSV-2</td>
</tr>
<tr>
<td>(cloned by Dr. M. van Niekerk)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP5(1)-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>EcoRI - EcoRI</td>
<td>AHSV-4 AHSV-4(1)</td>
</tr>
<tr>
<td>VP5(13)-pCMV-Script</td>
<td>analyze cytotoxicity and membrane permeabilization in Vero cells</td>
<td>EcoRI - EcoRI</td>
<td>AHSV-4 AHSV-4(13)</td>
</tr>
<tr>
<td>NS1-pCMV-Script</td>
<td>non-cytotoxic viral protein control</td>
<td>BamHI - EcoRI</td>
<td>AHSV-6</td>
</tr>
<tr>
<td>NS3-eGFP-pCMV-Script</td>
<td>determine localization properties</td>
<td>BamHI - HindIII</td>
<td>AHSV-3</td>
</tr>
<tr>
<td>(cloned by Dr. V. van Staden)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure 3.4 A  pCMV-Script plasmid map showing the cytomegalovirus (CMV) promoter, the multiple cloning site (MCS) and the SV40 polyadenylation signal. Figure taken from pCMV-Script Vector instruction manual (Invitrogen).

Figure 3.4 B  Restriction endonuclease analysis by agarose gel electrophoresis, of wild type and recombinant pCMV-Script plasmids (B). Molecular weight marker III (Roche) is shown for size comparison (lanes 1 and 18). Wild type pCMV-Script (lane 2) is linearized with EcoRI (lane 3). Undigested VP5-pCMV-Script (lane 4) and the VP5 inserts of the AHSV-4(1) (lane 5) and AHSV-4(13) (lane 6) virus isolates excised from pCMV-Script with EcoRI are shown. Undigested NS3-pCMV-Script (lane 7) and the NS3 inserts of AHSV-4(1) (lane 8) and AHSV-4(13) (lane 9) isolates, S2REF-82/61 (lane 10) and S2 Vaccine-125 (lane 11) excised with BamHI and EcoRI are shown. Undigested eGFP-pCMV-Script (lane 12) is indicated, with the eGFP insert excised with HindIII and Xhol (lane 13). Undigested serotype 3 NS3-eGFP-pCMV-Script (lane 14) can be seen with the insert excised with BamHI and HindIII (lane 15). Undigested serotype 6 NS1-pCMV-Script (lane 16) is shown with the insert excised with BamHI and EcoRI (lane 17).
3.3.3 Optimization of the transfection procedure

Transient expression of proteins in mammalian cells has the disadvantage that it is difficult to distinguish between transfected and nontransfected cells. This problem is addressed by the use of eGFP, which can be used effectively to optimise the transfection procedure. This optimization ensures that the maximum number of cells are transfected with the least transfection-related damage. This also makes it possible to determine at what time post transfection the highest protein concentration is reached.

For optimization of the transfection procedure, Vero cells were transfected with a range of concentrations of eGFP-pCMV-Script and DOSPER Liposomal Transfection Reagent. Fluorescence levels and cell viability were compared visually using a fluorescence microscope. The optimal plasmid concentration for transfection of one well of a 6 well plate (962mm² area containing approximately 1.2×10⁶ cells) was estimated to be approximately 500-750ng of DNA (Fig. 3.5). From visual observations this concentration range appeared to produce an upper limit of fluorescence with no further increase in fluorescence observed for transfection with lower DNA concentrations. The best cell viability levels observed together with high transfection levels were obtained using 10µg of the DOSPER Liposomal Transfection Reagent per 6 well. Following the visual optimization, relative fluorescence values for the previously determined plasmid concentrations were obtained using the fluorometer. These readings were highest for 500ng of plasmid DNA per well of a 6 well plate (Fig. 3.6). Lower DNA concentrations were not tested, however, the results obtained from the fluorometer indicate that lower DNA concentrations may have provided improved fluorescence.

To determine at what time post transfection a high level of expressed protein is expected, a number of wells of 24 well plates (200mm² area containing approximately 0.2×10⁶ cells) were transfected with eGFP-pCMV-Script plasmid DNA, and fluorescence readings from three wells taken every 3 hours, from 9 hours post transfection to 78 hours post transfection. The fluorescence readings of eGFP-pCMV-Script transfected cells increased steadily for about 36 hours after which they levelled off, remaining fairly constant until 78 hours post transfection (Fig. 3.7).
Figure 3.5  Vero cells transfected with (A) 500ng, (B) 750ng, (C) 1µg, (D) 2µg and (E) 4µg of eGFP-pCMV-Script 48 hours post transfection, viewed under the fluorescence microscope.
Figure 3.6  Graph showing the relative fluorescent unit (R.F.U.) values of Vero cells from a 6 well plate transfected with a range of concentrations of the eGFP-pCMV-Script plasmid. Values taken 48 hours post transfection.

Figure 3.7  Graph showing the relative fluorescent unit (R.F.U.) values of Vero cells over 78 hours from a 24 well plate transfected with the eGFP-pCMV-Script plasmid. Fluorescence readings of three wells were taken every three hours. The average of the three readings are plotted on the graph and standard deviation bars are shown.
3.3.4 Expression of NS3 and VP5 in Vero cells

Before assessing the cytotoxicity or membrane permeabilization properties of NS3 and VP5 in mammalian cells, it was necessary to confirm expression of the proteins. After optimization of transfection, most cells were transfected, but there were still only low levels of protein produced in the cells. eGFP fluorescence is easily detected at low levels, even when eGFP expression cannot be detected on a Coomassie blue stained protein gel. NS3 and VP5 may have cytotoxic properties in mammalian cells, which may result in inhibition of expression, resulting in even lower protein levels than the non-cytotoxic protein eGFP. Therefore, Western blot analysis was used for protein expression confirmation. A commercial GFP antibody (Sigma) and the IgY produced against β-gal-NS3 and β-gal-VP5 were used in the immune detection of the proteins.

The Western blotting procedure was first optimized using the anti-GFP antibody to detect eGFP expressed in Vero cells transfected with eGFP-pCMV-Script. A weak signal was obtained at 24 hours and a fairly strong signal at 48 hours post transfection (Fig. 3.8 A lanes 2 and 3), using an antibody dilution of 1:1000. All NS3 and VP5 expressing cells to be used for Western blot analysis were subsequently harvested at 48 hours post transfection.

The Western blot analysis of NS3 and VP5 displayed weak signal for the proteins from transfected cells, but strong signals for NS3 and VP5 from AHSV-infected cells. This indicates low protein concentration from transfected cells. The antibody dilution had to be decreased to 1:50 in order to detect the expressed NS3 and VP5 in transfected cells. Expression was, however, confirmed for NS3(1), NS3(13) and VP5(1) (Fig. 3.8 B lanes 1 and 2 and C lane 1, respectively). The faint bands observed in the Western blots indicate low expression levels of these proteins in this system compared to the protein expressed in AHSV-4 infected cells. In Fig. 3.8 C smaller proteins were detected in the lanes showing expressed VP5 in addition to the full length VP5 protein. Expression of the serotype 2 NS3 proteins and the serotype 6 NS1 protein were not confirmed by Western blot analysis.
Figure 3.8 A  Western blot of eGFP-pCMV-Script transfected Vero cells using a commercial GFP antibody (Sigma) to detect expressed eGFP. Protein molecular weight marker sizes are indicated on the left hand side of the membrane. Cells transfected with the eGFP-pCMV-Script plasmid and harvested at 4 hours post transfection (lane 1), 24 hours post transfection (lane 2) and 48 hours post transfection (lane 3), as well as cells transfected with pCMV-Script (lane 4) and untransfected Vero cells (lane 5) are shown. Arrowheads indicate expressed eGFP.

Figure 3.8 B&C  Western blots of (B) NS3-pCMV-Script transfected Vero cells using anti-β-gal-NS3 IgY and (C) VP5-pCMV-Script transfected Vero cells using anti-β-gal-VP5 IgY. Protein molecular weight marker sizes are indicated on the left hand side of each membrane. The membranes show cells transfected with NS3(1)-pCMV-Script (lane 1 B) and NS3(13)-pCMV-Script (lane 2 B), VP5(1)-pCMV-Script (lane 1 C) and VP5(13)-pCMV-Script (lane 2 C), as well as cells transfected with pCMV-Script (lanes 3 B&C), untransfected Vero cells (lanes 4 B&C) and AHSV-4 infected Vero cells as a positive control (lanes 5 B&C). Arrowheads indicate targeted proteins NS3 (B) and VP5 (C).
3.3.5 Membrane permeabilization by NS3 and VP5

3.3.5.1 CellTiter-Blue assay

In order to determine whether NS3 and VP5 have cytotoxic properties when expressed in Vero cells, the cell viability of transfected cells was examined with the CellTiter-Blue kit (Promega). This kit is a cell viability assay measuring the production of resorufin, a fluorescent product, via resazurin reduction by viable cells. The higher the fluorescence, the higher the number of viable cells present. Therefore, a reduction in fluorescence measurement would indicate a loss of cell viability.

Vero cells in 96 well plates were transfected with approximately 35ng of plasmid DNA per well; two wells were transfected with each construct. Two wells were infected with the AHSV-4 as a control to indicate dying cells. The CellTiter-Blue assay was carried out 48 hours post transfection. Fluorescence readings with excitation wavelength 544nm and emission wavelength 590nm were taken at 2 and 4 hours after the start of the assay. The background fluorescence was measured in triplicate wells with culture medium containing no cells. An average of these was subtracted from the experimental wells. An average of the values obtained for the two wells was calculated for each construct (Fig. 3.9). Cells expressing NS3, from both serotype 2 and 4, and VP5 showed similar patterns of viability compared to the non-cytotoxic controls (pCMV-Script, eGFP and NS1) and untreated Vero cells. The only obvious loss of viability was seen with AHSV-infected cells, which were used as a positive control for dying cells.

3.3.5.2 CytoTox-ONE assay

In order to determine whether NS3 and VP5 have membrane permeabilization properties when expressed in Vero cells, the membrane integrity of transfected cells was measured using the CytoTox-ONE kit (Promega). This kit is a homogenous membrane integrity assay, which measures the leakage of lactate dehydrogenase (LDH) into the culture medium through disrupted cell membranes. The LDH in the culture medium converts the lactate and NAD\(^+\) supplied in the substrate mix to pyruvate and NADH. The NADH is then used by the diaphorase in the substrate mix in the conversion of Resazurin to the fluorescent product, Resorufin. Therefore, the higher the fluorescence, the more permeabilized cells present.

Vero cells in 96 well plates were transfected with approximately 35ng of plasmid DNA per well. Each construct was transfected in one well and one well was infected with AHSV-4 as a control to show dying cells. The CytoTox-ONE assay was carried out 24 hours post
transfection and repeated 48 hours post transfection. Fluorescence readings with excitation 544nm and emission 590nm were taken and background fluorescence was measured in triplicate wells containing cell-free culture medium. An average of these was subtracted from the values obtained for the rest of the wells to compensate for background fluorescence. A well of Vero cells was lysed with the Lysis Solution provided, 30 minutes before CytoTox-ONE Reagent was added, for a maximum LDH release control.

Cells expressing serotype 2 and 4 NS3 and serotype 4 VP5 had no obvious increase in membrane disruption compared to control cells, such as pCMV-Script and eGFP-pCMV-Script transfected cells (Fig. 3.10 A). However, the transfection procedure seems to have a greater effect on membrane integrity than AHSV infection. The AHSV-infected cells show more cytopathic effects than the transfected cells (Fig. 3.10 B and C), but AHSV infected cells show less membrane disruption, as measured by the assay, possibly indicating that AHSV cytopathology in Vero cells is not interrelated with membrane permeabilization at this stage of infection.

3.3.6 Membrane targeting of an NS3-eGFP fusion protein
The expression of an NS3-eGFP fusion protein in Vero cells and the observation of these cells under the fluorescence microscope were employed in determining whether NS3 exhibits membrane localization properties in Vero cells.

Vero cells were transfected with NS3-eGFP-pCMV-Script (for cloning procedure see paragraph 3.3.2 and Table 3.1) and viewed under the fluorescence microscope at 24 hours post transfection. Fluorescence was observed in the cells, confirming expression of the NS3-eGFP-pCMV-Script construct. The green fluorescence resulting from the expressed NS3-eGFP fusion protein showed evidence of localization, possibly with membranous components within the cells (Fig. 3.11 A, B, C, D). These were compared to cells transfected with eGFP-pCMV-Script as a control where the expressed eGFP was more or less evenly distributed throughout the cell as seen by a roughly uniform distribution of green fluorescence in the cell (Fig. 3.11 E). Cells exhibiting distinctive localization were rare events. Better microscopic techniques are required for clearer images and clarification of NS3 localization. The results indicate that it is possible that the NS3-eGFP protein exhibits membrane localization properties and/or perinuclear localization in Vero cells. This putative association with membranous components may be further investigated by confocal microscopy in order to clarify which cellular components NS3-eGFP localizes to.
Figure 3.9  Graph showing the relative fluorescent unit (R.F.U.) values of Vero cells analysed for viability with CellTiter-Blue in a 96 well plate. Fluorescence measurements were taken at 2 and 4 hours after the addition of the assay substrate, 48 hours post transfection. Cells infected with AHSV-4 were included as a cell-death control. Untreated Vero cells were included as a healthy cell control.

Figure 3.10 A  Graph showing the relative fluorescent unit (R.F.U.) values of Vero cells analysed for membrane permeabilization with the CytoTox-ONE kit in a 96 well plate. The assay was carried out at 24 hours, and again at 48 hours post transfection (p.t.) on all plasmids used for analysis. Cells were infected with AHSV-4 at the same time as cells were transfected. Untreated Vero cells were included as a healthy cell control. Vero cells lysed with Lysis Solution 30 minutes before assay substrate addition were included as a total cell membrane disruption control.
Figure 3.10 B, C, D  Vero cells analysed for membrane permeabilization with the CytoTox-ONE kit observed under the light microscope. pCMV-Script transfected cells (B), AHSV-4 infected cells (C), and untreated Vero cells (D) as observed at 48 hours post transfection/infection.
Figure 3.11  Vero cells expressing the NS3-eGFP fusion protein (A, B, C and D) showing evidence of localization, or unequal distribution within cells, and Vero cells expressing the eGFP protein (E) showing equal distribution throughout the cells, as viewed under the fluorescence microscope.
3.4 Discussion

The primary objective of this chapter was to investigate whether there is any cytotoxic effect of AHSV-4 VP5 and NS3 on mammalian cells when the proteins are expressed within mammalian cells. These proteins have been found to be cytotoxic in other systems as mentioned in the introduction. AHSV can persistently infect and replicate in insect cells with no obvious cytopathic effect, as happens with BTV (Wechsler et al., 1989). AHSV also infects Culicoides midges with no known pathogenic effect, whereas infection of horses is highly pathogenic, often resulting in death. Mammalian cell lines such as Vero cells support AHSV replication, but the infection results in cytopathic effects and cell death (Coetzer and Guthrie, 2004). The effect of AHSV VP5 and NS3, expressed outside the context of virus infection within mammalian cells, had previously not been examined. To determine their effect on mammalian cells they were successfully inserted into a mammalian expression vector and transiently expressed in Vero cells.

Optimal conditions for the best expression levels obtained in this study were determined using eGFP. Surprisingly, lower concentrations of plasmid DNA resulted in higher transfection levels. However, the transfection levels obtained did not provide expression levels of viral proteins that were high enough for easy detection and function studies. The viral proteins were detected immunologically using the IgY produced for this purpose, but the antibodies as well as the transfected cells had to be used at very high concentrations resulting in a lot of background reaction on the membrane. Due to the low levels of expression a sensitive or strong tag would be useful for confirmation of transient expression in mammalian cells. Stratagene have produced an updated version of the pCMV-Script vector that contains three Histidine tags fused to each other for easier detection. In this study eGFP fused to AHSV-3 NS3 was useful in confirming expression and in observing possible localization properties of NS3 in mammalian cells.

When NS3 and VP5 were expressed under the optimized conditions, no obvious signs of cytotoxicity were observed. Commercial kits measuring cell viability, CellTiter-Blue, and membrane integrity, CytoTox-ONE, were employed to investigate possible cell death or membrane permeability. Using these kits, expression of NS3 or VP5 in Vero cells was found to have no detectable effect on cell viability or membrane integrity. A possible weakness of the CellTiter-Blue assay for use with transiently transfected cells may be that background non-transfected cells are viable and can grow to replace any cells possibly affected by expressed proteins. The CytoTox-ONE assay does not have this drawback, but the transfection procedure was shown to cause some membrane permeabilization,
possibly masking any effect caused by the expressed proteins. There may also be a number of other reasons for this lack of cytotoxicity. As no conclusions on NS3 cytotoxicity could be drawn from these experiments, it was not possible to compare the effect of the AHSV-2 NS3 proteins, which were included in the experiments, to the AHSV-4 NS3 proteins.

The low expression levels of the proteins in the mammalian cells may result in an amount of protein that is too low to cause membrane damage or to have an effect on cell viability. The concentration of the proteins in the transfected cells is far less than that of cells infected with AHSV-4, as can be seen in Fig. 3.8. These low expression levels may be due to inefficient transfection levels resulting in low copy numbers of plasmid DNA per cell and low mRNA levels, or it may be due to low levels of translation.

The eGFP protein was more readily detectable by Western blot analysis. This may be due to higher expression levels or better antibody strength or specificity. Possible higher levels of eGFP compared to the viral proteins may be due to non-cytotoxic nature of eGFP compared to the possible cytotoxic properties of NS3 and VP5 in the cells. As NS3 and VP5 have been shown to have cytotoxic properties in insect and bacterial cells, they may have similar properties in mammalian cells, in which case their expression may be inhibited in the cell, reducing the protein level compared to a non-cytotoxic protein such as eGFP which would not be inhibited.

Alternatively, the possible higher expression levels of eGFP compared to the viral proteins may result from an optimal Kozak sequence around eGFP’s start codon (ACCATGG), as opposed to suboptimal Kozak sequences around the VP5 (GCCATGG), and especially the NS3/NS3A (GTCATGA/ACCATGC) initiation codons. The optimal sequence, encompassing the start codon, for translation initiation in eukaryotic cells is A/GCCATGG. The purine at the -3 position and the G at the +4 position have the greatest effects on translation initiation, with an A being preferential to a G at the -3 position (Kozak, 1986; Kozak 1991). Roner et al. (1989) indicate that translation efficiency of reovirus mRNA is influenced by the 5’ untranslated region. In addition, Doohan and Samuel (1993) have shown that the extent of ribosome pausing at the initiation codons of reovirus is affected by the sequences flanking the initiation codon. However, viral mRNA from infected cells produce larger amounts of protein, but have the same Kozak sequences, therefore the lower translation levels are probably due to lower quantities of mRNA produced in transfected cells as opposed to infected cells. Zou and Brown (1996) found that the reovirus protein µ2 was expressed at
lower levels in stably transfected mammalian cells than in infected cells, although expression levels could be increased by the insertion of a stronger promoter element.

Given that Vero cells support AHSV replication, and that NS3 and VP5 are produced during the virus life cycle without the cells undergoing immediate cell death, it is likely that low levels of NS3 and VP5 would not be very cytotoxic in Vero cells. In BTV, Hyatt et al. (1989) found that virus extrusion through the cell membrane did not seem to result in an obvious disruption of membrane integrity during the time of maximum viral release. This is consistent with the results observed for lack of membrane permeabilization in AHSV-4 infected Vero cells (Fig. 3.10 A). Viral replication is supported by Vero cells and early membrane permeabilization would interfere with continued virus replication. Owens et al. (2004) have proposed that the ratio of NS1 to NS3 in infected cells may affect the mechanism of virus release after finding that reduced NS1 tubule formation resulted in budding rather than cell lysis. AHSV infection shows late permeabilization of Vero cells, which may be due to accumulation of NS3 over a critical level, or which may be due to an accumulation of NS3 in the extracellular environment, and not intracellular NS3. Previously, extracellular addition of NS3, produced in Sf9 cells, to Vero cells resulted in membrane permeabilization (Meiring, 2001). Extracellular addition of BTV VP5 to mammalian cells, as well as to insect cells, was also found to permeabilize cell membranes (Hassan et al., 2001).

BTV NS3 transfected into COS-1 cells has been shown to permeabilize those cell membranes using a Hygromycin B assay (Han and Harty, 2004). This is possibly a more sensitive assay, which is more suited to analysing transfected cells. Only Vero cells were investigated in this study, so the possibility of cytotoxicity or membrane permeabilization in other mammalian cell lines or the possibility of cytotoxicity or membrane permeabilization properties being detectable using other assays cannot be ruled out.

The Western blots of AHSV-4 infected Vero cells (Fig. 3.3 and Fig. 3.8) show reactivity of the anti-β-gal-VP5 antibodies with a protein corresponding to the size of full length VP5 (estimated molecular weight: 57 kDa) as well as with proteins corresponding to truncated VP5 products previously described by Filter (2000) for in vitro translations of AHSV-3 VP5. Translation of these truncated VP5 proteins may be initiated from in frame start codons. Martinez-Torrecuadrada et al. (1994) observed a smaller protein (50 kDa) in the serotype 4 VP5 gene products expressed in Sf9 cells. Grubman and Lewis (1992) also identified a second, smaller protein product of a serotype 4 VP5 gene by in vitro translation.
Another aim of this chapter was to determine whether NS3 exhibits membrane localization properties in Vero cells. The eGFP gene was fused to the C-terminal of AHSV-3 NS3 in order to visualize the protein in the cells under the fluorescence microscope. Fluorescence from the NS3-eGFP fusion protein was not evenly distributed throughout the cells indicating localization within the cells. This localization may have been with membranous components within the cells, although further investigation is required to confirm this. Van Staden et al. (1995) found evidence of perinuclear localization of NS3 in infected Vero cells, and the NS3-eGFP fusion protein has been shown to be putatively targeted to membranous regions in Sf9 cells (T.-L. Hatherell, unpublished results). These initial investigations into NS3 localization were partially successful in showing possible localization within the cell and will most likely lead to more sensitive microscopic techniques being used, such as confocal microscopy comparing NS3 localization to cellular markers, to determine precise localization.
Chapter 4:
Concluding Remarks

This project focused on the role of AHSV NS3, VP5 and VP2 in the virulence phenotype of the virus, the molecular aspect of the process of attenuation of AHSV and virus plaque size. To investigate this, differences between these proteins from a low passage isolate, AHSV-4(1), and subsequent passage virus of that isolate, AHSV-4(13), were compared on DNA and amino acid sequence levels. The sequence variation relating to viral plaque size was also investigated. In addition, the membrane permeabilization and cytotoxic properties of NS3 and VP5 from the virus variants, expressed within Vero cells, were investigated. This was done in order to ascertain whether these proteins exhibit cytotoxic properties when expressed within mammalian cells as they do in bacterial and insect cells, and if so, to correlate any differences in cytotoxic properties between proteins of the virulence variants with the sequence differences between the variants.

The NS3, VP5 and VP2 sequences of the AHSV-4(1) virus and the AHSV-4(13) isolate were determined. In addition, NS3 and VP5 sequences from independently serially plaque-purified lines derived from AHSV-4(1) were determined and the sequences were compared. No sequence variation was observed in the regions of the proteins already known to be associated with cytotoxicity and membrane permeabilization, such as the hydrophobic domains of NS3 and amphipathic helices of VP5. However, differences were observed in the C-terminal region of NS3, which is likely to be associated with VP2 in a mechanism of virus release, similar to what has been shown for BTV NS3 (Beaton et al., 2002). Differences were also found in the antigenic regions of the outer capsid proteins. Some amino acid differences between virulent and avirulent BTV isolates have also been found within a neutralization epitope of VP2 (Bernard et al. 1997). These regions are probably exposed on the outer surface of the virus particles, and may therefore be involved in attachment of the virus to the cell. This has been shown to be a function of BTV VP2 (Hassan and Roy, 1999). Variation in the VP2 antigenic regions may also play a role in NS3-VP2 binding in the virus release mechanism described by Beaton et al. (2002) where the N-terminal region of NS3 interacts with a cellular protein involved in an exocytosis pathway and the C-terminal region interacts with VP2. The variation found in the outer capsid proteins may influence virus virulence in the horse by affecting tissue tropism through antigen variation. The variation in NS3 and VP2 may be involved in changing the rate of virus release from a cell, and cell-to-cell spread, due to possible variation in NS3-VP2 interaction in a virus release mechanism.
The range of plaque sizes observed for AHSV-4(1) suggests that this isolate is essentially a pool of viruses, exhibiting some phenotypic variation. The variation in AHSV-4 plaque size and the change from small to large plaques during plaque-to-plaque transfers could not be correlated with any sequence variation in NS3 or VP5. Plaque size may be influenced by VP2 sequence differences involved in cell tropism or the rate of cell entry and exit, or possibly by other viral proteins involved in the rate of virus replication. One would expect the variation in plaque size to be directly correlated to some aspects of genetic variation of the different viruses, as the conditions in a cell-culture plate should be invariable.

The nucleotide and amino acid sequence variation within the virus variants, evidence of which was found in sequences of cloned genes and PCR amplicons, indicated genetic heterogeneity within the viruses. The emergence of a single amino acid change in NS3 in two independently passaged lines also indicated a virus population with a quasispecies structure. New ideas on the role of quasispecies structure in virus virulence have recently emerged. Sauder et al. (2006) found that changes in the level of heterogeneity at certain nucleotide sites were correlated to a decrease of virulence in an attenuated mumps virus obtained by passage in cell culture. No definite mutations, or nucleotide sites containing only one nucleotide in the virulent virus and another nucleotide in the avirulent virus were found. Furthermore, Vignuzzi et al. (2006) found that reduced genetic diversity in poliovirus lead to a loss of neurotropism and a highly attenuated phenotype. They confirmed that the attenuated phenotype was not due to a mutation in the viral polymerase causing reduced genetic diversity, nor was the change in neurotropism due to any defined mutations. This indicates that genetic variability within the viral population, rather than specific mutations, was related to a higher level of pathogenicity. Their results were consistent with the idea that selection occurs at the level of the virus population rather than acting on individual viruses.

It is possible that there may be a loss of heterogeneity during passage in cell culture. A virus or a viral population that is adapted to the specific cell environment during the passaging process will have no need for the variability that allows for adaptation to different tissues or the non-static environment of the regular host. Therefore, there may be a reduction in adaptability, which may lead to a reduction in pathogenicity as described by Vignuzzi et al. (2006). The identification of a virulence determinant would be more complicated on the quasispecies level than the observation of definite mutations between sequences. To gain more clarity on the population structure of the AHSV-4 virus isolates, it
may be of interest to sequence more clones of the viral genes encoding VP2, VP5 and NS3 to determine the distribution of mutants within the virus population.

In this project, only VP2, VP5 and NS3 were investigated for variation between the AHSV-4(1) and AHSV-4(13) virus isolates. They were considered to be the most likely candidates to show variation and they are thought to be involved in virus virulence. However, sequence data of other genome segments of AHSV-4(1) and AHSV-4(13) may be worth investigating. It may give an indication of any other proteins that may be involved in the virulence phenotype. For example, NS1 may play a role in the mechanism of virus release from cells, and influence cellular pathogenesis in a similar way to BTV NS1. It was found that BTV NS1-tubules play a role in virus release, as the prevention of NS1-tubule formation resulted in virus budding instead of cell lysis and a major reduction of cytopathic effect of virus infected cells (Owens et al., 2004).

The effects of the mutations observed in AHSV VP5 and NS3 were investigated by transient expression in mammalian cells. A system was established to express these proteins in Vero cells, and the assessment of their membrane permeabilization properties and cytotoxic effect was attempted. The genes encoding NS3 and VP5 from both AHSV-4(1) and AHSV-4(13), as well as control genes were inserted into the pCMV-Script vector and transfected into Vero cells in order to express these genes in a mammalian system. The assays carried out on Vero cells expressing low levels of NS3 and VP5 showed no detectable cytotoxic effect and no obvious increase in membrane permeabilization. This lack of demonstrable membrane damage or cytotoxicity meant no differences between the virulent and attenuated variants could be detected in this way.

The absence of these effects on the cells may be due to a number of reasons. Firstly, the assays used may not be optimal for detecting effects in cells expressing the proteins in a transient manner, and any non-transfected cells may mask the effects of the proteins on the transfected cells. An alternative assay for examining the permeability of cells expressing NS3 or VP5 is a Hygromycin B assay. This assay detects the inhibition of translation by Hygromycin B in cells with permeabilized membranes, through which small molecules such as Hygromycin B can pass. Han and Harty (2004) used this method, in conjunction with immune-precipitation of BTV NS3, to detect a reduction in translation of BTV NS3 in mammalian cells expressing the protein, thus showing that cells expressing BTV NS3 were permeabilized. Secondly, the protein levels within the cells may have been too low to have an effect on cell viability or to cause detectable membrane damage. In normal AHSV infection of Vero cells, cell death does not occur immediately. It is therefore
logical that protein levels similar to, or lower than those found in the earlier stages of virus infection of cells, would not cause a great extent of damage to the cell. Any cytotoxic effects of the proteins may also be more readily detected in other mammalian cell lines, such as equine endothelial cells.

To further investigate expression of NS3 in Vero cells an NS3-eGFP construct was used to visualize the fusion protein within mammalian cells. When observing cells expressing the NS3-eGFP fusion protein, some cells showed evidence of possible localization to membranous components. Possible localization in perinuclear regions was also observed in NS3-eGFP expressing cells, suggesting that AHSV NS3 may be endoplasmic reticulum (ER) and Golgi associated, as NS3 of BTV has been found to be (Wu et al., 1992; Han and Harty, 2004). NSP4 of rotavirus, a cognate protein of NS3, has also been found localized to, and retained in the ER (Bergmann et al., 1989; Mirazimi et al., 2003). In order to obtain clearer images of the cells and possibly confirm ER and Golgi localization, confocal microscopy may be useful in matching the localization of NS3 to cellular markers for the ER and Golgi complexes.

The use of eGFP in the construction of fusion proteins for easy detection can also be used for VP5, and other proteins to be expressed in mammalian cells. Furthermore, eGFP fusion proteins produced in another system allowing higher expression levels, can be added exogenously to mammalian cells to observe the effects of the proteins on the cell membrane in conjunction with cytotoxicity and membrane permeabilization assays.

Through establishing a system for the expression of proteins within mammalian cells, it was found that lower concentrations of plasmid DNA resulted in higher transfection levels. Even so, the transfection levels achieved did not provide protein levels that were easily detectable by any means tested other than fluorescence. Thus the use of eGFP to optimise expression proved to be valuable. Immunological detection of expressed proteins by Western blotting was inefficient with both eGFP and the viral proteins being relatively difficult to detect. Where fluorescence cannot be used to confirm expression, it may be useful to employ a strong tag to improve detection, or to use a more sensitive immunological method such as immune-precipitation of radiolabelled target proteins, or a combination of a fused tag and immune precipitation.

An alternative to the kind of expression vector used in this study, i.e. a transiently transfected plasmid allowing constitutive expression, is an inducible mammalian expression system that can control the level of expression of the recombinant protein. Such
a system has been shown to produce higher expression levels than a constitutive cytomegalovirus promoter (Jones et al., 2005). Viral vectors, e.g. alphavirus vectors have also been used in research to study protein function. They can produce high expression levels and are flexible in that they can allow transfection of replicative RNA or infection with recombinant viruses (Berglund et al., 1996). Alternatively, protein function can be studied through a reverse genetics system, the likes of which have been developed for reovirus and rotavirus (Roner and Joklik, 2001; Komoto et al., 2006). A similar system could be developed for the related orbiviruses, but such systems are technically challenging to produce and recovery of recombinant viruses can be inefficient. An alternative to a reverse genetics system is the use of siRNA to inhibit protein synthesis, thus creating gene “knock outs”. RNAi has been effectively used to study rotavirus proteins function (Déctor et al., 2002; Arias et al., 2004; López et al., 2005; Cuadras et al., 2006), and recently BTV protein function (Wirblich et al., 2006). The effectiveness of RNAi is also dependant on the efficiency of the transfection of the siRNA (Arias et al., 2004).

The AHSV-4 virus isolates, i.e. AHSV-4(1) and AHSV-4(13), investigated in this project showed plaque size differences as well as sequence differences between their respective NS3, VP5 and VP2 proteins. Certain sequence changes may have an influence on virus entry into cells and exit from cells. No noticeable signs of cytotoxicity were observed in Vero cells expressing NS3 and VP5. This requires further investigation, possibly in the baculovirus expression system, which has been used previously for cytotoxicity analysis, as the lack of cytotoxicity may be due to low transfection and expression levels. However it was shown that NS3 is may be localized to membranous components of Vero cells, also calling for further investigation using more powerful techniques to gain greater understanding of exact localization.
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**VP5 nucleotide sequence alignment**

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AHHSV-4(1)         GGGGCGGCGA ACGTTATCGC CACAACCCGC GCAATACAGG GGGGGTTAAA ACTAAAGGAA ATTGTTGATA AGCTTACGGG CATAGATTTG AGCCATTTGA AGGTGGCCGA CATTCATCCA    840
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AHHSV-4(1)         CAACATATTGA AAAGGCAATG CTACGTGAT ACTGTAACGG ACAAAGATTT GGCGATGGCA ATTAAGTCAA AAGTGGATGT AATTGACGAG ATGAACGTAG AAACGCAGCA CGTAATCGAT    960
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AHHSV-4passage8c   ----------- ---------- --------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------    960
AHHSV-4passage8d   ----------- ---------- --------- ---------- ---------- ---A------ ---------- ---------- ---------- ---------- ---------- ----------    960

AHHSV-4(1)         GCCGTTCTACC GATAGTTAAA CAAGAATATG ATAACAAATA TCATGTTAGG ATCCCAGGTG CATTGAAGAT ACATTCAGAG CACACGCCTA AGATACATAT ATATACGACC   1080
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AHHSV-4(13)-clone  ----------- ---------- --------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   1080
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AHHSV-4(1)         CCATGGGATTC GGATAGCGTC TTCATGTGT AGAGCCATTG CACCGCATCA TCAACAACGA AGCTTTTTCA TTGGATTTGA TCTAGAAATT GAATATGTCC ATTTTGAAGA TACTTCAGTT   1200
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AHHSV-4(13)        ----------- ---------- --------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   1200
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