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Characterisation of the African horse sickness virus NS4 protein

Yi Jin

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in Infection and Immunity

Institute of Infection, Immunity & Inflammation

College of Medical, Veterinary & Life Sciences University of Glasgow

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I. ABSTRACT

African horse sickness is one of the most deadly infectious diseases of horses. The disease is caused by African horse sickness virus (AHSV), an arbovirus transmitted by culicoides midges. AHSV is classified within the genus *Orbivirus*, family *Reoviridae*. The AHSV genome is composed by ten segments of double stranded RNA (dsRNA) encoding seven structural and at least four non-structural (NS) proteins.

AHSV shares structural and functional similarities with Bluetongue virus, the ‘prototype’ species of the genus *Orbivirus*. An alternative open reading frame (ORF), overlapping the main ORF encoding the VP6, has been identified in segment 9 in both AHSV and BTV. This additional ORF encodes the non-structural protein NS4. The BTV NS4 localises in the nucleoli of the infected cells. NS4 is an interferon antagonist and a determinant of virus virulence.

In this thesis, I aimed to characterise the AHSV NS4. Unlike the BTV NS4, the AHSV NS4 are relatively variable among different virus strains. I have divided these proteins into four different subtypes: NS4I, NS4-IIα, -IIβ, and IIγ based upon their sequence similarity and on the presence of N-terminal or C-terminal truncations. In contrast to BTV, all four of these NS4 types localise in the cytoplasm of either transfected or infected cells. In addition, in transient transfection assays all the NS4 types show the ability to hamper gene expression, with NS4-IIβ being the most efficient.

In order to further understand the biological significance of the AHSV NS4 we used reverse genetics to generate viruses expressing the four types of NS4 (AHSV-NS4-I, AHSV-NS4-IIα, AHSV-NS4-IIβ, AHSV-NS4-IIγ) and the corresponding NS4 deletion mutants (AHSV-ΔNS4-I etc.).

Deletion of NS4 did not affect virus replication kinetics in either KC cells or interferon incompetent cells such as the BSR cell line. Similarly, both AHSV-NS4-IIβ and the corresponding ΔNS4 mutant showed similar replication kinetics in the interferon competent E. Derm cell line and in primary horse endothelial cells. In contrast, AHSV-NS4-I, AHSV-NS4-IIα, and AHSV-NS4-IIγ replicated more efficiently...
than the corresponding ΔNS4 viruses in these horse cells. Interestingly, the defects in replication of the ΔNS4 viruses were removed after treatment with an inhibitor of the JAK/STAT pathway. Indeed, we observed that primary horse cells infected with the ΔNS4 mutants released higher levels of type I interferon (IFN) than cells infected with the corresponding NS4 expressing viruses. In addition, we found the NS4 to be a determinant of virus virulence in vivo in NIH-Swiss mice infected with the viruses described above.

Collectively, the data described in this thesis suggest that the NS4 is one of the proteins used by AHSV to modulate the IFN response.
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Thank you every one.
VI. Author’s declaration

I, Yi Jin, declare that, except where explicitly reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Printed name ______________________________

Signature ________________________________
VII. List of Abbreviations

Ab  antibody
aa  amino acid
AHS  African horse sickness disease
AHSV  African horse sickness virus
AP-1  activating protein 1
APCs  antigen-presenting cells
BHK21  Baby hamster kidney cell line
BSR  Baby hamster kidney cell line, derived from BHK21
BT  Bluetongue disease
BTV  Bluetongue virus
bp  base pairs
CARD  caspase activation and recruitment domain
CD4  cluster of differentiation 4
CD8  cluster of differentiation 8
CMI  cell-mediated immunity
CO₂  carbon dioxide
CPE  Cytopathic effect
CPT Tert  sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT)
CTD  C-terminal domain
CTL  cytotoxic T-lymphocytes
DNA  deoxyribonucleic acid
cDNA  complementary DNA
DAPI  4’, 6-diamidino-2-phenylindole
cDC  conventional dendritic cell
pDC  plasmacytoid dendritic cell
DMEM  Dulbecco’s modified eagle medium
E. Derm  Equine dermis fibroblast cell line
EMCV  Encephalomyocarditis virus
EHDV  Epizootic haemorrhagic disease virus
ELISA  Enzyme linked immunosorbent assay
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>interferon-α/β receptor</td>
</tr>
<tr>
<td>eIFN</td>
<td>recombinant equine interferon alpha 1</td>
</tr>
<tr>
<td>uIFN</td>
<td>recombinant universal interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factors</td>
</tr>
<tr>
<td>ISGs</td>
<td>IFN-stimulated genes</td>
</tr>
<tr>
<td>ISGF3</td>
<td>ISG factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>IFN-stimulated response element</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus tyrosine kinase/signal transducer and activator of transcription protein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat extracellular domain</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma Differentiation-Associated protein 5</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>mix of the 4 deoxynucleotide triphosphates; dATP, dCTP, dGTP &amp; dTTP</td>
</tr>
<tr>
<td>rNTP</td>
<td>mix of the 4 ribonucleotide triphosphates; rATP, rCTP, rGTP &amp; rUTP</td>
</tr>
<tr>
<td>NS</td>
<td>non-structural</td>
</tr>
<tr>
<td>OAS</td>
<td>oligoadenylate synthetase</td>
</tr>
<tr>
<td>OIE</td>
<td>World organisation for animal health</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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</tbody>
</table>
PAMPs  pathogen associated molecular patterns  
PBS  phosphate buffered saline  
PCR  polymerase chain reaction  
p.i.  post-infection  
PKR  protein kinase R  
RG  reverse genetics system  
polyI:C  Polyinosinic:polycytidylic acid  
PRRs  pattern recognition receptors  
RIG-I  retinoic acid inducible gene 1  
RRV  rhesus rotavirus  
RT  reverse transcriptase enzyme  
RT-PCR  reverse-transcriptase polymerase chain reaction  
RNA  ribonucleic acid  
rpm  rounds per minute  
ssRNA  single stranded RNA  
dsRNA  double stranded RNA  
mRNA  messenger RNA  
SDS  sodium dodecyl sulphate  
TCID$_{50}$  tissue culture infectious dose of 50%  
TIR  Toll/interlukin-1 receptor domain  
TLRs  the Toll-like receptors  
TNF  tumor necrosis factor  
TRAF  TNF receptor associated factors  
TRIF  TIR-domain-containing adapter-inducing interferon-β  
TYK2  tyrosine kinase 2  
UTR  Untranslated region
VIII. List of Units

kD  kilodaltons
°C  Degrees celsius
rpm revolutions per minute
x g  gravity
  g  gram
mg  milligram
ug  microgram
ng  nanogram
pg  picogram
  M  molar
mM  millimolar
uM  micromolar
nM  nanomolar
  l  litre
ml  millilitre
ul  microliter
v/v  volume/volume
w/v  weight/volume
  %  percentage
  V  Volts
  h  hour
min  minute
  s  seconds
Chapter 1. Literature review
1. Literature review

This review of the literature will cover initially the historical outbreaks, epidemiology, and the different clinical manifestations of African horse sickness virus (AHSV) infection. This information highlights the importance of the disease and why studies such as the one presented in this thesis, which investigate the role of viral proteins in modulating the host immune response, are important. In addition, an introduction into the concepts of viral virulence and how the virus interacts with the host innate immune response will be also given. In several instances, due to the relatively little information available directly on AHSV, parallels have been based on bluetongue virus (BTV), the prototype member of the Orbivirus genus.

1.1 African horse sickness

African horse sickness (AHS) is one of the most devastating infectious diseases of equids. The mortality rates vary between different equid species affected by the disease. In horse, the mortality rate can be up to 95%, whereas in zebra, there are no significant clinical signs when infected (Awad, Amin et al. 1981). The causative agent of the disease is African horse sickness virus (AHSV). The virus is non-contagious and infection is spread between the mammalian hosts by insect vectors. AHSV has a greater capacity to infect a range of different insect vectors in comparison to BTV which to date only infects Culicoides spp. To date, 9 serotypes of AHSV have been identified in field (Howell 1962).
1.1.1 Historical outbreaks and epidemiology of African horse sickness

Traditionally, AHS disease was endemic in the sub-Sahara area, especially in the tropical and sub-tropical areas, spreading to South Africa (Mellor and Boorman 1995). African horse sickness is an ancient disease. As early as in 1327, a disease resembling AHS was reported in Yemen and over 200 years later, in 1569, there was a documented report in East Africa of horses becoming affected by AHS after they were imported from India (MacLachlan and Guthrie 2010). In southern Africa, this disease was first recognised in 1657 when the horses were initially introduced in the region.

The first major breakout of AHS occurred in 1791, and during that breakout over 1,700 horses were killed due to the pathology associated with AHSV (Mellor and Boorman 1995). In the subsequent following centuries, there have been more than 10 major outbreaks that have swept through Africa and southern Europe. The largest recorded breakout occurred in South Africa between 1854 and 1855 when 70,000 animals died, accounting for 40% of the local population (Guthrie, Quan et al. 2009). The outbreak of AHS in 1959-60 was the largest documented in recent times spreading throughout the Middle East and as far west as South-West Asia with death of more than 300,000 equids (Anwar and Qureshi 1972). In recent times, there have been more recorded outbreaks and several cases indicating that AHS spread from Africa into southern Europe around the Mediterranean basin. In 1965, AHS was first reported in Morocco before spreading throughout Algeria and Tunisia and crossing into Spain via the Strait of Gibraltar in 1966 (Lubroth 1992).

The second incursion of AHSV into Europe occurred in 1987, when infected animals were imported into Spain. Although an extensive vaccination program was undertaken, the virus spread into southern Spain and arrived in Portugal in 1989. The last clinical cases were reported in 1990 (Zientara, Sailleau et al. 1998). There have been outbreaks occurring north of the Sahara, the natural barrier of AHSV spread with reported outbreaks in Ethiopia in 2003 and 2008 (Aklilu, Batten et al. 2014). These two outbreaks killed more than 2,000 of horses. In 2007, the disease was reported in Nigeria, Senegal and Gambia in the west of Africa indicating the
virus had moved from the southern Africa into both the east and west of Africa with a greater frequency of outbreaks (Diouf, Etter et al. 2013, Fall, Fall et al. 2015).

To date, AHS breaks out frequently and severely in southern Africa. The Sahara Desert, to date, has proved to be an effective barrier that prevented the disease to spread geographically. However, with the human activities and global climate changing, AHS started to extend to Northern Africa and other places of the world (Baylis, Mellor et al. 1999, Harvell, Mitchell et al. 2002), including countries surrounding the Mediterranean (Spain and Portugal) (Rodriguez, Hooghuis et al. 1992, Portas, Boinas et al. 1999), Arabian Peninsula (Wilson, Mellor et al. 2009), and South Asian subcontinent including India and Pakistan (Mellor 1993). Although, not as successful as BTV, which is now endemic in Europe after spreading from southern Africa, the similarities in the rate and types of incursion routes are high.

1.1.2 Host range of African horse sickness

Equids are the susceptible species of AHS, including horses, mules, donkeys and zebras. Horses are the most susceptible species and infection with AHSV general results in the development of acute and subacute forms of the disease with very high mortality rates. Donkeys and mules, in comparison develop an infection that is often subclinical and resolves while infection in zebras is asymptomatic. Zebras are postulated to be the main reservoir of AHSV in the wild as their habitat and the epidemic area of the disease overlap (Baylis, Mellor et al. 1999). It is believed that zebras also play an important role in allowing virus persistence in the sub-Saharan area. Virus and/or viral antibodies have been detected in other species, including camels, elephants and rhinoceroses (Moustafa and El-Bakry 1984, Barnard, Bengis et al. 1995, Van Vuuren and Penzhorn 2015), however their biological role in allowing epidemics and transmission is insignificant. Dogs are reported to develop a very severe and short clinical disease, which is fatal, due to the ingestion of the contaminated horse meat indicating that the dog may be another susceptible species. Traditionally, it was hypothesised that dogs may not play a role in disease transmission (Binepal, Wariru et al. 1992, Alexander, Kat et al. 1995), but a recent report has shown the direct transmission of AHSV to dogs without ingesting contaminated meat (Van Sittert, Drew et al. 2013).
1.1.3 Clinical signs of African horse sickness

The nature of disease and the outcomes of infection are often dependent on several factors that are determined by the host-virus interplay including virus strain, breed etc. (Howell 1963, Burrage and Laegreid 1994). The incubation period prior to the onset of disease varies from 3 to 15 days depending on the virus and the host. Unlike BTV or Epizootic haemorrhagic disease virus (EHDV), which have a similar clinical outcome that has a sliding scale of severity, AHSV infection results in several clinically different outcomes. The 4 principal manifestations of AHS, in order of increasing severity are horse sickness fever, cardiac form, mixed form and a pulmonary form. There are not known viral determinants or host-associated factors that determine the severity of the clinical manifestations. It is reported that the mixed form is the most frequently occurring clinical outcome (reviewed by (Zientara and Lecollinet 2015)). However, a retrospective study on data from the 2009–2010 outbreak in Ethiopia reported all forms of the disease with cardiac the most prevalent form (54.8%), followed by horse sickness fever, pulmonary (7.1%) and 2.4% with the mixed form (Aklilu, Batten et al. 2014). This highlights the differences in the reporting of prevalence of the clinical manifestation.

Horse sickness fever is the mild form and generally not lethal. Infection results in a mild to moderate increase in body temperature between 40–40.5 °C and general malaise for 1 or 2 days. This form is frequently observed in partially immune (vaccinated) horses or in response to the live attenuated strains such as the vaccine in the susceptible equid species (horse) or in donkeys or zebras (Mellor and Hamblin 2004, Zientara and Lecollinet 2015). In the cardiac form, which is sometimes referred to as the subacute form, clinical signs usually appear 7 to 12 days p.i and is characterised by high body temperature lasting for weeks (Mellor and Hamblin 2004, Zientara and Lecollinet 2015). The cardiac form of AHSV infection results in a swollen head and neck due to oedema, which is particularly noticeable around the supra-orbital fossa region of the eye. Conjunctivitis, abdominal pain and shortening of breath/laboured breathing are also common clinical signs of this form. The mortality rate ranges between 50% and 70% with surviving animals recovering within 7 days (Howell 1963, Mellor and Hamblin 2004, Zientara and Lecollinet 2015).
The pulmonary form is the most severe and has the highest case fatality rate: up to 95% of infected horses will die 7 days p.i. (Mellor and Hamblin 2004). The onset of disease is very rapid and it is common for horses to die without displaying any previous signs of disease. Clinical signs include depression, increased body temperature (39-41°C), and followed by respiratory distress and severe dyspnoea. Spasmodic coughing and severe sweating occurs and at the terminal stage frothy fluid oozes out of the nostrils. The mixed form of disease is reported as the most common clinical outcome to AHSV infection of horses. It is a combination of the pulmonary and cardiac form with mortality rates around 70%. Death occurs 3-6 days after the onset of fever (Howell 1963, Mellor and Hamblin 2004, Kazeem, Rufai et al. 2008, Van Sittert, Drew et al. 2013, Zientara and Lecollinet 2015).

1.1.4 Vaccine of African horse sickness

As clinical signs of AHS develops very fast and causes badly tissue damage and highly death rate, one of the most important methods for the disease control and prevention is vaccination.

To date, the only commercially available vaccines are polyvalent attenuated vaccines from Onderstepoort Biological Products (OPB) in South (Mellor and Hamblin 2004). The vaccines are first attenuated by multiple inoculating in mouse brain, and then are further attenuated through passage in cell culture. These vaccines are used in southern Africa currently and formulated into two vials which contains AHSV-1, -3 and -4, and AHSV-2. -6, -7, and -8 respectively. It is recommended that the two vials of the vaccines should be administrated separately for at least three weeks, but no more than four weeks annually in late winter or early summer (von Teichman, Dungu et al. 2010). AHSV-9 is not included as this serotype has strong cross protection with AHSV-6(von Teichman, Dungu et al. 2010) and AHSV-9 is rarely epidemic in southern Africa historically (Mellor and Hamblin 2004). AHSV-5 is not included as well, because it is difficult to get attenuated strains and severe reactions and death have been reported after vaccination (Molini, Marucchella et al. 2015).

A monovalent attenuated AHSV-9 vaccine that produced by National Laboratory of Senegal is widely used in West Africa where this serotype is widely epidemic and
causes serious outbreaks (Oura, Ivens et al. 2011). Whereas in Europe, an attenuated AHSV-4 monovalent vaccine which was produced by OPB was used also used in Spain and Portugal during 1987 to 1990 when AHSV-4 broke out in these areas (Portas, Boinas et al. 1999) as this is the only serotype that breakouts in Europe historically.

Although, the live attenuated vaccines successfully help to control the endemic of AHSV, the commercial vaccines are still not licenced to use in the EU. There are still concerns for these vaccines usage. The vaccines are generated in South Africa, and using the vaccines world widely would introduce the viruses into other ecosystems. Furthermore, the attenuated virus genome could potentially reassort with field virus strain genome and generate the reassortant virus (von Teichman and Smit 2008). The virulence reversion and further reassortment are not able to control in field, which may cause potential risk.

Inactivated vaccines are also developed. However, due to the difficulty of ensure complete inactivation of the vaccine and the expensive price, this vaccine is withdrawn from the market after AHSV annihilation from Europe (Mellor and Hamblin 2004).

Recently, a new AHSV antigen delivery system using modified vaccinia Ankara (MVA) has been described (Calvo-Pinilla, de la Poza et al. 2014). The MVA replication in mammalian cells delivers the AHSV VP2 as a foreign antigen and induces the host cellular and humoral immune responses to generate neutralisation antibodies.

1.2 African horse sickness virus
1.2.1 AHSV is a member of the Reoviridae

The Reoviridae is the largest and most diverse family of dsRNA viruses and are classified as a Group III virus by the International Committee on Taxonomy of Viruses (ICTV). The virions have icosahedral symmetry but may appear spherical in shape and have a segmented genome consisting of 10 to 12 segments (Mertens 2004). Fifteen distinct genera have been identified within Reoviridae including the genus Orbivirus. This genus is the largest one, which consists of over 35 species including BTV, AHSV, EHDV and Equine encephalosis virus (EEV).
The name Orbivirus derives from the Latin word ‘orbis’, which means ring or circle, referring to the structure at core particles (see section 1.2.5 for description). The structural morphology is similar among different orbiviruses. The genome of orbiviruses is composed of 10 linear dsRNA segments. Although, the structure overall and virus activities are conserved among members of this genus, there is very little genetic similarity within and among genera of the *Reoviridae*.

### 1.2.2 The AHSV genome

The ~19.5 kb AHSV genome is composed of 10 dsRNA segments (Bremer, Huismans et al. 1990, Potgieter, Steele et al. 2002). The size of each segment varies from 3,965 bp (segment 1) to 765 bp (segment 10) and the segments are named segment 1 (largest) to segment 10 (smallest) based on gel migration (Gumm and Newman 1982, Mertens, Burroughs et al. 1987, Bremer, Huismans et al. 1990). The profile of AHSV genome is similar but distinct from that of BTV or EHDV (Figure 1.1). Each AHSV segment contains 5’ and 3’ untranslated regions (UTRs) at both sides of the ORF. The length of the UTRs is variable. At 5’ end, the length of the UTR ranges from 12 bp (segment 2) to 25 bp (segment 5), while at the 3’ end range from 29 bp (segment 6) to 100 bp (segment 7). The sequences of UTRs of each segment show reverse complementarity, which suggests that secondary structures of the mRNAs that are transcribed could be formed (Roy 1989). The six terminal nucleotides are conserved and present on the 5’ and 3’ end of each segment of the AHSV genome (Roy 1989, Potgieter, Steele et al. 2002). The consensus sequence of this hexanucleotide sequence is found on all genome segments and is unique for each member e.g., AHSV (5’-GUU(A/U)A(A/U)……AC(A/U)UAC-3) and BTV (5’-GUUAAA…..(A/G)CUUAC-3’). This may be important for viral packaging (Burkhardt, Sung et al. 2014) and specific translation of viral proteins in the infected host (Wilkie, Dickson et al. 2003, Boyce, Celma et al. 2012).
Figure 1.1 Migration pattern of the dsRNA genome of different orbiviruses. Native acrylamide gel of dsRNA segments of BTV-10, EHDV-1 and AHSV-4 (Roy, Mertens et al. 1994). The orbivirus genome is composed of 10 distinct dsRNA segments that can be separated by size. However, the size of the genome segments differs among different genera. Reproduced with permission.

The segments are mostly monocistronic, although second functional, in-frame initiation codons or additional protein-coding ORFs have been identified in the AHSV genome as well as in BTV. The non-structural protein 4 (NS4) was initially identified by bioinformatics analysis in several different orbiviruses (BTV, GIV, etc.) and its expression has been confirmed in AHSV infected samples (Firth 2008, Belhouchet, Jaafar et al. 2011, Ratinier, Caporale et al. 2011, Zwart, Potgieter et al. 2015). Furthermore, another alternate ORF was identified in segment 10 of AHSV and BTV (Stewart, Hardy et al. 2015), as well as encoding proteins from alternate frames, the main ORFs in segment 9 and 10, NS3 and VP6, can be expressed from an in-frame AUG downstream of the initiating AUG forming NS3a and VP6a maybe due to leaky scanning (Hyatt, Zhao et al. 1993, van Niekerk, Smit et al. 2001).
1.2.3 AHSV virion

AHSV is non-enveloped double-layered icosahedron virus of ~80 nm in diameter (Bremer 1976, Roy, Mertens et al. 1994, Manole, Laurinmäki et al. 2012) (Figure 1.2). The virion is composed of 7 structural proteins (VP1 to VP7) (Bremer 1976, Burroughs, O'Hara et al. 1994, Burroughs, Grimes et al. 1995, Manole, Laurinmäki et al. 2012) similar to BTV (Hewat, Booth et al. 1992, Grimes, Jakana et al. 1997, Grimes, Burroughs et al. 1998, Zhang, Boyce et al. 2010). Some have reported the structure as a triple-layered particle but the subcore, consisting of the structural protein VP3, is highly fragile and for BTV has been shown to rapidly ‘fall’ apart (Kar, Ghosh et al. 2004). The cryoEM structure of AHSV has recently been solved (Manole, Laurinmäki et al. 2012).

![Diagram of AHSV virion structure](image)

Figure 1.2 Schematic cartoon of the structure of the AHSV particle. The outer layer of AHSV is composed of the VP2 and VP5 proteins. The core of the particle packages the viral dsRNA genomic segments and the minor structural proteins VP1, VP4 and VP6. The layer of the core is formed by the VP3 and VP7 proteins. VP3 forms the scaffold of the layer on which VP7 is arranged and interacts with the outer layer (Mertens and Diprose 2004). Reproduced with permission.

Three-D reconstruction of the AHSV virion, highlighted the structural similarities with BTV particularly within the core (VP3, VP7 inner capsid) (Manole, Laurinmäki...
et al. 2012). The only striking difference is in the central region of the triskelion VP2 outer capsid (Nason, Rothagel et al. 2004, Manole, Laurinmäki et al. 2012). The inner core (subcore) consists of 120 VP3 (~103 kDa) molecules in a T=1 arrangement, that is coated by 780 molecules (or 260 trimers) of VP7 (~38 kDa) arranged in a T=13 pattern. Five trimers of VP7 form an asymmetric unit (Manole, Laurinmäki et al. 2012) and each of the trimers in this unit is labelled (P, Q, R, S and T) based on the BTV nomenclature (Manole, Laurinmäki et al. 2012) (Grimes, Burroughs et al. 1998) (Figure 1.3). These asymmetric VP7 units form rings of pentamers and hexamers around the particle. AHSV VP7 has been shown to from sheets which consist solely of the 6-ringed formation (Burroughs, Grimes et al. 1995).

![Figure 1.3 The structure of the core particle of BTV. A. VP7 assembles the outer surface of the core. B. VP3 forms the inner surface (Grimes, Burroughs et al. 1998). Reproduced with permission.](image)

The AHSV inner capsid shell (~72 nm) is also called the core particle and packages the AHSV dsRNA genome complexed with the minor structural/enzymatic proteins (VP1, ~140 kDa; VP4 ~72 kDa; and VP6, ~36 kDa). Each viral particle contains 12, 24 and 72 copies of VP1, VP4 and VP6 respectively (Van Dijk 1982). Each copy of VP1 associates with a dimer of VP4 and hexamer of VP6 to form 12 transcription complexe units which are located at the 5-fold axes of the inner surface of core capsid (Grimes, Burroughs et al. 1998, Gouet, Diprose et al. 1999, Manole, Laurinmäki et al. 2012)
VP2 and VP5 form the outer capsid of the virion including 180 copies of VP2 and 360 copies of VP5 molecules forming a T=13 lattice (Fig 1.3a). VP2 and VP5 together form a continuous layer and cover the viral core particle (Hewat, Booth et al. 1992, Manole, Laurinmäki et al. 2012) (Figure 1.4). VP2 molecules form triskelion trimers that site on the 260 VP7 trimers. The 120 VP5 trimers form a globular structure that is positioned around VP7 in the 3-fold axis and sitting below VP2. VP5 interacts with both VP2 and VP7, which is consists to what has be observed with BTV (Hyatt, Zhao et al. 1993, Mertens, Diprose et al. 2004, Zhang, Boyce et al. 2010, Manole, Laurinmäki et al. 2012).

Figure 1.4 Three-dimensional reconstruction of AHSV virus structure. VP2 trimers locate on the surface of the viral particle (Manole, Laurinmäki et al. 2012). Reproduced with permission.

1.2.4 Outer capsid proteins: VP2 and VP5

VP2 and VP5 are involved in cell attachment and membrane penetration (Hassan and Roy 1999, Hassan, Wirblich et al. 2001). VP2 is the most variable AHSV protein and determines the virus serotype. The neutralizing epitopes of AHSV have been mapped to a few discreet regions on the VP2 molecule (Burrage, Trevejo et al. 1993,
Martinez-Torrecuadrada, Iwata et al. 1994, Bentley, Fehrsen et al. 2000). AHSV exists in nature as 9 distinct serotypes (AHSV-1 to -9) based on differences in VP2 (McIntosh 1958, Howell 1962, Martinez-Torrecuadrada, Iwata et al. 1994, Scanlen, Paweska et al. 2002). As VP2 sits on the surface of the viral particle and is the target of neutralising antibodies, it is proposed that high immune pressure results in the development of antigenic variants positive selection and serotype diversity (Roy 1992, Nason, Rothagel et al. 2004, Calvo-Pinilla, de la Poza et al. 2014). Although there are 9 distinct AHSV serotypes, there is cross-reactive of neutralising antibodies detected. Serotypes pairs that have exhibited cross-neutralising \textit{in vitro} included AHSV-3 and -7, -5 and -8, and -6 and -9 (Mellor and Hamblin 2004, Kanai, Van Rijn et al. 2014).

The VP2 protein of orbiviruses also has haemagglutinating activity, suggesting that the protein has a role in determining host range (Cowley and Gorman 1989, Cowley and Gorman 1990). Previous studies on BTV demonstrated that VP2 is involved in virus entry and exit of the cell via its interaction with NS3 (Hassan and Roy 1999, Beaton, Rodriguez et al. 2002, Meiring, Huismans et al. 2009). Structural analysis of BTV VP2 revealed a sialic acid binding domain in the hub of VP2 (Zhang, Boyce et al. 2010) and studies by Manole et al. (Manole, Laurinmäki et al. 2012) indicate that AHSV may have a similar region. They suggest that VP2 binds the oligosaccharides in cell surface glycoprotein to enable entry. Cleavage of VP2 by proteases blocks the virus haemagglutinating activity, however, the infectivity of infectious subviral particles (ISVP) is higher for insects in both \textit{in vitro} and \textit{in vivo} studies for BTV and AHSV (Mertens, Burroughs et al. 1987, Burroughs, O'Hara et al. 1994, Marchi, Rawlings et al. 1995).

VP5 protein, the second outer capsid protein, is postulated to be a type 1 fusion protein based on its high structural similarity to BTV-1 VP5 (Manole, Laurinmäki et al. 2012). Studies on BTV VP5 have indicated that VP5 has pH-dependent fusion activity (Forzan, Wirblich et al. 2004) and is activated in the low pH environment of the late endosome allowing the virus core to enter the cytosol (Patel, Mohl et al. 2016). Expression studies of AHSV VP5 demonstrated that it was cytotoxicity and had membrane destabilising capacity, similar phenotype to BTV (du Plessis and Nel 1997, Stassen, Huismans et al. 2011). Mutagenesis of AHSV VP5 highlighted that the
predicted two amphipathic α-helices at the N-terminal of the protein were essential for its fusogenic activity (Stassen, Huismans et al. 2011). Structural studies confirmed that the protein did, indeed contain at least amphipathic α-helices (Manole, Laurinmäki et al. 2012). Although VP5 is less exposed than VP2, it can elicit neutralising antibodies (Martínez-Torrecuadrada, Langeveld et al. 1999).

### 1.2.5 Core proteins: VP3 and VP7

The core particle is a highly stable structure that does not disassociate in the cytoplasm of the infected cell (Mertens and Diprose 2004). This prevents the dsRNA genome from exposure to pattern recognition (PAMPs) by the host cell. Expression of VP3 and VP7 in baculovirus indicates that it could autoassemble into core-like particles without the requirement of the transcription complex or genomic RNA (Maree, Durbach et al. 1998). The VP3 and VP7 layer interact via their hydrophobic surface (both AHSV VP3 and VP7 contain several alanine, methionine and proline residues). AHSV VP7 protein is highly hydrophobic and less soluble than BTV or other orbivirus VP7 proteins (Roy, Hirasawa et al. 1991).

During infection and in *in vitro* studies, AHSV VP7 is highly expressed, and unlike VP7 from other orbivirus, it forms flat hexagonal crystal sheets or particles that can be clearly visualised (Burroughs, O'Hara et al. 1994). Previous studies reported that AHSV VP7 is a group-specific immunogenic antigen (Chuma, Le Blois et al. 1992) and this is often used in ELISAs designed to differentiate among different orbivirus infections. X-ray crystallography reveals that there are two distinct domains in AHSV VP7 monomer. The formation of the VP7 trimer structure does not change the distribution of the two domains in the particle; the upper interacts with VP2 and the lower with VP3 (Basak, Grimes et al. 1997). The β sheet structure of the upper domain also contains an Arg-Gly-Asp (RGD) tripeptide motif. The RGD motif has been shown to be important for viral entry via integrin receptors (e.g., FMDV (Logan, Abu-Ghazaleh et al. 1993, Kotecha, Wang et al. 2017) and JEV (Fan, Qian et al. 2017)). It is postulated that the VP7 RGD motif assists entry of orbiviruses into insect host cells (Basak, Grimes et al. 1997, Tan, Nason et al. 2001).
VP3 is essential for the formation of the core and viral particles as VP7 does not have the ability to form the core without VP3 and cannot associate with dsRNA or the transcription complex. Other than the structural studies there is very little published information on AHSV VP3. Drawing parallels with BTV VP3, we can postulate that AHSV VP3 interacts with the transcription complex and ssRNA to enable the formation of the core (Grimes, Burroughs et al. 1998, Lourenco and Roy 2011).

### 1.2.6 Transcriptional complex proteins: VP1, VP4 and VP6

Three of the minor core proteins, VP1, VP4 and VP6 form the replicase complexes that locate in the core of the AHSV viral particle. As with other AHSV proteins, very little functional work has been undertaken and thus assignment of function is based on BTV. AHSV VP1 is a putative RNA dependant RNA polymerase that is encoded by segment 1 of AHSV (Van Dijk 1982, Roy, Mertens et al. 1994, Vreede and Huismans 1998). Several motifs required for RNA-dependent RNA polymerase activity have been identified in AHSV VP1 (Sankar and Porter 1992).

It is the most conserved protein not only in the genus *Orbivirus* but also in the *Reoviridae* (Vreede and Huismans 1998, Attoui, Billoir et al. 2000, Attoui, Stirling et al. 2001). This is highlighted when comparing AHSV-9 and BTV-10 VP1 proteins; these proteins share 55.6% and 73.2% aa similarity at the nucleotide and amino acids level respectively. The GDD sequence required for activity is in a different region in comparison to BTV VP1 (Vreede and Huismans 1998). The biological significance of this is still unknown.

VP4 is also conserved between AHSV and BTV, with 48.5% aa similarity (Noriko, Kenichi et al. 1993). VP4 is a putative capping protein, encoded by segment 4 and similarly to other AHSV proteins the function assignment is based on its similarity to BTV. BTV VP4 was one of the first proteins to be characterised that was able to catalyse all of the sets of the capping process to generate a Cap1 (m7GpppG-2’-O) at the 5’ end of the nascent viral transcripts (Martinez-Costas, Sutton et al. 1998, Ramadevi, Burroughs et al. 1998, Stewart and Roy 2015). BTV VP4 possess RNA triphosphatase, guanylyltransferase, guanine-N7-methyltransferase and nucleoside-2’-O-methyltransferase activity (Ramadevi, Burroughs et al. 1998, Sutton, Grimes et
al. 2007). This cap is identical as eukaryotic mRNAs, (Mertens and Diprose 2004). The first step of the capping pathway, RNA triphosphatase activity, has been described for AHSV recombinant VP4 (Van den Bout 2005).

As the AHSV genome is composed of double-stranded RNA, it requires a protein with helicase activity to unwind the strands to allow VP1 to synthesize new transcripts. AHSV VP6 is able to bind specifically dsRNA (de Waal and Huismans 2005) and is hypothesised, based on similarity to BTV VP6, to have helicase activity. BTV VP6 has affinity for both dsRNA and other nucleic acids (Roy, Adachi et al. 1990) and ATPase and helicase activity (Stäuber, Martinez-Costas et al. 1997). BTV VP6 is a hydrophilic, basic protein. The aa sequence of VP6 is conserved at the C-terminus where the RNA binding domain locates. Sequencing analysis indicates that AHSV VP6 contains this conserved domain at the same location (Turnbull, Cormack et al. 1996).

1.2.7 Non-structural proteins of AHSV: NS1, NS2, NS3 and S10-ORF2

Besides the 7 structural proteins, AHSV has been shown to encode up to 4 non-structural proteins. These 4 non-structural proteins, NS1, NS2, NS3 and NS4, play essential roles in the viral life cycle.

NS1 is encoded by segment 5 and it is the largest and most abundantly expressed non-structural protein of orbivirus with up to 25% of the total viral proteins in BTV-infected cells (Huismans and Els 1979). The aa sequence of NS1 is highly conserved among Orbivirus members: 50-60% aa similarity among BTV, AHSV and EHDV (Van Dijk and Huismans 1988, Wilson 1994). AHSV NS1 sequences share up to 96% aa similarity (Maree and Huismans 1997). AHSV NS1 forms tubules structures which are ~23 nm in diameter and 4 microns in length (Maree and Huismans 1997). This is half the size of the 52.3 nm tubules structure formed by BTV NS1. In each turn of the BTV tubule there are 22 NS1 dimers (Hewat, Booth et al. 1992, Hewat, Booth et al. 1992). NS1 tubules assembly seems to be independent, as the tubules are observed when NS1 is expressed separately in the cell from other viral proteins for both AHSV and BTV (Urakawa and Roy 1988, Maree and Huismans 1997). Mutational analysis of the analysis of BTV NS1 revealed that the conserved cysteine residues in NS1 at aa 337 and 340, and N’ and C terminal of the protein are critical for tubule formation.
(Monastyrskaya, Gould et al. 1995). These two cysteine residues are also conserved in the same position in the AHSV NS1 sequence.

NS1 is predominantly expressed in the cytoplasm of infected cells, whereas a small amount is found in viral inclusion bodies (Huismans and Els 1979, Brookes, Hyatt et al. 1993). In BTV-infected cells, NS1 forms a tubule structure within the cytoplasm (Urakawa and Roy 1988). NS1 is abundantly translated early in virus replication, therefore their association with intermediate filament components of the cellular cytoskeleton (Eaton, Hyatt et al. 1987) and association of virion and core particles with tubules (Eaton, Hyatt et al. 1988), suggest that NS1 may be involved in virus replication and/or virus translocation (Eaton, Hyatt et al. 1990). Knock-down of BTV NS1 decreased virus induced CPE and virus budding via the plasma membrane has led some to hypothesis that it is a viral virulence factor (Owens, Limn et al. 2004) and indeed, reassortment of different NS1 proteins increases viral virulence (Meiring 2011). Recently, BTV NS1 has been shown to increase viral protein synthesis by binding the UTRs of viral mRNA (Boyce, Celma et al. 2012). BTV NS1 appears to provide a translational advantage of viral transcripts translation over host mRNAs. This may explain, in part, the shutdown of host protein synthesis after BTV infection.

NS1 has also been shown to stimulate the immune response in the host however, it is not involved in antibody neutralisation (Roy, Urakawa et al. 1990). A recent study showed that AHSV NS1 is a key protein in vaccine composition for protection of IFNAR (-/-) mice (de la Poza, Calvo-Pinilla et al. 2013). This is different to vaccine studies with BTV, probably due to the strong CTL response raised by AHSV NS1.

NS2 is a highly conserved non-structural protein that is highly expressed in orbivirus-infected cells (van Staden and Huismans 1991). NS2 is encoded by segment 8 and is the only phosphorylated viral protein translated by BTV and AHSV (Devaney, Kendall et al. 1988). It is a major component of viral inclusion bodies (VIBs) (Brookes, Hyatt et al. 1993). Expression of AHSV NS2 from recombinant baculovirus in insect cells results in the formation of dense granular bodies or VIBs (Uitenweerde, Theron et al. 1995). The phosphorylation state of BTV NS2 has been shown to be critical for the formation of the VIBs. Dephosphorylation of NS2 is hypothesised to ‘release’ the
subcore and to complete assembly and egress of the particle (Modrof, Lymperopoulos et al. 2005, Kar, Bhattacharya et al. 2007).

VIBs are also rich in viral ssRNA and core structural proteins, thus they are thought to be the site of virus replication and assembly (Brookes, Hyatt et al. 1993) (Thomas, Booth et al. 1990). AHSV and BTV NS2 both have ssRNA binding ability and are thought to recruit viral ssRNA to the VIBs (Uitenweerde, Theron et al. 1995). The ability to bind ssRNA was found to be via the N-terminus of the protein in VIBs, this allows NS2 to form dimers (Fillmore, Lin et al. 2002, Kar, Bhattacharya et al. 2007). More in-depth RNA binding studies of BTV NS2 demonstrated it had the capacity to discriminate between viral and host cell ssRNA based on the unique RNA structures in the viral RNA species (Lymperopoulos, Noad et al. 2006).

The smallest dsRNA segment of virus genome, segment 10, encodes two forms of NS3, NS3 and NS3A (van Staden and Huismans 1991). NS3A translation is initiated to the secondary start codon, 10 aa downstream of NS3 and it is proposed to occur via leaking scanning (van Staden and Huismans 1991). There are conserved regions within AHHSV NS3/NS3A, which are also found in other orbivirus NS3/NS3A proteins (Bansal, Stokes et al. 1998, Van Niekerk, Freeman et al. 2003, Celma and Roy 2009). The conserved regions include: a proline rich region, a highly conserved region, two hydrophobic/transmembrane domains and a highly variable domain which is flanked by two transmembrane domains.

The expression of recombinant NS3 proteins or in infected cells is lower than the other non-structural proteins NS1 and NS2 (French, Inumaru et al. 1989, Van Staden, Stoltz et al. 1995, Celma and Roy 2009). In addition, for BTV NS3, the amount synthesised is cell type dependent (French, Inumaru et al. 1989). NS3/NS3A localises to the cell membrane of infected cells (Van Staden, Stoltz et al. 1995) and as AHHSV NS3 localises to sites of virus release, it is proposed to have a role in membrane destabilisation to allow the virus to exit (Stoltz, Van der Merwe et al. 1996). BTV NS3/NS3A has shown to have a role in egress of the virus and release via budding (Hyatt, Zhao et al. 1993, Wirblich, Bhattacharya et al. 2006, Celma and Roy 2009). Although, BTV has a late lytic cycle to allow release of the virus, the early budding from mammalian cells is thought to assist in cell to cell spread early in infection.
(Celma and Roy 2009) (Bhattacharya and Roy 2010). It may also have a role in virus egress in insect cells as the virus infection is non-lytic. It is also proposed that AHSV NS3/NS3A is a viral virulence factor. Overexpression of AHSV NS3 in insect cell was cytotoxic and increased cell membrane permeability (Stoltz, Van der Merwe et al. 1996). The cytotoxic nature of AHSV NS3 is mediated by the two transmembrane domains (van Niekerk, Smit et al. 2001).

AHSV NS3/NS3A is the second most variable viral protein after VP2 with up to ~28% to ~37% aa difference across the different serotypes (van Niekerk, Smit et al. 2001). The driving force behind the high level of variation in NS3 is unknown as expression is low in infected cells. Sequence analysis of the NS3 aa acid sequences revealed that they cluster into 3 distinct phylogenetic clades (α, β and γ) (Sailleau, Moulay et al. 1997, Martin, Meyer et al. 1998, van Niekerk, Smit et al. 2001, Quan, Van Vuuren et al. 2008). These clades have been implicated in the potential virulence of AHSV (Martin, Meyer et al. 1998, O’Hara, Meyer et al. 1998). Viral reassortment studies, prior to the development of a reverse genetics system for AHSV, verified that AHSV NS3 had a key role in virus release and permeabilization of the cell membrane (Meiring, Huismans et al. 2009). Meiring and colleagues demonstrated that viruses expressing NS3γ had the greatest ability to permeabilise the cell membrane followed by NS3β and NS3α. This effect was only observed in mammalian cells and not in insect cells. Although, AHSV NS3 has been shown to have a role in virus release, reverse genetic-engineered viruses that did not express NS3 could replicate efficiently in BSR cells but not in the insect vector cell line (Van de Water, van Gennip et al. 2015). The mutational analysis of NS3 also supported previous findings that NS3 had a role in virus cytotoxicity (Van de Water, van Gennip et al. 2015).

The most recent putative non-structural protein to be identified was segment 10 ORF2 which was identified using bioinformatics (Sealfon, Lin et al. 2015, Stewart, Hardy et al. 2015). An ORF was identified in all 230 AHSV segment 10 sequences analysed, ranging from 60–83 aa in length (Stewart, Hardy et al. 2015). No further analysis has been published studying the function of this ORF in AHSV although in BTV is not required for replication in mammalian or insect cell lines and may have a role in host cell protein shutdown (Stewart, Hardy et al. 2015).
1.2.8 Non-structural protein 4, NS4

NS4, originally referred to as ORFX, was identified by bioinformatics in +1 reading frame of segment 9 of several orbiviruses (AHSV, BTV, GIV, PALV, PHSV, SCRV and YUOV) overlapping the major ORF encoding VP6 (Firth 2008, Belhouchet, Jaafar et al. 2011). Initial bioinformatics data identified only one form of putative ORF (ORFX) in the AHSV genome, whereas Zwart et al. analysis revealed that this ORF split into 2 distinct phylogenetic clades and referred to the products as NS4-I and NS4-II. This nomenclature was based on the equivalent protein in BTV (Zwart, Potgieter et al. 2015) as no experiments have been published demonstrating that AHSV NS4 is a non-structural protein. Both types of AHSV NS4 have been detected in the spleen, lung and heart from infected animals and in vitro expression studies demonstrated that it localised predominantly to the cytoplasm but was also detected in the nucleus (Zwart, Potgieter et al. 2015). This was slightly different to BTV NS4, which only has one form and localises predominantly to the nucleolus (Ratinier, Caporale et al. 2011). Zwart et al. also demonstrated that NS4 was able to protect DNA from specific DNase treatment but not dsRNA, although there were no controls using protein known to protect the dsRNA or DNA and no interaction assays such as EMSA were undertaken to prove an interaction. The main functional role of AHSV NS4 is still unknown.

BTV NS4 has been studied in greater depth thanks to the development of a reverse genetic system. This allows the study of viral proteins without the other factors affecting the outcome i.e. the viruses all have the same parental backbone. NS4 is expressed at early stages during virus replication (4 h post-infection (Belhouchet, Jaafar et al. 2011)) and localised in cell nucleus (Ratinier, Caporale et al. 2011). NS4 was shown to be dispensable for viral replication in the insect vector and mammalian cells in vitro and in vivo (Ratinier, Caporale et al. 2011), however it was shown to confer a replicative advantage in interferon competent cell lines and animals (Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016). Moreover, in vitro studies demonstrated that BTV NS4 downregulated the expression of a firefly luciferase from several different promoters and this activity specifically localised to the nucleolus of the cell (Ratinier, Shaw et al. 2016). The data for BTV NS4 suggest that it is an interferon antagonist and may have a role in viral pathogenesis (Ratinier,
Caporale et al. 2011, Ratinier, Shaw et al. 2016). The aim of this thesis is to investigate the biological functions of AHSV NS4.

1.3 AHSV replication

Different viruses use various mechanisms to enter the cell. Viruses penetrate the plasma membrane and ultimately release viral genome into cytoplasm directly. Enveloped viruses fuse their envelopes with cell membrane to release the viral particle into cytoplasm. While non-enveloped viruses enter cells via clathrin-mediated uptake, caveolae-mediated endocytosis, lipid raft-mediated endocytosis, receptor-mediated endocytosis or macropinocytosis (Pelkmans and Helenius 2003).

The first step of virus infection is the attachment of virions onto the receptors of the cell surface (Figure 1.5). No specific receptor(s) have been reported for any orbivirus while these viruses have been shown to entry and replicate in a wide range of different cells. It is suspected that BTV VP2 interacts with sialic acid on the cell surface to initiate entry (Zhang, Boyce et al. 2010) via clathrin-mediated endocytosis (Eaton, Hyatt et al. 1990, Hassan and Roy 1999, Forzan, Wirblich et al. 2004, Forzan, Marsh et al. 2007) in HeLa and Vero cells. Further studies of BTV in BHK cells also demonstrated that it was able to enter cells via clathrin and cholesterol-independent mechanisms that also required dynamin (Gold, Monaghan et al. 2010) indicating that it may enter via macropinocytosis. Until recently the entry strategy of AHSV was unknown and inferred based on BTV. Recently AHSV has been shown to enter BSR cells via macropinocytosis (Vermaak, Conradie et al. 2016) and does not require caveolin- or clathrin-dependent endocytosis. Entry was shown to be dependent on dynamin in BHK-21 cells. Recent reverse genetic experiments including truncations of AHSV VP2 demonstrated that it is not required for entry into BSR, Vero or Kc cells (Van Gennip, van de Water et al. 2017) indicating that there are multiple entry strategies used by AHSV to infect cells.
The mechanism used by AHSV to leave the endosome is still unknown and is inferred based on BTV studies. Once the particle enters the endosome, VP2 is shed in the early endosome and virus particles containing VP5 are trafficked to late endosomes (Du, Bhattacharya et al. 2014) for further transfer to liposomes (Patel, Mohl et al. 2016). The low pH of liposomes causes VP5 to change conformation allowing the release of core particles into the cytoplasm (Hassan, Wirblich et al. 2001, Forzan, Wirblich et al. 2004, Patel, Mohl et al. 2016, Zhang, Patel et al. 2016).

Like other reoviruses, BTV and AHSV replicate in the cytoplasm of infected cells. The AHSV core is a transcriptionally active (Matsuo, Celma et al. 2010) and the genomic dsRNA is not released into the cytosol stopping early activation of the host cell anti-viral system and triggering IFN production (Marcus and Sekellick 1977). The core particle of AHSV contains all the necessary enzymes to initiate transcription and cap the nascent transcripts. Before transcription initiation, the genome dsRNA must be unwound by the viral helicase, VP6 (de Waal and Huismans 2005), which
allows the RNA dependent RNA polymerase (VP1) to start producing the transcripts (Vreede and Huismans 1998). The transcription is asymmetric and only positive sense RNA molecules are synthesised from the negative strands of viral genome dsRNA. These newly synthesised ssRNAs are capped and methylated at 5’ end by VP4, which is the capping enzyme (Van den Bout 2005), and become ‘mature’ mRNAs. The materials and substrates required for the transcription and capping activities, including S-adenosylmethionine (SAM) nucleotide triphosphates (NTPs), and the by-products, for example S-adenosyl-l-homocysteine (SAH) and adenosine diphosphate (ADP) are transferred into and out of the core particle by alternative channels (Diprose, Burroughs et al. 2001) (Figure 1.6). It is hypothesised, based on studies using rotavirus, that each transcript is simultaneously made and exits the core particle from transcript specific pores at the 5-fold axis of the particle (Periz, Celma et al. 2013).

Figure 1.6 Schematic cartoon representing BTV transcription in the core particle. The dsRNA segment is transcribed by a transcription complex and mature capped mRNA is extruded via the translocation portal into the cytoplasm. The substrates, including SAM and NTP, and the by-products, including SAH and ADP, of the transcription machinery are transferred by alternative pores (Mertens and Diprose 2004). Reproduced with permission.
After released into the host cell cytoplasm from the viral core particle, the capped viral mRNAs start to be translated by recruitment of host cell translation complex at the capped 5’ end. In mammalian cells, the mature mRNA contains a polyadenylated poly(A) tail at the 3’ end, which interacts and is bound to a poly(A) binding protein (PABP). PABP interacts with the translation complex to stabilise the mRNA and regulate translation (Drummond, Armstrong et al. 1985, Gallie 1991). mRNAs of AHSV and other reoviruses do not contain a poly(A) tail, however in BTV, the inverted repeats in UTR region of viral mRNA possibly interact with PABP and allow the viral mRNA to be translated (Markotter, Theron et al. 2004). Recent work with BTV NS1 indicates that it aids with the up-regulation of viral protein translation (Boyce, Celma et al. 2012), therefore NS1 is hypothesised to interact with cellular PABP or elongation.

During replication, large VIB is consisting of mainly NS2 (Huismans and Els 1979, Venter, Van der Merwe et al. 2014). These VIB are viral ssRNA and the viral proteins VP1, VP3, VP4, and VP6 and are proposed to be the site of sub-core assembly (Roy 1996). For BTV, it has been demonstrated that VP6 recruits ssRNA. This allows the interaction of VP6 with the inner surface of the VP3 decamer and then it recruits VP1 and VP4 to assemble the particle. Synthesis of the dsRNA genome occurs within the sub-core (Grimes, Burroughs et al. 1998, Nason, Rothagel et al. 2004, Lourenco and Roy 2011). The exact mechanism by which the ssRNA is packaged is still unknown, however it has been demonstrated for BTV that VP6 is essential for ssRNA packaging (Matsuo and Roy 2009) and that ssRNA is recruited in a segment- and sequence-specific manner (Burkhardt, Sung et al. 2014, Sung and Roy 2014, Boyce, McCrae et al. 2016). Once the sub-core has been released from the VIB, it acts as a scaffold for the addition of VP7.

The assembly of the mature particle and egress of AHSV is also based on BTV and there have only been a couple of studies examining this process (Bhattacharya, Noad et al. 2007, Bhattacharya and Roy 2008). After the sub-core particle leaves the VIB, VP7, VP5 and VP2 are assembled in succession to form the outer capsid of the virus and form mature progeny virus (Roy 1996). VP7 has been detected around the VIB and it is thought that the interaction between VP3 and VP7 is via hydrophobic interactions. VP5 is associated with lipid rafts and its integrity is critical for the
assembly of the virion (Bhattacharya and Roy 2008). The interaction between BTV VP2 and vimentin is involved in the transport of BTV viral particles cell microtubules within the cytoplasm (Bhattacharya, Noad et al. 2007), while NS3 mediates viral particle movement to the cell surface (Beaton, Rodriguez et al. 2002, Celma and Roy 2009). AHSV NS3 has been shown to increase cell membrane permeability (Meiring, Huismans et al. 2009), which contributes to the release of the viral particle to the environment from the host cell. Furthermore, NS3 also recruits the ESCRT-I protein and interacts with the vacuolar sorting pathway (Hurley and Emr 2006), which results in the budding of BTV a temporary envelope (Stoltz, Van der Merwe et al. 1996).

Once released, the new mature viral particles can infect new cells to start a new replication cycle or ‘superinfect’ the same cell (Hyatt, Eaton et al. 1989).

1.4 Immune response to AHSV infection

Currently, the knowledge of the host immune response to AHSV infection is limited. As AHSV and BTV belong to the genus Orbivirus and share similarities in their viral structures and protein functions, the results from BTV studies will be used to infer the information of AHSV.

1.4.1 Innate immune response

The innate immune response constitutes the first line of defence against virus infection. The mechanism is not specific to any given pathogen and relies on cellular sensors to detect foreign or non-self objects in the cell and it occurs rapidly after the onset of infection. There are several different types of innate immunity including intrinsic innate immunity where existing cellular factors directly restricts virus replication (for example, Vif and APOBEC (Malim 2006)). To date no one has published any information of host cell restriction factors against either AHSV or BTV. Another arm of the innate immune response is the indirect restriction of viral infection by inducing interferons and up-regulating the expression of other antiviral
molecules. Apoptosis, pyroptosis and necroptosis are also mechanisms used by the cell after sensing virus infection in order to restrict replication and virus spread.

Cell death by necroptosis and apoptosis has been reported for both AHSV and BTV (DeMaula, Jutila et al. 2001, DeMaula, Leutenegger et al. 2002, Mortola, Noad et al. 2004, Nagaleekar, Tiwari et al. 2007, Stewart and Roy 2010, Stassen, Huismans et al. 2012, Vermaak and Theron 2015). In the case of BTV, outcome seems to be highly dependent on cell type with primary cells undergoing necrosis (DeMaula, Jutila et al. 2001, DeMaula, Leutenegger et al. 2002) and transformed cell lines undergoing apoptosis (Mortola, Noad et al. 2004, Stewart and Roy 2010). Unlike BTV, where exposure of cells to the outer capsid proteins was sufficient to trigger an apoptotic response, AHSV-induced apoptosis required entry of the virus into the cytosol (Vermaak and Theron 2015). Both the intrinsic (caspase-9) and extrinsic (caspase-8) pathways have been shown to be triggered by orbiviruses (Stewart and Roy 2010, Stassen, Huismans et al. 2012).

Infection of animals with BTV has been demonstrated to initiate a cytokine storm (DeMaula, Jutila et al. 2001, DeMaula, Leutenegger et al. 2002, Hemati, Contreras et al. 2009, Drew, Heller et al. 2010). Experiments using PMBCs isolated from horse and infected with AHSV-4 were also demonstrated to release a number of cytokines indicating that a cytokine storm is probable during the initial phase of AHSV infection prior to the induction of the adaptive immune response (Sánchez-Matamoros, Kukielpka et al. 2013). BTV infection activates the production of inflammation and immune cytokines in the host cells, for example interleukins (IL)-1, IL-6, IL-12 and tumour necrosis factor α (TNFα) (Hemati, Contreras et al. 2009, Drew, Heller et al. 2010). Further, BTV dsRNAs are also recognised by host cell toll-like receptors (TLRs) and/or retinoic acid inducible gene (RIG-I)-like family triggering downstream cell signalling pathways and inducing the production of interferons (IFN) and pro-inflammation cytokines (Levy, Marié et al. 2011).

The induction of IFN in response to AHSV infection has not been studied. To date, there is only one report eluding to the role of IFN in controlling AHSV infection (de la Grandière, Dal Pozzo et al. 2014). BTV is shown to induce a strong type-I IFN production both in vivo and in vitro (MacLachlan and Thompson 1985, Russell,
O’Toole et al. (1996) and it is hypothesised that AHSV infection could also induce a strong IFN response. In BTV-infected endothelial cells, type-I IFN production is activated following RIG-I and MDA5-mediated signalling (Chauveau, Doceul et al. 2012). BTV infection is non-lethal in IFN competent adult mice, whereas in IFN receptor knockout mice (IFNAR−/−) mice, both BTV and AHSV infections are lethal (Calvo-Pinilla, Rodríguez-Calvo et al. 2009, de la Grandière, Dal Pozzo et al. 2014).

The innate immune system does not provide a long-lasting immune protection for the host. In experimentally infected sheep, IFN starts to be detectable in serum at about 4-days post p.i. and the IFN concentration reaches a peak between 4 and 8 days p.i. Then, the concentration decreases rapidly at about 10 days p.i. (Foster, Luedke et al. 1991). But the type-I IFN that is produced by the innate immune system further triggers B cell responses and therefore promotes humoral immunity to generate antibodies against BTV (Deal, Lahl et al. 2013).

1.5 Cellular immune response

Cell-mediated immunity (CMI) limits virus spread by targeting and destroying infected cells. Antibodies are not part of the cellular immune response. CD4+ cells provide protection against exogenous pathogens and guide the transformation of naive T cells into activated effector T cells. T cells respond to antigens presented on major histocompatibility molecules (MHCs), including from professional antigen-presenting cells (APCs) such as dendritic cells (Iwasaki and Medzhitov 2004). BTV-specific CD8+ T-cells develop in both sheep and mouse following BTV infection. In general, the CD8+ T cell population increases after 10-15 day p.i. (Ghalib, Schore et al. 1985, Sanchez-Cordon, de Diego et al. 2015), whereas short-lived anti-BTV cytotoxic T-lymphocytes (CTL) develop at 12 to 14 days p.i. (Jeggo, Wardley et al. 1984). Previous studies demonstrated that transfer of CTLs from the thoracic duct of sheep previously infected with BTV to recipient sheep can provide partial protection from either homologous or heterologous serotypes of BTV (Jeggo, Wardley et al. 1984).

The BTV VP2 and NS1 proteins have been identified as major targets for the host CMI response in sheep, with the NS1-specific CTL response playing a primary role in
serotype cross-protection (Andrew, Whiteley et al. 1995, Janardhana, Andrew et al. 1999). In contrast, both NS1- and NS2-specific CTLs develop in BTV infection of mice (Jones, Williams et al. 1997).

1.6 Humoral immune response

Antibodies to both structural and non-structural proteins are produced following infection by BTV and AHSV (Richards, MacLachlan et al. 1988, Hamblin, Anderson et al. 1992). In particular, neutralising antibodies block virus infection by targeting epitopes within the outer capsid protein VP2 of BTV and AHSV. However, as VP2 is the most antigenically diverse protein of BTV and AHSV, there is a lack of cross protection against infection across different serotypes. For BTV, the presence of common epitopes has been proven and neutralising antibodies are found to exert a level of cross reactivity among several BTV serotypes (Rossitto and MacLachlan 1992). The heterotypic antibody response is not efficient during co-infections with multiple viruses (Jeggo, Wardley et al. 1984).

1.7 Type I IFN production in response to virus infection

IFNs are composed by a group of proteins that are involved in cell signalling pathway regulation. IFNs are released by host cells in response to pathogens, including virus, bacteria, and parasites. To date, three major types of IFN have been identified based on the type of receptor. Amongst these, type I IFN is the most important against virus infections (Parkin and Cohen 2001). Type I IFN proteins in human comprise several subclasses but share the same cell surface receptor, IFN-α/β receptor (IFNAR) (Novick, Cohen et al. 1994). In human, Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω (de Weerd, Samarajiwa et al. 2007). IFN proteins themselves do not have antiviral functions, however, they induce the transcription of downstream genes which act as antiviral effectors (Pestka, Krause et al. 2004).

When viruses infect cells, IFN production is induced. The production of IFN is triggered following the recognition of pathogen associated molecular patterns (PAMPs). Generally, there are three types of nucleic acid-based PAMPs during pathogen infection: dsRNA, ssRNA and non-methylated CpG DNA. Moreover, there is
one type of lipid-based PAMP: lipopolysaccharide (LPS) (Brentano, Kyburz et al. 2005). These PAMPs are detected by pattern recognition receptors (PRRs). These receptors are involved in distinguishing self from non-self-material and promoting the antiviral response to exogenous pathogens. To date, two major families of PRRs have been characterised in virus detection: Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (Akira, Uematsu et al. 2006).

TLRs are activated in response to extracellular and endosomal PAMPs, and trigger a variety of downstream defence mechanisms based on cell type and receptors. To date, 13 TLRs have been identified in human and mouse (Thompson and Locarnini 2007). TLRs are mainly expressed by antigen presenting cells, for example macrophages, whereas other cell types also express a subset of TLRs (Akira, Uematsu et al. 2006). TLRs share a common organization, including a leucine-rich repeat extracellular domain (LRR), a transmembrane domain, and a Toll/interlukin-1 receptor domain (TIR). The LRR domain is involved in PAMP recognition, whereas the TIR domain interacts with TIR domain-containing adaptors (Yamamoto, Takeda et al. 2004). Activation of TLRs results in the dimerization of receptors and the recruitment of adaptors, in turn triggering multiple downstream signalling pathways, resulting in the synthesis of IFN and pro-inflammatory cytokines (O’Neill and Bowie 2007). Of the recognised TLRs, TLR3, TLR7/8 and TLR9 are the most important ones for the host innate immune response against viral infection.

TLR3 recognises dsRNA as a PAMP. Double-stranded RNA is considered as the most important PAMP of infections by Group III viruses such as orbiviruses. During viral infection, dsRNAs sometimes appear as intermediates of virus replication or structured RNA, whereas the host cell is not able to generate dsRNA. By binding and interacting with dsRNA, TLR3 can recruit and activate the downstream adaptor TRIF, subsequently activating the transcription factors IRF-3/7, NF-κB and AP-1 (Yamamoto, Sato et al. 2003, Vercammen, Staal et al. 2008). IRF-3/7 is essential for IFN-β expression (Sato, Suemori et al. 2000). By interacting with TRAF3, TRIF activates TBK1 and IKKε kinases. The resulting phosphorylation and dimerization leads to the activation of the transcription factors IRF-3/7. Activated IRF-3 and -7 then translocate into the cell nucleus and induce the transcription of the IFN-β gene (Panne, Maniatis et al. 2007). IRF-3 and IRF-7 play different roles in IFN-α/β gene
transcription. Briefly, IRF-3 expression is engaged at an early stage of viral infection, whereas IRF-7 is involved in IFN release and plays an important role in the IFN positive feedback loop (Honda, Yanai et al. 2005, Hiscott 2007). TRIF also interacts with TRAF6 and RIP1 to activate NF-κB and AP-1, which are both regulators of transcription of the various inflammatory cytokine genes. Briefly, TRAF6 and RIP1 activate the MAPK pathway, which results in activation of AP-1. This MAPK pathway also regulates the IKK complex, the activation of which results in the phosphorylation and degradation of the IκBα inhibitor. The released NF-κB can translocate into the cell nucleus to induce the inflammatory response (Alexopoulou, Holt et al. 2001, Peng, Yuan et al. 2010).

When TLR7/8 binds ssRNA in the endosomal compartments, they recruit and activate the adaptive molecule MyD88 via their TIR domain. The activated MyD88, combined with IRAK-1 and IRAK-4 kinases, forms a complex to induce IRF-7 phosphorylation and activate the NF-κB pathway with TRAF6 (Oganesyan, Saha et al. 2006). The PAMP of TLR9 is non-methylated CpG DNA that originates from bacterial or viral genomes (Eisenächer, Steinberg et al. 2008). Similar to TLR7/8, TLR9 also induces the upregulation of IFN-α/β expression via IRF-7 activation and provokes the activation of the NF-κB pathway (Fiola, Gosselin et al. 2010).

1.7.1 Receptors of viral infection in the cytoplasm

The RIG-I-like receptor pathway is considered to be an alternative cellular innate immune system for the detection of intracellular viral infections. It is reported that the helicases RIG-I and MDA5 are able to detect intracellular dsRNA and act as PRRs in order to induce IFN-β production and trigger inflammatory cytokine expression (Yoneyama, Kikuchi et al. 2004, Chauveau, Doceul et al. 2012). These RIG-I-like receptors (RLRs) share a similar structure, including a helicase domain, a C-terminal domain (CTD) and two CARD domains at the N-terminus. RIG-I additionally has as a repressor domain that is absent in MDA5 (Takahasi, Kumeta et al. 2009). RIG-I and MDA5 are expressed and trigger IFN production in most types of cells, including fibroblast and conventional dendritic cells (cDCs). However, in pDCs, where TLRs are highly expressed, the cells preferentially use the TLR system (Luber, Cox et al. 2010) (Kato, Sato et al. 2005).
Although the aa sequence similarity between RIG-I and MDA5 is high, the PAMPs of these two proteins are different. RIG-I has a higher affinity for ssRNA as opposed to dsRNA (Saito and Gale 2008, Takahasi, Yoneyama et al. 2008), specifically ssRNA with an exposed 5′-triphosphate (Takahasi, Yoneyama et al. 2008, Schmidt, Schwerd et al. 2009). As 5′-triphosphate RNAs are not presented in non-infected cells, this RNA motif is a strong hallmark of viral infection. Moreover, RNA secondary structures, for example base-paired stretches, can also influence the activation of RIG-I. The combination of a double strand stretch of RNA, and a 5′-triphosphate forms a sufficient PAMP for RIG-I recognition (Schmidt, Schwerd et al. 2009, Takahasi, Kumeta et al. 2009). Despite its lower affinity for dsRNA, RIG-I is also associated and activated by short dsRNA (<2kb) (Kato, Takeuchi et al. 2008). Unlike RIG-I, MDA5 does not bind 5′-triphosphatate ssRNA. However, it has a high affinity for dsRNA, in particular long dsRNA (>2kb). Indeed, long dsRNA molecules are necessary for MDA5 activation (Kato, Takeuchi et al. 2008).

In non-infected cells, the activity of RIG-I is maintained in an inactive conformation by the RD domain. When the CTD domain of RIG-I binds viral RNA, the conformation of the protein is altered and the CARD domain is exposed, thus activating RIG-I. After activation, RIG-I interacts with the mitochondrial antiviral signalling protein (MAVS) via the CARD domain (Seth, Sun et al. 2005). Following activation by dsRNA, MDA5 forms dimers and filaments, which also interact with MAVS (Berke and Modis 2012). Similarly, to TRIF, activated MAVS interacts with TRAF3 to activate TBK1 and IKKe, subsequently activating the IRF-3/7 pathway to trigger IFN-β expression (Saha, Pietras et al. 2006). Activated MAVS is able to trigger the NF-κB pathway and downstream inflammatory response by associating with TRAF6 (Seth, Sun et al. 2005).

1.7.2 Cell response to extracellular type I IFN

IFN secretion triggers the Janus tyrosine kinase/signal transducer and activator of transcription protein (JAK/STAT) pathway in most cell types (reviewed by (Aaronson and Horvath 2002, Rawlings, Rosler et al. 2004, Quintás-Cardama and Verstovsek 2013)). The JAK/STAT pathway is well characterised and activation of this pathway results in the downstream gene expression of IFN-stimulated genes (ISGs). The
proteins encoded by ISGs play critical roles on cellular functional processes, including the innate and adaptive immune systems, cell proliferation and survival, and cellular protein synthesis in order to establish an antiviral state. The cell surface receptor IFNAR is the receptor for extracellular IFN-α/β. IFNAR is composed of two subunits, IFNAR1 and IFNAR2, which are both transmembrane proteins and expressed in most of the cell types (de Weerd, Samarajiwa et al. 2007). Binding either IFNAR1 or IFNAR2 with the type I IFN leads to the recruitment of the other subunit to form a dimer. After the dimerization, IFNAR1 and IFNAR2 associate with different adaptors. IFNAR1 associates with and phosphorylates tyrosine kinase 2 (TYK2), which belongs to the JAK family (Gauzzi, Velazquez et al. 1996), whereas IFNAR2 interacts with JAK1 and STAT2 (Uddin, Chamdin et al. 1995, Gauzzi, Velazquez et al. 1996). The activated JAK1 in turn phosphorylates the IFNAR1 and IFNAR2 on the tyrosine residues (Zhao, Lee et al. 2008). This allows the STAT2 protein to bind the IFNAR1 on the phosphorylated tyrosine residues. Following STAT2 phosphorylation, STAT1 is further phosphorylated by binding STAT2 (Yan, Krishnan et al. 1996). The phosphorylated STAT1 and STAT2 dimerise and the STAT1/2 dimer is released from IFNAR2 into the cytoplasm. In the cytoplasm, the heterodimer interacts with IRF9 to form a protein complex referred to as the transcription factor ISG factor 3 (ISGF3) which transfers into the cell nucleus to regulate ISG transcription by binding the promoter element ‘IFN-stimulated response element’ (ISRE). STAT is a big protein family. Protein STAT1 to STAT6 are all associated with, and can be activated via, IFN pathway. However, variation in STAT usage is cell type dependent (Meinke, Barahmand-Pour et al. 1996, Matikainen, Sareneva et al. 1999) (Figure 1.7).
1.7.3 Viruses regulate the host cell type I IFN response

In order to successfully infect and proliferate in the host, viruses have evolved a variety of mechanisms to escape the host immune system. Detection of viral PAMPs by cellular PRRs is the first step in generating a host immune response. To avoid viral PAMPs being detected by cellular sensors, a frequent strategy for many RNA viruses is to protect and hide their genomic RNA inside the viral- or cellular-derived
structures such that the genome is not visible to the cellular sensors. Multiple ways of blocking PAMP detection have evolved in different viruses. Vaccinia virus expresses two proteins, A46 and A52, which are strong antagonists of TLR receptors. The A46 viral protein contains a Toll/interlukin-1 receptor domain, and associates the adaptors of TLRs, including MyD88, TRIF and TRAM, to prevent the TIR domain of the TLRs associating with their adaptors competitively (Stack, Haga et al. 2005). On the other hand, the A52 protein of vaccinia virus binds to TRAF6 and IRAK-2 in order to stop the NF-κB pathway activation (Harte, Haga et al. 2003). In the infected cells, the RIG-I pathway is one of the most important PAMP detection mechanisms. As a result, RIG-I-like receptors (RLRs) and their adaptors are common targets of viral antagonism. For example, the Influenza A viral protein NS1 binds to the viral RNA genome to prevent the viral RNA from being detected by RIG-I (Guo, Chen et al. 2007). IRF is another target for viral proteins to inhibit the IFN response. The Borna disease virus P protein is phosphorylated by TBK1 and acts as a decoy for host IRF-3 phosphorylation (Unterstab, Ludwig et al. 2005).

To stop the host cell response to extracellular IFN and block antiviral protein synthesis, the JAK/STAT pathway is also targeted by viruses. Many viral proteins act as antagonists of this pathway to obstruct antiviral response. For example, rhesus rotavirus (RRV) is able to block STAT1 and STAT2 translocation to the nucleus in order to inhibit the response to type I IFN (Holloway, Truong et al. 2009). Viruses can also disrupt IRF9 from forming the ISGF3 complex in order to down regulate the JAK/STAT pathway. The μ2 protein from a non-myocarditic strain of reovirus results in the accumulation of IRF9 in order to prevent formation of the ISGF3 complex (Zurney, Kobayashi et al. 2009), and inhibit downstream ISG expression. By blocking the JAK/STAT pathway, viruses also inhibit ISG expression, and subsequently affect the positive feedback loop of the IFN response.

Proteins that are encoded by ISGs play a variety of roles in the antiviral mechanism. Therefore, to promote viral replication, viruses inhibit the IFN response. ISGs, including protein kinase R (PKR) and oligoadenylate synthetase/RNase L (OAS/RNase L), are important host antiviral proteins. Some viral proteins can target these proteins to either block their function or induce their degradation. For example, the E2 protein of hepatitis C virus contains a homologous sequence to PKR and shares
the same phosphorylation sites. The E2 protein is in turn able to target PKR and
prevent its phosphorylation and activation (Taylor, Shi et al. 1999), whereas human
immunodeficiency virus (HIV) infection upregulates the expression of RNase L
inhibitor (RLI), which leads to the downregulation of RNase L activity (Martinand,
Montavon et al. 1999).

1.8 BTV interactions with the IFN pathway

Most viruses will induce the IFN response to a greater or lesser extent during
infection. BTV has been shown to be a strong inducer of type I IFN, possibly as a
result of its dsRNA genome (Tytell, Lampson et al. 1967, Huismans 1969). IFN
synthesis during BTV infection has been demonstrated both in vivo and in vitro.
When primary embryonic murine cells were infected with BTV-10A, an attenuated
American vaccine strain of BTV-10, IFN could be detected in cell supernatants by 5
h p.i, and increased until 24 h p.i (Huismans 1969). When infecting other cell types,
including human cells, similar results were obtained (Jameson and Grossberg 1981).
However, it was found that field strains, including BTV-2, -4 and -6, induced higher
amounts of IFN compared to the attenuated strain (Lyons, Schoub et al. 1982). Later
studies also demonstrated that field strains of BTV were strong IFN inducers in vitro
(Chauveau, Doceul et al. 2012).

IFN synthesis in vivo was also demonstrated by intravenous injection of $4\times10^8$ PFU of
purified BTV-10 V, a virulent strain derived from North America, into mice and IFN
was assessed in blood samples collected at different time points. IFN was detected
at 4 h p.i and reached its maximum concentration between 8 to 12 h p.i. Then the
titer decreased and became undetectable at 24 h p.i (Huismans 1969). However, in
another experiment, injection of $10^{6.8}$ PFU of BTV into mice induced a rapid IFN
production that reached a peak at 8 h p.i (Jameson, Schoenherr et al. 1978).
Experiments have also been conducted in sheep and cattle, natural hosts of BTV.
Infection in bovine foetuses with BTV-10 resulted in IFN detection in both serum and
tissues (MacLachlan, Schore et al. 1984). On the other hand, when sheep were
infected with BTV-10, -11, -13 or -17, IFN was detected in serum at 5 d.p.i, reaching
a peak at 6 d.p.i (Foster, Luedke et al. 1991).
1.8.1 Induction of type I IFN by BTV

PAMPs of BTV are the keys for the virus to induce host IFN response. As a dsRNA virus, the genome itself is a strong potential PAMP for the IFN response. It was shown that the injection of 10 ug of purified BTV-10A genomic dsRNA into mice induced IFN synthesis in vivo after 2 h, reaching a peak 6 h following injection (Eksteen and Huismans 2016). Further experiments showed that transfection of BTV dsRNA into cells also activated IFN-β (Vitour, Doceul et al. 2014). These data suggest that the BTV viral genome can act as PAMPs. However, later experiments revealed that UV-inactivated BTV virus also induced IFN production both in vivo (mice) and in vitro (rabbit cells) (Jameson, Schoenherr et al. 1978, Taylor and O’Brien 1985). However, another study suggested that BTV-8 replication is a requirement for IFN-β expression, as in BTV-8 infected A549 cells and MDBK cells, the level of mRNA of IFN-β increased synchronously with BTV viral RNA synthesis. On the contrary, the level of IFN-β mRNA in cells infected with UV-inactivated virus was close to background levels (Chauveau, Doceul et al. 2012).

1.8.2 Modulation of the IFN pathway by BTV

Previous data suggests that BTV-8 infection inhibits the IFN-β promoter activity after stimulation of the RIG-I-like pathway (Vitour, Dabo et al. 2009). The non-structural protein NS3 shows the strongest capability to inhibit IFN-β promoter activity. NS3 from both field and attenuated strains of different serotypes induce a similar response. Furthermore, NS3 reduces the level of IFN-β mRNA, which indicates that transcription of IFN-β is also affected by the NS3 protein. However, NS3 does not affect the translation of in vitro transcribed mRNA, suggesting that NS3 may not play a role in regulating translation. Moreover, the CMV promoter is not affect by BTV NS3, indicating that NS3 may not inhibit a broad range of promoters (Chauveau, Doceul et al. 2013).

NS4 is encoded by the alternative ORF in BTV segment 9 and is also reported to be an IFN antagonist (Ratinier, Shaw et al. 2016). Sheep endothelial cells infected with a BTV-8 NS4 deleted virus (BTV-8ΔNS4) released a much greater amount of IFN into the supernatant compared to wild-type BTV-8 (BTV-8wt) (Ratinier, Shaw et al.
Moreover, the growth of BTV-8wt in IFN-competent cells is more efficient than BTV-8ΔNS4. These results suggest BTV-8 NS4 is a strong IFN antagonist that blocks IFN synthesis in host cells. However, RNAseq experiments revealed that cells infected with either BTV-8wt or BTV-8ΔNS4 infected cells both induced the transcription of ISGs. This indicates that although NS4 inhibits host IFN production to a certain extent, NS4 is not able to entirely shutdown the host immune response. Similarly, with BTV NS3, BTV-8 NS4 also inhibits IFN-β promoter activity, as well as ISRE promoter. As BTV-8 NS4 it localises to the nucleolus and has a putative nucleic acid binding domain at the C-terminal, it was suggested that BTV-8 NS4 may regulate host transcription.

1.9 Aims

AHSV and BTV are related members of the genus Orbivirus. These two viruses share a similar structure and viral protein functions. BTV NS4 has been shown to be an IFN antagonist and is a determinant of virulence. The presence of an AHSV NS4 has also been suggested and identified (Zwart, Potgieter et al. 2015). However, unlike BTV NS4 which is conserved across serotypes, AHSV NS4 is divided into two distinct groups and the amino acid sequences are much longer than that of BTV NS4. However, the functions of AHSV NS4 remain to be described.

Reverse genetics systems are powerful tools for the study of viral proteins and virus replication. Reverse genetics systems for orbivirus are well developed (Boyce, Celma et al. 2008, Kaname, Celma et al. 2013, Van de Water, van Gennip et al. 2015). The goal of this project was to develop a reverse genetics system for AHSV and to study the function(s) of AHSV NS4 protein. During the course of this study, a reverse genetics system for AHSV was developed and published (Conradie, Stassen et al. 2016).

The specific aims of this project are the following:

- To analyse the sequences of AHSV segment 9 and perform phylogenetic analysis of the AHSV NS4 protein.
- To establish a reverse genetics system for AHSV in order to rescue wild type
and NS4 deletion mutants of AHSV.

- To study the function(s) of the AHSV NS4 protein and characterise its role(s) in viral replication.
Chapter 2.  Materials and Methods
2. Materials and methods

2.1 In silico analyses

Bioinformatics analysis to identify ORFs in segment 9 of AHSV occurred on two separate occasions. The initial analysis was performed on 38 sequences downloaded from GenBank (as of July 2014). The second set of analysis were performed on 207 AHSV sequences available in GenBank (as of August 2016) and included full genome sequences to allow reassortment/segment linking studies as well as to confirm the initial results. Bioinformatics analysis was carried out using CLC Genomics Workbench, Version 6.5. There are a number of programs in CLC Genomic Workbench (https://www.qiagenbioinformatics.com/). Initially, ORFs were identified and the NS4 coding sequences were extracted and aligned using ClustalW. Sequence motifs were examined using MOTIF SEARCH (http://www.genome.jp/tools/motif/) and NoD (http://www.compbio.dundee.ac.uk/www-nod/HelpDoc.jsp).

Dr Joseph Hughes (CVR Bioinformatics Service) aligned each ORF using MAFFT (Katoh, Kuma et al. 2005). Duplicate and truncated sequences or sequences containing gaps or missing start codons were removed from the alignments. Selection of substitution model was performed using jModeltest (Posada 2008) and maximum likelihood phylogenies were reconstructed (1,000 bootstraps) using RAxML 8.2.8 (Stamatakis 2014).

2.2 Antibodies

The primary antibodies used in immunofluorescence and western immunoblot were a rabbit anti AHSV NS4-I, a rabbit anti-NS4-II, a rabbit anti-AHSV VP7 antibody (Proteintech Group, Inc.) and rabbit anti-tubulin (Sigma T3320). Antibodies to detect the AHSV proteins had to be made commercially. Anti-NS4-I the antibody was raised against the entire sequence of NS4-I, anti-NS4-II was raised against the entire sequence of NS4-IIa and anti-AHSV VP7 was raised against the entire sequence of AHSV-1 VP7. The antigen was tagged with Glutathione S-transferase (GST) tag and expressed in bacteria (Proteintech Group, Inc.). Rabbits were immunized at least 4 times prior to bleeding for sera collection.
The secondary antibodies used for immunofluorescence included: Alexa Fluor 594 goat anti-rabbit (Life Technologies, A11037) and Alexa Fluor 488 goat anti mouse (Life Technologies, A21202). The secondary antibody used for western immunoblots was a donkey anti-rabbit HRP conjugate (GE, GENA9340V).

2.3 Cell lines

BSR cells, derived from the baby hamster kidney cell line BHK-21 (kindly provided by Dr. Conzelmann), were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 5% foetal bovine serum (FBS) (Life Technologies). E. Derm cells, a fibroblast cell line obtained from dermis of a four-year-old female horse (ATCC number CCL-57), were kindly provided by Dr. Murcia (CVR, UK). E. Derm cells were grown in DMEM supplemented with 15% FBS and 1% MEM non-essential amino acids (Invitrogen). A549 cells (Dr Elliott, CVR), a human alveolar basal epithelial cell line derived from andenocarcinoma, were grown in DMEM supplemented with 10% FBS. A549-NPro a cell line derived from human A549 cells that constitutively expresses the bovine viral diarrhoea virus NPro (a gift from Dr Randall, University of St Andrew’s, UK) were grown in DMEM supplemented with 10% FBS and 2 ug/ul puromycin. HEK-293T cells were cultured in DMEM supplemented with 10% FBS. CPT-Tert cells (kindly provided by David Griffiths) are sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT). The cells were grown in Iscove’s modified Dulbecco’s medium (IMDM), supplemented with 10% FBS (Arnaud, Black et al. 2010). All mammalian cells were grown at 37 °C in a 5% CO₂ humidified atmosphere.

Kc, a cell line derived from larvae of Culicoides sonorensis larvae (Wechsler, McHolland et al. 1991) were grown in Schneider’s Drosophila Medium supplemented with 15% FBS and incubated at 28 °C.

Cells were grown to confluency in a 75-150 cm² flask, washed with PBS, trypsinised with ATV and resuspended in the appropriate media supplemented serum as previously described above. Cells seeded to the appropriated density to reach 50% to 80% confluence the following day (i.e., a 12-well plate was seeded with 1×10⁵ BSR cells for transfections to rescue virus by reverse genetic). Cells were seeded onto 13
mm circular coverslips (Cover glass Thickness No. 0, VWR) if the transfected samples were to be used for immunofluorescence and confocal microscopy. Prior to use, cover slip were sterilised by soaking in 75% (v/v) ethanol (Sigma) for a minimum of 30 min. Coverslips were then air dried in the MSC hood and placed into each well of the respective plate.

2.3.1 Generation of equine primary cells

Horse aorta endothelial primary cells were isolated from horse aortas supplied by Mr. Richard Irvine (post-mortem department, University of Glasgow, UK). Batch 1 and batch 3 primary cells were from the aortas of 1-year-old female horses. The aortas were prepared by removing the connective and adipose tissue on the external side of the aortas. The cleaned aortas were washed with PBS containing 1% (v/v) penicillin-streptomycin (Invitrogen). Aortas were cut into ~5 cm×5 cm pieces and 1 ml of 2 mg/ml collagenase (Sigma) diluted in FBS-free DMEM was added to the endothelial side of the aorta. The aorta and collagenase were incubated for 1 h at 37 °C. The cells that had been incubating with the collagenase were then scraped from the surface and transferred into fresh media. The harvested cells were gently centrifuged and resuspended in human large vessel endothelial cells basal medium (Cell Work) supplemented with 15% FBS, 1% Penicillin-Streptomycin, 100 U/ml Nystatin (Sigma), 2% Large Vessel Endothelial Cell Growth Supplement (Cell Work) and 0.1% Amphotericin B/Gentamycin (Cell Work). Cells were placed into a 12 well-plate and grown in a low oxygen environment. (3% oxygen, 5% CO₂, 37 °C humidified atmosphere).

2.3.2 Screening of cell lines for pestiviruses and mycoplasma

All the cell lines were routinely screened for mycoplasma and pestivurses contamination by Aislynn Taggart using commercial available kits.
2.4 Viruses

The nine AHSV reference strains (AHSV-1 to AHSV-9) were obtained from the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise “Giuseppe Caporale” (Teramo, Italy). Encephalomyocarditis virus (EMCV) was kindly provided by Dr. Randall (University of St Andrew’s, UK).

2.5 Preparation of virus stocks

Rescued viruses were passaged twice after rescue to generate stocks for subsequent experiments. From rescue (p0) in a 6-well plate, 100 ul of the supernatant was used to infect BSR cells were seeded in a T25cm² flask to generate a p1 stock. The virus inoculum was left on the cells until visible CPE. Care was taken to ensure the medium did not acidify. The supernatant was harvested and cell debris pelleted by centrifugation. The passaged virus was stored at 4 °C while an aliquot was stored at -80 °C. The cell pellet was kept to purify the dsRNA as described in section 2.21. To generate a p2 stock, 200-300 ul of the p1 stock was used to infect a T225cm² flask. As for the p1 stock, cells incubated until visible CPE. The supernatant was harvested and cell debris pelleted by centrifugation. The passaged virus was stored at 4 °C and an aliquot was stored at -80 °C.

2.6 Virus Quantification

The cell-free virus stocks were quantified using plaque assays and end-point dilutions (TCID50/ml) in BSR cells.

2.6.1 Plaque Assay

Plaque assay is a method to calculate a virus titre based on the number of plaque forming units (PFU) in a standard cell culture system. It relies on the ability of the virus to induce visible CPE (cell death) that leaves holes in the monolayer. We titrated the viruses in BSR cells since Kc, E. Derm and primary horse aorta endothelial cells were not amenable to the formation of plaques. Ten-fold serial dilution of the viruses were made in DMEM without supplements and 500 ul of virus
dilutions were added to a 80% confluent monolayer of BSR cells and incubated for 1.5 h at 37 °C in a humidified 5% CO2 environment. Afterwards, the inoculum was aspirated, the cells washed and overlayed with 1.5% low melting point agarose in MEM supplement 4% FCS. Cells were incubated at 37 °C in a humidified 5% CO2 environment for 72 h when they were fixed with 4% formaldehyde. Plaque overlay was removed and the monolayer stained with 1.5% crystal violet and the plaques counted. Plaque assays were performed in duplicate. Virus titre was calculated as the number of PFU/ml.

2.6.2 Endpoint dilution assay

Tissue culture infectious dose (TCID$_{50}$) is an endpoint dilution assay that allows virus quantification based on the dilution that has less than 50% CPE in the cell monolayer. Ten-fold serial dilution of each virus was made in quadruples in a 96-well plate. One hundred ul of 1×10$^4$ BSR cells were then added to each well. Plates were incubated for 96 h and the presence of CPE in each well was scored using a light microscope. Viral titers were calculated by the method of Spearman and Karber and expressed as log$_{10}$ TCID$_{50}$/ml.

2.7 Virus growth curves.

BSR, E. Derm, Kc and primary horse aorta endothelial cells were seeded in 12-well plates and infected by the indicated viruses at a MOI of 0.01 (BSR, E. Derm and Kc cells) or 0.1 (primary horse aorta endothelial cells). The MOI used for E. Derm, Kc and primary horse aorta endothelial cells was based on virus titers in BSR cells. The virus inoculum was incubated with the cells for 1.5 h at 37 °C in a 5% CO2 environment and then aspirated and washed in medium. Fresh medium was added to the cells and cell culture supernatants were collected at 12, 24, 48 and 72 h post infection (p.i.). The virus titer (expressed as TCID$_{50}$/ml) was determined by endpoint dilution as described in section 2.6.

Virus growth was assessed in cells in the presence of 1,000 U of universal type I interferon. Universal type I Interferon (uIFN) was obtained from PBL InterferonSource. E. Derm cells were treated with 1,000 U of uIFN 4 h prior infection...
with AHSV at an MOI equivalent of 0.01. One and half-hours after infection, the virus was aspirated, cells washed with once with medium and fresh medium without uIFN. Cell supernatants were collected at 12, 24, 48 and 72 h post-infection and virus infectivity was subsequently estimated by endpoint dilution analysis on BSR cells (see section 2.6).

Virus growth in interferon competent primary horse aorta endothelial cells in the presence of ruxolitinib (rux), an inhibitor of the IFN pathway, was also assessed. Rux was obtained from Selleckchem and diluted in DMSO. For comparative purposes, DMSO was added to the control infected cell at same final concentration as rux-treated cells to ensure the effect was not due to the DMSO. Horse aorta endothelial cells were treated with rux or DMSO for 4 h prior to infection with AHSV at a MOI equivalent of 0.1 for 1.5 h at 37 °C. Rux or DMSO was added to the medium during infection. Afterwards, the inoculum was aspirated, cells were washed with medium and fresh medium containing either rux or DMSO was added. Supernatants were collected at 12, 24, 48, 72 and 96 h post-infection and virus infectivity was subsequently titrated by endpoint dilution analysis on BSR cells (see section 2.6).

2.8 Commercial plasmids

The construction of the plasmids to generate the T7 ssRNA for the reverse genetic system for AHSV was based on the design used for BTV (Boyce, Celma et al. 2008, Ratinier, Caporale et al. 2011). An exact copy of each segment of AHSV-4 (GenBank accession numbers JQ796724-JQ796733) and segment 9 for AHSV-1, AHSV-6 and AHSV-7 (GenBank accession numbers AM883170, AHU33000 and JQ742012) were commercially synthesised (Genscript). The T7 sequence was placed at the 5’ end such that it allowed the start of the transcript at the first G of all the AHSV segments. To ensure that the transcript finished at the last C of the segments, a unique restriction site was incorporated. The restriction sites were Bbs1 (Segment 1), Bsa1 (Segments 2, 3, 5, 6, 8, 9 and 10) and Sap1 (Segments 4 and 7).

pCI plasmids that contained the alternative ORF of segment 9s of AHSV-1, AHSV-4, AHSV-6 or AHSV-7 were commercially synthesised (Genscript. GenBank accession numbers are the same as above). pCI vector was from Promega. pCI BTV-10 NS2
(GenBank accession number NC_006007) and pCl BTV-8 NS4 (GenBank accession number JX680455) have been previously described (Stewart, Hardy et al. 2015, Ratinier, Shaw et al. 2016).

Mutant segment 9s of AHSV-1, AHSV-4, AHSV-6 and AHSV-7 were commercially synthesised to delete NS4 ORF, the putative methionine initiation codons of NS4 were removed and several stop codons were added. None of the introduced mutations changed the amino acid (AA) residue in the VP6 ORF. For AHSV-1 mutations included: G209A, T305C, G413A, T449C, T452C and G569A. For AHSV-4 mutations included: T215C, T260C, T296C, T395C and C491G. For AHSV-6 mutations included: T149C, G209A, T305C, T401C and T470A. For AHSV-7 mutations included: T149C, T179C, G209A, T305C, T377C, G413A and T470A. We also replaced bases in AHSV-7 segment 9 including T149C and T179C to express an AHSV-1 NS4-like protein and C577T to express AHSV-6 NS4-like protein. However, in order to express AHSV-6 like-NS4 protein, an amino acid residue in the AHSV-7 VP6 ORF, tyrosine at position 187, had to be replaced to isoleucine.

2.9 Plasmid transformation and purification

All plasmids were transformed into either XL-1blue (Agilent) or Stbl2 (Invitrogen) chemically competent E.coli as per manufacturer’s protocol. After transformation XL-1 Blue-transformed cells were grown at 37 °C while Stbl2 at 30 °C.

Plasmids were extracted from transformed E.coli using an alkaline lysis-based extraction with a QIAgen kit according to manufacturer’s protocols.

2.9.1 Polymerase chain reaction

Amplification of NS4-I was performed for cloning into pCl empty. Primers were designed flanked with restriction endonuclease sites to allow ligation into the vector (Table 2-1). The PCR reaction mix consisted of 1 x PfuUltra II Reaction Buffer (final concentration of 2 mM MgCl₂), 0.25 mM dNTPs, 20 pmol of each primer, 1ul of
PfuUltra II fusion HS DNA polymerase, between 50-250 ng of vector and made to a final volume of 25 ul with ultrapure water. Cycle condition consisted of an initial denaturation of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 55-60 °C for 20 s, 72 °C for 30 s and a final extension at 72 °C for 3-5 mins. For annealing temperatures, please see Table 2-1.

Table 2-1 PCR primers used for the amplification of NS4. The restriction endonuclease site is highlighted in red. The position in the segment is given.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ - 3’)</th>
<th>Position in the genome (5’-3’)</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS4-IF</td>
<td>TAT CTCGAG</td>
<td>184-203</td>
<td>53.7/61.5</td>
</tr>
<tr>
<td></td>
<td>CGGAAAAGGTAGGAAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS4-IR</td>
<td>TGT GTCGAC</td>
<td>692-672</td>
<td>52.5/61.6</td>
</tr>
<tr>
<td></td>
<td>AACTCCAACCTTCTTCTGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.10 Agarose electrophoresis

Agarose gel electrophoresis was performed on horizontal gels consisting of 1.0% or 1.5% (w/v) agarose (Invitrogen), TAE buffer (40 mM Tris-HCl; 20 mM glacial acetic acid; 2 mM EDTA; pH 7.0) and 1 x SybrSafe (Invitrogen). PCR products, DNA and dsRNA samples were loaded into the agarose gel in 6× loading dyes and electrophoresed in TAE buffer. DNA and PCR products were run for 30-60 min at 90-110 V in a Minisub DNA cell (BioRad). dsRNA samples were run at 4°C for either 4 h at 90-110 V or 30 V overnight in a Minisub DNA cell (BioRad). The size of the bands was determined by comparison with a 1 Kb plus molecular weight marker (Invitrogen). Gels were visualised by UV illumination at 260 nm.

2.11 Generation of pCI expression constructs

The pCI vector was to express viral proteins in mammalian cells. Vectors that expressed NS4-I alone and either AHSV VP1, VP3, VP4, VP6, VP7, NS1 or NS2 for the first round of transfections for AHSV rescue were required.
2.11.1 Preparation of vector and inserts

Empty pCI (pCI.empty) and pUC57AHSV51, pUC57AHSV53, pUC57AHSV54, pUC57AHSV55, pUC57AHSV57, pUC57AHSV58 and pUC57AHSV59 were cut with EcoR1 and Sal1 to either linearize the vector or ligate an insert that contained the complete segment. The reaction conditions consisted of 1 x Cutsmart buffer (NEB), 100 U EcoR1 (NEB), 100 U Sal1 (NEB) and 100 μg of plasmid. The samples were incubated at 37 ℃ for 1 h and run on a 1% agarose gel in TAE buffer.

2.11.2 Gel purification of vector and insert

The DNA bands of the correct size were excised from the agarose gels using a sterile scalpel blade and the DNA extracted using the QIAquick Gel Extraction Kit (QIAGEN). The quantity of the DNA was measured using a NanoDrop (Thermo).

2.11.3 Ligation

Restriction digested PCR-generated products and full-length AHSV segments digested from the pUC57 vector were directly ligated into the prepared linear pCI vector with compatible ends. Ligation reactions were performed using the Rapid DNA ligation kit (Roche) using vector to insert molar ratios of 1:5 or 1:10 and incubated at room temperature overnight.

2.12 Mutagenesis of the NS4 coding sequence in segment 9

Mutations were made in either the coding regions of NS4-Ilβ or γ to identify the determinants of protein shutdown. Mutations were introduced in a pCI plasmid expressing wild type AHSV NS4 gene by site-directed mutagenesis (Table 2-2). Sets of overlapping or complementary primers were designed that would allow the introduction of the mutations into segment 9 to stop expression of NS4 (See Table X). The mutagenesis reaction consisted of 1 x reaction buffer, 15 pmol of each primer, 0.2 mM dNTP, 2.5 U PfuUltra HF DNA polymerase and 50-100 ng of plasmid DNA. To ensure that no other mutations were introduced the number of cycles was
kept to a minimum. The reaction conditions were as follows: one cycle at 95 °C for 30 s followed by 15 cycles at 95 °C for 30 s, 55 °C for 1 min and 68 °C for 4 min.

The parental DNA plasmid was then digested with Dpn1, which only recognises methylated DNA rather than DNA generated by PCR. Chemically competent XL-1Blue E.coli was transformed with 1 ul of the Dpn1-treated samples according to section 0.

Table 2-2. Primers used to truncate NS4-IIβ and γ to ensure that they only express the conserved core/central domain of NS4-II

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Pair</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal deletion of NS4-IIγ</td>
<td>F-CGAGAATTTCAGGGCTGGGGAGAAGAAGAACAAG</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td>R-CTTGTCTTTCTTCTCCCCAGACTGAATTCTCG</td>
<td>69.5</td>
</tr>
<tr>
<td>C-terminal deletion of NS4-IIβ</td>
<td>F-GAACCATTGGATAGGATAGGCCTGCAGC</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>R-GCTGCAGCCGCCTATCCTATCCAATGGTTC</td>
<td>70.9</td>
</tr>
</tbody>
</table>

2.13 Quantification of nucleic acids

The concentration of DNA, ssRNA, dsRNA samples were measured in Nanodrop 2000 (Thermo Fisher).

Qualification of ssRNA samples (described in section 2.18) transcription was tested in Agilent 2100 Bioanalyzer with chip supplied. Gel and RNA dye were equilibrated in room temperature and mixed well, and were loaded into the chip by plunger supplied. Then load another 9 ul of gel into each well of the chip. The dsRNA samples were diluted ~1 ng/ul. 5 ul of the RNA ladder and 9 ul of dsRNA samples were loaded
in each well. The chip was vortexed for 1 min at 2,400 rpm then analysed by an Agilent 2100 Bioanalyzer following manufacturer’s instructions.

2.13.1 Sequence analyses

The sequencing of DNA (PCR products or/and plasmid constructs) was outsourced to Source Bioscience. The chromatograms were analysed using CLC Genomics Workbench. Miscalled bases were corrected, the ends trimmed and the sequence aligned to reference sequence. The samples were occasionally subjected to blastn on the NCBI website (https://blast.ncbi.nlm.nih.gov/). All edited sequences were analysed using the following programmes: BlastN v2.0 to 2.29 and BlastP v2.0 to 2.2.9 (Altschul, Gish et al. 1990, Gish 1993, Madden, Tatusov et al. 1996, Altschul, Madden et al. 1997, Zhang and Madden 1997, Zhang, Schwartz et al. 2000, Morgulis, Coulouris et al. 2008, Camacho, Coulouris et al. 2009, Boratyn, Schäffer et al. 2012), Translate (CGC Accelrys, Inc.), Flip ORF (FLIP: a Unix C program) and Frames (CGC Accelrys, Inc).

2.14 Transfections

Lipofectamine2000 (Invitrogen), a lipid-based reagent, was used to facilitate the transfection of recombinant plasmids and ssRNA into the different eukaryotic cell types.

A lipid:DNA complex was prepared on the day of transfection by adding 2.5 µl of Lipofectamine Transfection Reagent to 1 µg of DNA or ssRNA in optimum media following the manufacturer’s instructions. Prior to transfection the cells were washed in the appropriate media, the lipofectamine:nucleic acid mix was added drop-wise and incubated at 37 ºC in a 5% CO₂-in-air atmosphere for 2-6 h when the media was replaced and the cells were placed at 37 ºC in a 5% CO₂-in-air atmosphere.

2.15 Luciferase assays

We established a luciferase reporter system to measure the influence of AHSV NS4 on host cell protein synthesis using the change of Firefly luciferase (Fluc) as an
indicator of protein regulation. CPT-Tert cells were co-transfected (see section 2.14) with a 100 ng pGL3Flu, a CMV-driven expression plasmid which expressed firefly luciferase, and 100 ng of pCI expression plasmids for either AHSV NS4-I, NS4-IIα, β or γ or BTV NS4 or BTV NS2 and 300 ng of pCI.empty to normalise the DNA amounts. As a control, 400 ng of pCI.empty were co-transfected with pGL3Fluc. Twenty-two hours post-transfection the cell culture supernatant was aspirated and 100 ul of passive lysis buffer (Promega) was added. Cell monolayers and lysis buffer were incubated at room temperature 15 min on an orbital shaker. The sample was harvested, centrifuged to pellet cellular debris and the supernatant used to assess luciferase activity. Five microliters of the supernatant were added to 50 ul of LARII (Promega), mixed and read immediately on a GLOMAX luminometer (Promega). Data is expressed as relative light units (RLU).

2.16 Apoptosis assay (caspase 3/7 pathway)

To determine if NS4 had a role in inducing apoptosis, we measured the activation of caspase3/7 in transfected cells using the Caspase-Glo 3/7 Assay Systems (Promega). Briefly, cells were transfected as in section 2.14 with the slight modification that the pGL3.CMVFluc plasmid was omitted. In particular, cells were grown in 96-well plates, transfected with 6.67 ng of the vector of interest and 26.67 ng of control pCI or 33.33 ng of pCI-empty. At 3, 6, 9, 12, 15 and 22 h post-transfection the media was aspirated and cells lysed with Passive Lysis Buffer (Promega). After 15 mins, 5 ul of the cell lysate was added to 50 ul of the Caspase-Glo® 3/7 Substrate and incubated at room temperature for of 1 h. The substrate is Caspase-3/7 DEVD-aminoluciferin that is cleaved if active caspase 3 or 7 is present in the substrate and results in a proluminescent that can be measured. The samples were measured using a Promega GLOMAX luminometer.

2.17 Immunofluorescence and confocal microscopy.

Immunofluorescence followed by confocal microscopy was performed on infected and transfected cells to examine the localisation of NS4 and monitor virus replication. Cells were seeded onto coverslips as described in section 2.14. BSR cells were either transfected as in section 2.14 with 100 ng of the indicated NS4
expression plasmid (NS4-I, NS4-IIα, β or γ) or infected with the indicated viruses at an MOI of 0.1. Cells were fixed with 4% formaldehyde for 20-30 min. 24 h post-transfection or infection.

Cells were permeabilized with 1% (v/v) Triton X100 in PBS for 15 mins, blocked in 0.4% fish gelatine, in PBST (PBS and 0.1% Tween 20) for 10 min followed by a second block in 2.5% goat sera in PBST for 10 min. These steps were performed at room temperature on an orbital shaker. The primary antibody was diluted in 2.5% goat sera in PBST and samples were incubated overnight at 4 °C on an orbital shaker. Secondary antibodies conjugated with either Alexa Fluor 488 (Invitrogen, Molecular Probes) or Alexa Fluor 594 (Invitrogen, Molecular Probes) were diluted in 2.5% goat sera in PBST and incubated for 1 h at room temperature in a dark environment. The sample was washed 5 times in PBST and once in PBS. Slides were mounted using VECTASHIELD Mounting Medium with DAPI (4′, 6-diamidino-2-phenylindole, Vector Laboratories). Slides were analysed using a Leica GMIR2 confocal microscope.

2.18 Western blotting.

Expression of viral and cellular proteins was monitored by western immunoblot. Briefly, cells were lysed with Laemmli Buffer and proteins separated in pre-cast 4%-12% Tris-gel (NuPage, Thermo) in MES buffer at 200 V for 60 min. Proteins were transferred to a 0.45 µm nitrocellulose membrane (Thermo) or PDVF (Amersham/GE Healthcare) using a mini Trans-Blot apparatus (BioRad) in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol). Transfer was performed at at 150 V for 30 min at room temperature for each apparatus, respectively.

The membranes were blocked in 5% skimmed milk in PBST for a minimum of 1 h at room temperature on an orbital shaker. Primary antibodies were diluted in 5% skimmed milk in PBST and incubated at 4 °C overnight on an orbital shaker. Membranes were washed 5 times in excess PBST and peroxidase-labelled secondary antibodies were added and incubated for 1 h at room tempereate. The signal was visualised using a ChemiDoc XRS+ scanner (Biorad).
2.19 *In vitro* transcription

Plasmids containing the AHSV genome segments were linearized at the last nucleotide of the 3'UTR of the segment (see section 2.11 for specific restriction endonuclease) using restriction enzymes. Linearized plasmids were purified using phenol-chloroform.

Linearized plasmids were used as template of *in vitro* transcription using mMESSAGE mMACHINE T7 ultra kit (Ambion) as per manufacturer’s protocol. The ARCA-capped ssRNAs were then extracted by phenol-chloroform extraction and then excess dNTPs and ARCA cap were removed from the ssRNA by size exclusion through Illustra Micropin G25 columns (GE Healthcare). The quality of the RNA was assessed by electrophoresis analysis (as described in section 2.10) and Bioanalyzer (Agilent).

2.20 Rescue of AHSV-4 and AHSV reassortants

AHSV-4 and reassortants containing segment 9 (S9) from different strains of AHSV and the backbone of AHSV-4 were rescued by reverse genetics as previously described for BTV and AHSV (Boyce, Celma et al. 2008, Matsuo, Celma et al. 2010, Ratinier, Caporale et al. 2011, Van Rijn, Van de Water et al. 2016). BSR cells at a confluency of 70% to 90% were co-transfected with lipofectamine 2000 as described in section 2.14 with 150 ng of each of the six mammalian expression plasmids for AHSV segments 1, 3, 4, 5, 8 and 9. At 18h post-transfection, cells were co-transfected again with $1 \times 10^{11}$ molecules of each of the 10 AHSV ssRNA segments made *in vitro*. Three to four h post-transfection, the medium was replaced with fresh DMEM containing 5% FBS. Cells were monitored daily and supernatants were collected when CPE was evident.

The reference strain of AHSV-4 (GenBank accession numbers JQ796724-JQ796733) was used as a parental virus for the segment 9 reassortants described below. The S9 of the reference strain of AHSV-4 encodes an NS4 “type I” (NS4-I) and therefore, the resultant virus is referred to as AHSV NS4-I. Three additional reassortants were also rescued that contain nine genomic segments (the “backbone”) of the AHSV-4 reference strain and S9 derived from three different viral strains and are referred
to as: AHSV NS4-IIα, AHSVNS4-IIβ and AHSV4NS4-IIγ. NS4-deletion mutants are referred to as AHSV4ΔNS4-I, AHSV4ΔNS4-IIα, AHSV4ΔNS4-IIβ and AHSV4ΔNS4-IIγ.

2.21 dsRNA extraction

dsRNA was extracted from infected cell as already described (Boyce and Roy 2007). Infected cell pellets (either BSR, Kc, E. Derm or horse aorta endothelial cells) were resuspended in Trizol (Invitrogen) and processed according the manufacturer’s protocol. Lithium chloride was added to the total RNA to a final concentration of 2 M and incubated overnight at 4 °C to precipitate ssRNA. Viral dsRNA was precipitated using 100% isopropanol in the presence of sodium acetate, washed twice in 70% (v/v) ethanol and resuspended in RNase free water.

2.22 RT-PCR of AHSV segment 9

All reactions were performed using the SuperScript III reverse transcription kit (Life Technologies) according to the manufacturer’s protocol. The RT step was performed using AHSV segment 9 UTR specific primers (Table 2-3). Each reaction consisted of 1 x FS RT Buffer, 1 mM of each dNTP, 18–25 pmol of primer, 200 U of SuperScript® III RT, 10 mM DDT, 40 U of RNaseOUT and ~1 µg of dsRNA. Each RT reaction was performed in duplicate. In one reaction, SuperScript® III RT was eliminated to determine whether there was DNA contamination of the RNA samples. The RT reaction conditions were slightly modified from the manufacturer’s recommendation to include a step to denature the dsRNA. The conditions were as follows: the dRNA, primers and dNTPs were heated on ice 95 °C for 5 min and placed on ice for 2-3 min. The first strand buffer, enzyme and RNase inhibitor were added and the reaction incubated at 50 °C for 1 h, the RT enzyme was denatured at 85 °C for 15 min and the reaction was placed at 4 °C or at -80 °C until the PCR reaction was performed.

The PCR reaction mix consisted of 1 x PfuUltra II Reaction Buffer (final concentration of 2 mM MgCl₂), 0.25 mM dNTPs, 20 pmol of each primer, 1ul of PfuUltra II fusion HS DNA polymerase and 50-250 ng of vector. Cycle conditions consisted of an initial denaturation of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s and a final extension at 72 °C for 3-5 mins.
Table 2-3 Primers for the RT and PCR amplification of AHSV segment 9.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ - 3’)</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>As9_RT_Seq_F</td>
<td>GTTGTCATCTGCCG</td>
<td>53.6</td>
</tr>
<tr>
<td>As9_RT_Seq_R</td>
<td>CGTTTACCCTGGACC</td>
<td>52.3</td>
</tr>
</tbody>
</table>

2.23 Antiviral cytokines protection assays

To determine whether AHSV infection of primary cell stimulated the production of IFN, the amount of IFN present in the media of infected cells was estimated using an IFN protection assay. Primary horse aorta endothelial cells were infected with an MOI equivalent of 2 for 1.5 h after which cells were washed twice in warm medium and fresh medium added. The supernatants were harvested 16 h post infection and stored at -80 °C. Live viruses were inactivated by UV light exposure (20 mins). One hundred microliters of two-fold serial dilutions of UV-treated primary horse aorta endothelial supernatants were added to CPT-Tert cells (an indicator cells line for equine IFN) and incubated for 24 h at 37 °C in a 5% CO2 humidified environment. The culture medium was aspirated and cells infected with EMCV. Cells were monitored for CPE for 48-72 h. The cells were then fixed in 4% formaldehyde and stained with 0.15% crystal violet to assess the integrity of the monolayer. A well was considered protected if greater than 75% of the monolayer was intact. As a control, commercially available equine interferon (eIFN, obtained from Kingisher Biotech) was used and as a calibrator, CPT-Tert cells were treated with 100 pg/ul of eIFN which was also two-fold serially diluted and infected with EMCV.

2.24 In vivo pathogenicity studies

All animal experimental procedures carried out in this study were approved by the ethical committee of the Istituto Zooprofilattico Sperimentale dell’Abruzzo e Molise “G. Caporale” (Teramo, Italy) (protocol no. 14040/2012) and further approved by the Italian Ministry of Health (Ministero della Salute) in accordance with Council Directive 131 86/609/EEC of the European Union and the Italian D.lgs 116/92. Animal
experiments were performed as previously described (Ratinier, Caporale et al. 2011). Litters of 4-day-old Swiss-NIH mice were infected intracranially with either 200 PFU of the indicated virus or mock-infected with cell culture media. Mice were examined for clinical signs daily until the experiment was terminated 14 days p.i. Survival plots were constructed using data collected from 9 experimental groups (n = 11-16 per each group).
Chapter 3. Identification of the AHSV NS4 protein
3. Identification of the AHSV NS4 protein

3.1 Introduction

In the last few years, a body of evidence has accumulated on the roles that “small” ORFs play in key biological processes in various genomes from viruses and bacteria to vertebrates and humans (Bazzini, Johnstone et al. 2014, Storz, Wolf et al. 2014) (Jaber and Yuan 2013, Slavoff, Mitchell et al. 2013). It is also reported that mitochondria in eukaryotes also contain overlapping ORFs (Clayton 1984, Fearnley and Walker 1986). For viruses, overlapping ORFs are a common feature and are assumed to be a strategy for viral genome compression, which allows the viruses to increase the potential protein repertoire without enlarging their genome size (Chirico, Vianelli et al. 2010). For example, in hepatitis B virus, a dsDNA virus within the Hepadnaviridae, over 63% of the length of the viral polymerase ORF harbours alternative overlapping ORFs (Miller, Kaneko et al. 1989). Overlapping ORFs are also found in RNA viruses such as in influenza A virus, whose genome is composed of 8 ssRNA segments and overlapping ORFs are found in segment 2 (Wise, Foeglein et al. 2009). Overlapping ORFs may originate in different ways, including loss of the initiation or stop codons and thus extending the ORF into contiguous areas downstream of upstream of the gene. Alternatively, point mutations inside or outside the original ORF may generate novel initiation or stop codons inside or outside the original ORF (Krakauer 2000). Viruses mutate very rapidly providing plenty opportunities for the generation of overlapping ORF that in turn can provide potential benefits to virus replication and/or in counteracting the in viral genome transcription and translation regulation and give advantages in virus evolution (Johnson and Chisholm 2004, Saha, Podder et al. 2016).

Viruses within the genus Orbivirus, including BTV, AHSV, GIV (Great Island virus) and EEV (Equine encephalosis virus) are composed of 10 segments of linear dsRNA. Until recently, it was thought that the 10 segments of dsRNA genome of the orbiviruses BTV and AHSV were monocistronic and encoded 7 structural, VP1 to VP7, and 3 non-structural proteins, NS1 to NS3 (Sangar and Mertens 1983, Mertens, Brown et al. 1984). These proteins were identified in BTV-infected cells or transfection of viral
genome RNA. However, older studies reported that when the BTV genome was translated \textit{in vitro}, some unknown small translation products were observed, but these were believed by-products of the \textit{in vitro} translation assays (Sangar and Mertens 1983).

However, in 2008 \textit{in silico} analysis of BTV and other orbiviruses indicated a putative short overlapping ORF of 77-79 amino acid residues in segment 9 of the genome (Firth 2008). Bioinformatics analysis indicated that this small ORF had a strong Kozak sequence in every strain identified and the amino acid sequence was conserved. Other orbiviruses, including AHSV, GIV, PALV (Palyam virus) and YUOV (Yunnan orbivirus) were also shown to contain a similar small ORF encoding 83 to 169 amino acid residues. This protein was named NS4.

The BTV and GIV NS4 were shown (Belhouchet, Jaafar et al. 2011) to localise to the nucleoli in BSR cells (Belhouchet, Jaafar et al. 2011, Ratinier, Caporale et al. 2011). In addition, they possess dsRNA and dsDNA binding activities. The BTV NS4 protein was also shown (Belhouchet, Jaafar et al. 2011, Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016) to repress host cell protein synthesis and act as an interferon antagonist (Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016). \textit{In vivo} experiments also showed that BTV NS4 is a determinant of virulence (Ratinier, Shaw et al. 2016). During the course of this PhD, a preliminary study focusing on the AHSV NS4 was published (Zwart, Potgieter et al. 2015). AHSV NS4 was found to be expressed in virus-infected cells (Zwart, Potgieter et al. 2015) and suggested that AHSV NS4 also localised to the nucleolus and possessed dsDNA (but not dsRNA) binding activity (Zwart, Potgieter et al. 2015). However, the function(s) of the AHSV NS4 were not explored.

In this chapter, we aim to confirm published data on AHSV NS4 and begin to understand some of the biological properties of this protein. In particular, we will:

a. Confirm and identify the presence of the NS4 ORF in AHSV segment 9 using bioinformatics approaches.

b. Confirm the expression and localisation of the AHSV NS4 in transfected an infected cells.
c. Investigate the effects of the AHSV NS4 protein on cell protein expression and apoptosis.
3.2 Results

3.2.1 Identification of alternative ORFs in AHSV segment 9

Our initial *in silico* analysis of 38 available sequences from GenBank (24/07/2014) confirmed a previous study (Zwart, Potgieter et al. 2015) showing that the AHSV segment 9 contained an extra ORF with respect to the ORF expressing the minor structural and helicase protein, VP6. All of these sequences were analysed to predict the +1 ORF within the sequences.

Similar to previous observations (Zwart, Potgieter et al. 2015), AHSV NS4 could be split phylogenetically into two separate clades, NS4-I and NS4-II (Figure 3.1). This situation is different from BTV, where only one NS4 type exists (Firth 2008, Ratinier, Caporale et al. 2011). Clustal X analysis of the putative ORF for NS4 supported Zwart, L., et al. finding that there were two distinct groups of NS4 (Zwart, Potgieter et al. 2015). Further, our analysis identified three different subtypes in the NS4-II clade (Figure 3.1B), which we named NS4-IIα, β and γ. The position of the start and stop codons differs among the four groups of NS4 resulting in proteins of different length. The NS4 ORFs starts at either nt 214, 148 or 193 of the NS4 ORF and terminates at nt 648, 657 or 576, encoding proteins of 144, 169, 154 or 143 amino acid residues (Figure 3.1). Unlike the BTV NS4 protein, which is highly conserved, the similarity at the amino acid level of the NS4 among different AHSV strains is relatively low and ranging between 45.6 and 89.4.
Figure 3.1 Schematic representation of AHSV segment 9 ORFs. A. Identification of AHSV NS4-I ORF. A 144 aa ORF was detected in the +1 ORF starting at nt 214. B. Identification of NS4-II ORF. Three different forms of NS4-II were identified in the +1 ORF including: NS4-IIα, -β and -γ. NS4-IIβ and γ show either an N- or C-terminal truncation compared to NS4-IIα respectively but they share either the same start or stop codon position.

Additional AHSV genomic sequences were made available in GenBank in August 2016. Analysis of these sequences further supported our initial findings. Interestingly, NS4-II represented approximately two thirds of more than 200 AHSV segment 9 sequences; NS4-IIβ represented more than 90% of the NS4-II type.

The amino acid similarity of NS4 among the NS4-I (Zwart, Potgieter et al. 2015) clade is between 94.5 to 100%; whereas, those sequences which belonged to clade II (NS4-II) show 84.7 to 100% similarity. We observed N- and/or C- terminal truncations in different sequences of the NS4-II group. Based on these truncations, NS4-II can be further divided (see Figure 3.1B) into three different types (NS4-IIα, β and γ). NS4-IIα full-length sequence is 169 amino acid residues in length, while NS4-IIβ protein has an N-terminal truncation of 15 amino acid residues and NS4-IIγ has a C-terminus truncation of 26 amino acid residues (Figure 3.2). There are two very well conserved
domains in NS4-I and NS4-II between residues 51-70 and 90-143 (in relation to sequence JQ742012) (Figure 3.2). In addition, a potential leucine zipper domain is present between amino acid residues 92 and 115 of the consensus sequence. We found a few NS4 sequences (6 out of the 207 available in GenBank) presenting premature stop codons and thus resulting in truncations of the resulting proteins. All the viruses with truncated NS4 proteins were either passaged in cell culture or in mice. Hence, the biological significance of this truncation is not known.

Figure 3.2. Alignment of representative aa sequences of the four identified NS4 proteins. Compared to NS4-IIα, NS4-IIβ had a 15 aa truncation at the N-terminus while NS4-IIγ had a 26 aa truncation at the C-terminus. The aa similarity of NS4-I and NS4-II ranged between 45.6% and 59.5%. Two conserved regions were identified from aa 51 to 70 and from aa 90 to 143.

Phylogenetic analysis of the available AHSV Seg-1, -3, -9 and -10 sequences (carried out by Joseph Hughes, using RAxML), revealed that there has been significant reassortment within the AHSV genome. Interestingly, the multiple truncations that occurred within the NS4-II clade were not linked to the lineages of the other proteins (Figure 3.3). It is possible that these NS4-II truncations have occurred due multiple separate events although this is not possible to determine based on the current analysis.
3.2.2 Expression of AHSV NS4 in vitro and in vivo

Specific antisera against the AHSV NS4 protein were produced commercially (Genscript) in order to verify that all four NS4 proteins were expressed in either transfected or infected cells. Due to the high level of sequence diversity observed between the two NS4 clades, two rabbit polyclonal antisera were generated against either full-length NS4-I or NS4-IIβ proteins. To verify that the antisera recognized specifically all forms of AHSV NS4, HEK-293T cells were transfected with a mammalian expression plasmid (pCI) for either NS4-I, NS4-IIα, β or γ. At 24 h post-transfection, whole cell lysates were harvested and analyzed by western blotting. Specific bands were detected in cell lysates collected from transfected cells but not from untransfected controls (Figure 3.4). The apparent size of NS4-I as judged by its mobility in SDS-PAGE was slightly higher than the predicted 16.6 kDa (Figure 3.4, top panel). On the other hand, NS4-IIα and γ migrated to the expected sizes of approximately 20 and 17 kDa, respectively. The apparent size of NS4-IIβ was lower than expected and closer to 16 kDa rather than the predicted 18.15 kDa (Figure 3.4, middle panel). α-tubulin was used as loading control for the western blotting (Figure 3.4, bottom panel).
Figure 3.4. NS4 expression in vitro. HEK-293T cells were transfected with PCI vectors expressing NS4-I, NS4-IIα, β or γ (Lanes 1 to 4), PCI-empty (Lane 5) or only treated with Lipofectamine 2000 (Lane 6). Whole cell lysates were harvested 24 h post-transfection and analyzed by western immunoblot using specific antibodies. NS4-I was detected in the upper panel and specific bands for NS4-IIα, β and γ were detected in the middle panel. There were no specific bands detected in PCI.empty transfected cells with either anti-NS4-I or anti-NS4-II antibodies. α-tubulin was used as loading control.

BSR cells were then infected with the 9 AHSV reference strains (AHSV-1 to -9) in order to ensure that NS4 was expressed in the context of viral infection. Whole cell lysates were harvested 24 h p.i. and analyzed by western immunoblot. NS4-I antisera detected the expression of NS4-I in two of the reference strains, AHSV-2 and AHSV-4, with a band observed at the predicted size of ~16.6 KDa in both (Figure 3.5A). The NS4-II antisera, as expected, detected the expression of the NS-IIα, β or γ forms by different AHSV strains. AHSV-3 and -6 expressed NS4-IIα; AHSV-2, -7 and -9 expressed NS4-IIγ while NS4-IIβ was detected in cells infected with AHSV-1 and -8 (Figure 3.5B).
Figure 3.5. NS4 expression in vivo. A. NS4-I was detected by antisera in AHSV-2 and -4 infected BSR cells, whereas NS4-IIα, β or γ were detected in AHSV-3 and -6, AHSV-1, and -8, and AHSV-2, -7 and -9. AHSV VP7 demonstrated infection and replication of all the viruses in the cell, and α-Tubulin was used as loading control of western blot. B. Summary of NS4 mixed expression in AHSV reference strains.

In some cases, we detected multiple bands for NS4 that were not detected in the lysates of mock-infected cells. These data may suggest either that NS4 undergoes post-translation modifications altering the migration pattern or that different transcripts are generated during infection encoding, for example, proteins with different initiation codons. However, we cannot rule out the possibility that the reference strains that we received could have mixed populations (see Figure 3.5B). For example, lysates obtained from cells infected with AHSV-2, seem to display both NS4-I and NS4-IIγ proteins. In cells infected with AHSV-5, we detected a band corresponding to a truncated NS-4 protein only when immunoblots were overexposed (see faint band in Figure 3.5). By sequencing AHSV-5, we confirmed the presence of
a premature termination codon that would result in a protein of 66 amino acid residues. This is consistent with the analysis described above showing the presence of a few sequences with a truncated NS4 in GenBank (section 3.2.1).

As expected, all 9 different serotypes were positive for VP7 expression while mock-infected cells were not positive for any form of NS4 or VP7. All lanes had equivalent proteins loaded as indicated by the detection of α-tubulin.

3.2.3 AHSV NS4 localises in the cytoplasm of infected cells

Bioinformatics analyses using either cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) or NoD (http://www.compbio.dundee.ac.uk/www-nod/HelpDoc.jsp#PredInfo) were not able to identify any NLS (nuclear localization signal) or NoLS (nucleolar localization signal) in the NS4 sequences available to us. On the other hand, Zwart and colleagues (Zwart, Potgieter et al. 2015) demonstrated by immunofluorescence that both NS4-I and NS4-II localized to the nucleus of cells infected with BSR cells.

We addressed these apparent discrepancies by immunofluorescence and confocal microscopy. Initially, BSR cells were transfected with plasmids expressing either NS4-I, NS4-IIα, β or γ. Twenty-four h post-transfection, cells were fixed and analyzed by confocal microscopy using the antisera generated against NS4-I or NS4-II. Both AHSV NS4 type I and II were diffused in the cytoplasm (Figure 3.6). No NS4 was found in the nucleus or nucleolus. These data was in contrast to the previous study published on AHSV NS4 (Zwart, Potgieter et al. 2015). In addition, the NS4 of the orbiviruses BTV and GIV also showed nucleolar localization (Belhouchet, Jaafar et al. 2011) (Ratinier, Caporale et al. 2011).
Figure 3.6. Immunofluorescence showing NS4 expression in the cytoplasm but not in the cell nucleus BSR cells were transfected with pCI vectors expressing AHSV NS4-I, NS4-IIα, β or γ separately. NS4-I (red) or NS4-II (red) proteins were detected by the corresponding antibody in the cytoplasm. The nuclei were stained with DAPI (blue). pCI-empty plasmid was used as mock transfection.

The same phenomenon was observed in BSR cells infected with the reference strains for either AHSV-4, -6, -1 and -7. These viruses were chosen because they represented the viruses expressing the different types of NS4. Cells were fixed 24 h p.i. and analyzed by immunofluorescence using confocal microscopy and antibodies for NS4 or VP7 (the latter used to confirm virus replication)(Figure 3.7). In every case, we found NS4 to localize in the cytoplasm and not in the nucleus. We also tried to look at the AHSV NS4 localisation in E. Derm cells, however, the results were the same (data not shown).
3.2.4 The effect of the AHSV NS4 on gene expression

In a previous study, it was shown that BTV NS4 has an effect on cellular protein expression (Ratinier, Shaw et al. 2016). Here, we also assessed the effect of AHSV NS4 on gene expression using a luciferase reporter system. CPT-Tert cells were co-transfected with a CMV-driven expression plasmid, which expresses firefly luciferase, and expression plasmids for either AHSV NS4-I, NS4-IIα, β or γ. The effect on luciferase production caused by AHSV NS4 was evaluated by measuring luciferase
signal (Figure 3.8A). The empty pCI plasmid was used to normalise the data (Figure 3.8B). BTV-NS4 was used as positive control while BTV-NS2 as negative control. NS4-IIβ had the greatest effect of luciferase expression and caused ~75% decrease in luciferase expression in comparison to the pCI empty plasmid, similar to what is observed with BTV NS4. In contrast, NS4-IIγ had nearly no effect on luciferase expression, like the negative control BTV NS2. AHSV Type I and IIα NS4 had similar effects on luciferase expression causing an approximately a 25% reduction of the luciferase signal.

Figure 3.8. Role of AHSV NS4 on cellular protein shutdown. 100 ng of each NS4 expression plasmid or pCI.empty plasmid together with 100 ng of CMV-driven firefly luciferase plasmid and 300 ng of pCI.empty plasmid were co-transfected into CPT-Tert cells. Luciferase activity was measured 22 h post-transfection. BTV8-NS4 acted a positive control and BTV10-NS2 was used as a negative control. A. Dot plot showing the RLU from three independent experiments highlighting the reproducibility of the assay and the differences among the different NS4 proteins. NS4-IIβ reduced luciferase expression whereas NS4-IIγ had little effect on luciferase expression. B. Bar plot showing luciferase activity relative to the pCI.empty control that was set at 100%. NS4-I, NS4-IIα, β or γ decreased luciferase expression by ~28%, 24%, 75% and 6% respectively. C. Dose dependent effect of NS4-IIβ and NS4-IIγ on luciferase activity. Decreasing
concentrations of NS4 expression vector were transfected with 100 ng of CMV-driven luciferase into CPT-Tert cells. D. Effect of N- and C- terminal NS4 deletions on luciferase activity. N-terminus NS4-IIβ deletion increased luciferase activity by 43% to 79%, whereas C-terminus NS4-IIγ deletion decreased luciferase activity from ~99% to 83%. Each experiment was performed three times, each time in duplicate.

In order to determine whether the effects on gene expression were dose dependent, different amounts of NS4-IIβ or IIγ expression plasmids were co-transfected with the luciferase reporter vector and the luciferase signals measured at 22 h post transfection. NS4-IIβ inhibited luciferase expression in a clear dose-dependent manner (Figure 3.8C). In contrast, there was no dose effect displayed by NS4-IIγ.

Considering the differences between the β and γ subtypes of AHSV NS4-II, we generated expression plasmids for NS4II-β lacking the C-terminus domain (NS4II-β-ΔC-ter) and NS4-IIγ lacking the N-terminal region of the protein (NS4-IIγ-Nter). When the C-terminus of NS4-IIβ was removed, its ability to reduce the luciferase expression was dramatically lost (Figure 3.8D) from ~40% to 21%; however when the C-terminus of NS4-IIγ was deleted, it had little effect on its ability to reduce luciferase activity.

3.2.5 AHSV NS4-IIβ and -IIγ expression induces apoptosis

We observed that BSR cells either transfected with AHSV NS4-IIβ expression plasmid or infected with AHSV-1 (which contains NS4-IIβ) by immunofluorescence showed morphological hallmarks of cell apoptosis (Figure 3.9). Cells expressing NS4 shrank, the cytoplasm appeared denser and nuclei had an abnormal morphology with obvious blebbing of the cell membrane. On the other hand, cells transfected with an expression plasmid for NS4-IIγ or infected with a virus expressing this protein did not show these characteristics.
Figure 3.9 Immunofluorescence showing morphological changes to BSR cells transiently transfected with expression vectors for NS4-IIβ or NS4-IIγ or infected with AHSV-1 reference strain. NS4-II (red) was detected using a specific antibody while the nuclei were stained with DAPI (blue). Cells expressing NS4-IIβ alone or in the context of virus infection (AHSV-1 reference strain) displayed hallmark signs of apoptosis. The abnormal shaped nucleus and membrane blobbing were not observed in mock transfected or infected cells.

In order to confirm these visual observations, and to verify that indeed transfected/infected cells were apoptotic, we measured the levels of activated caspase3/7 as a marker of apoptosis (Slee, Adrain et al. 2001) in NS4-IIβ or γ transfected cultures using a luciferase assay. Stautosporine was used as positive control, while pCI-empty was used as negative control (Bertrand, Solary et al. 1994).
A caspase-3/7 luciferase kit was used to measure the quantity of caspase-3/7 at 3, 6, 9, 12 and 22 h post-transfection. We detected activated caspase 3/7 in both NS4-IIβ and IIγ transfected cells from 6 h post-transfection (Figure 3.10). Even though there were morphological differences observed by confocal microscopy (Figure 3.9), there were no significant differences in the induction of caspase3/7 between NS4-IIβ and γ transfected cells (Figure 3.10B).

Figure 3.10 NS4-IIβ activation of caspase-3/7. BSR cells were transfected with pCI.NS4-IIβ, pCI.NS4-IIγ, pCI.empty plasmid or treated with staurosporine. Cell lysates were collected 3, 6, 9, 12 and 22 h post transfection. Caspase 3/7 activity was measured by the release of firefly luciferase after specific cleavage of the substrate. NS4-IIβ and NS4-IIγ expression induced the activation of caspase 3/7 overtime. A. RLU of firefly activity at different time points post transfection or after the addition of staurosporin. B. Influence of NS4-IIβ and γ on luciferase
activity. Values relative to the pCI.empty control are plotted. Each experiment was performed three times, each time in duplicate.
3.1 Discussion

This chapter reports that segment 9 from essentially all AHSV strains sequenced to date (with a handful of exceptions) express a NS4 protein. NS4 is encoded by a +1 alternative ORF overlapping the VP6 ORF and as expected follows the VP6 phylogeny. Previous studies revealed that the segment 9 of BTV and other orbiviruses such as ENDV-1, AHSV-3, PHSV, YUOV-1 and GIV is olycistronic (Firth 2008, Belhouchet, Jaafar et al. 2011, Ratinier, Caporale et al. 2011). Although some of the genome segments of the family Reoviridae were also found to be multicistronic (for example Phytoreovirus) (Suzuki, Sugawara et al. 1996), this +1 alternative overlapping ORF in segment 9 was only present in the genus orbivirus (Firth 2008). Although, no one has formally demonstrated that AHSV NS4 is a non-structural protein, due to the parallel drawn with BTV NS4, we and others have made that assumption by adopting the name NS4 (Ratinier, Caporale et al. 2011, Zwart, Potgieter et al. 2015).

Analyzing all the AHSV sequences available in GenBank, two types of NS4 were identified and named NS4-I and NS4-II. About a quarter of the AHSV strains express a NS4-I protein while the remaining possess a NS4-Iiβ protein. Further analysis indicated that NS4-II could be split into three subtypes, α, β and γ based on amino acid sequences and the presence of either N- or C-terminal truncations. Interestingly more than 90% of the NS4-II sequences were of β subtype. Phylogenetic analysis of NS4 suggested that it evolved independently from other segments and as expected was independent from virus serotype.

Reassortment is a common occurrence in the viruses whose genomes are composed of multiple segments, including reoviruses and orthomyxoviruses (Nomikou, Hughes et al. 2015) (Wenske, Chanock et al. 1985, Jones, Davies et al. 1987). AHSV is also shown to reassort in the field (Weyer, Quan et al. 2013). Zebras are considered the natural reservoir of AHSV, and it has been shown that the same animal can harbor different strains/serotypes simultaneously (Barnard 1993), therefore facilitating reassortment and the generation of viruses different from either parental strains. It is interesting to note that we detected multiple specific bands for NS4 in western blotting analysis of cells infected by the different South African strains of AHSV. The data may suggest either that NS4 undergoes post-translation modifications or use
different initiation codons. We cannot rule at this stage that the reference strains that we received could have a mixed populations containing different segment 9. In AHSV reference strain serotype 5, NS4 was truncated and composed of only 66 amino acids. Similar truncations were also observed in 6 of the 207 AHSV segment 9 sequences found in GenBank. However, these AHSV isolates have been passaged in cell culture (including Vero cells, BHK cells and BSR cells) and/or in mice over 5 times and may therefore simply represent cell-adapted strains.

Amino acid sequence analysis suggested that AHSV NS4 contains a putative leucine zipper-like domain at the C-terminus of the protein. This domain is also found in BTV NS4 (Ratinier, Caporale et al. 2011) and it is similar to domains found in the transcription factors of the bZip family (Vinson, Myakishev et al. 2002). Interestingly, a previous study suggested that AHSV NS4 binds dsDNA but not dsRNA (Zwart, Potgieter et al. 2015), whereas BTV NS4 has been found to bind both dsRNA and dsDNA (Belhouchet, Jaafar et al. 2011). Although it is hypothesized that the leucine zipper was involved in DNA binding, here we observed that the AHSV NS4 localizes in cytoplasm and not in the nuclei of infected cells. Thus, it is not immediately clear whether the DNA binding motif could actually function in the host genomic DNA.

BTV NS4 was shown to affect host protein synthesis. Here we observed using luciferase assays that the AHSV NS4 protein inhibits gene expression. The C-terminal domain of this protein is presumed to be important for this function. In comparison to other forms of AHSV NS4, AHSV NS4-IIγ has only a weak effect on gene expression and lacks the C-terminal 26 amino acid residues where the putative leucine zipper is located. NS4-IIβ lacks the N-terminal 15 amino acid residues but appeared to have the strongest inhibitory activity on cellular gene expression. It is not clear at this stage how AHSV NS4 blocks gene expression and if this effect can also be seen in the context of virus infection. The BTV NS4 protein appears to modulate expression of a variety of promoters including IFNβ and Interferon-stimulated response elements (ISREs). For these reasons, the BTV NS4 is considered an interferon antagonist (Ratinier, Shaw et al. 2016)

However, in our experiments the AHSV NS4 protein localizes to the cytoplasm, rather than the nucleus/nucleolus as BTV NS4 (Ratinier, Caporale et al. 2011, Zwart,
Potgieter et al. 2015). Indeed, we found no nuclear or nucleolar localization signals in any of the AHSV NS4 sequences. These data are in disagreement with a previous study where the authors found AHSV NS4 to localize in the nucleolus. We noticed that Zwart et al. generated antisera against NS4-I or NS4-II using the peptides QGAGLEGEEWAEWL or MIEEWRARNLREAD (Zwart, Potgieter et al. 2015). With these particular peptides we found homology (Using ProteinBlast in NCBI) to other cellular proteins including the nuclear transport factor 2 family protein and serine/threonine-protein phosphatase. Therefore, the antisera generated in that study could be detecting host rather than viral proteins.

In summary, in this chapter, we have identified four different forms of NS4 that are present in the AHSV genomes available in GenBank. Importantly, we found the AHSV NS4 protein to be expressed in infected cells. All the four forms of the AHSV NS4 proteins localized to the cytoplasm of infected cells. In the following chapters we will further characterize the function of this protein.
Chapter 4. Establishment of a reverse genetics system for AHSV
4. Establishment of a reverse genetics system for AHSV

4.1 Introduction.

The data generated in chapter 3 supported the previously published data from Firth, et al. and Zwart, et al. that an alternative ORF in segment 9 of AHSV encodes a small non-structural protein (termed NS4) (Firth 2008, Zwart, Potgieter et al. 2015). Furthermore, we showed that there are phylogenetically distinct clades of NS4 (I and II) and one of these clades can be further subdivided into three distinct groups on the bases of their initiation and stop codons. We showed that NS4 is expressed by all AHSV strains. Therefore, in order to systematically determine and study the function(s) of NS4 in the context of the viral life cycle, we set up to use a reverse genetic system in order to introduce modifications into the viral genome and obtain NS4 deletion mutants.

Reverse genetics systems (RG) have been used widely to study the role of viral proteins during virus replication. The first report of cDNA being used to recover infectious viral progeny was by Racaniello et al. for the rescue of poliovirus (Racaniello and Baltimore 1981). RG allowed the introductions of mutations into specific domains of viral proteins or into RNA structures important for the control of virus replication. Since this discovery, numerous reverse genetic systems have been developed to study a variety of RNA and DNA viruses. For example, the use of infectious molecular clones for HIV allowed to understand the biological function of many of its accessory proteins (Freed 2001).

The development of a RG system for dsRNA segmented viruses occurred relatively recently. A major breakthrough occurred after it was shown that reovirus serotype 3 (ST3) ssRNA generated in vitro using purified core particles was infectious after transfection into mammalian cells (Roner, Sutphin et al. 1990). BTV, the representative virus of genus Orbivirus, was first rescued in 2008 (Boyce, Celma et al. 2008) and since this date, there have been numerous systems developed including the use of helper viruses, plasmid only, two-step protocols with either ssRNA, DNA +ssRNA or DNA (Roner and Joklik 2001, Kobayashi, Antar et al. 2007, Matsuo and Roy 2009, Kaname, Celma et al. 2013, Conradie, Stassen et al. 2016). To date, the BTV
RG system has been used to answer key questions on the roles of structural proteins (VP2, VP4) and two of the non-structural proteins (NS3 and NS4). Advances in the BTV RG system allowed the application of this technology also to AHSV. In 2013, Kaname et al. (Kaname, Celma et al. 2013) published the recovery of AHSV using a similar method that had been used for BTV. Since then, Van de Water et al. (Van de Water, van Gennip et al. 2015) have published further refinements on the methods used to rescue AHSV while more recently, a plasmid-only method was successfully developed (Conradie, Stassen et al. 2016).

The development of a successful AHSV RG system will provide a platform on which to build a better understanding of the role of NS4 on virus biology. It could be used to address key questions on its function and determine if there is functional homology with the related orbivirus NS4 proteins.

This chapter aims to develop a RG system based on the AHSV-4 serotype. This technology will allow the rescue of virus reassortants containing genome segment 9 from other AHSV strains in order to develop all the reagents necessary to study the different types of NS4 proteins and corresponding deletion mutants.
4.2 Results

4.2.1 Establishment of a reverse genetic system for AHSV.

In order to establish a RG system for AHSV, the sequence of the AHSV-4 reference strain was downloaded from GenBank (accession No. JQ796724 to JQ796733), cDNA copies of each genome segment were synthesized commercially (Genscript) and inserted into plasmids that were designed similarly to what was already described (Boyce, Celma et al. 2008, Kaname, Celma et al. 2013). Briefly, the cDNA copy of the genome segment, including the untranslated region (UTR) and coding region, was placed downstream a T7 promoter (TAATACGACTCACTATAG) and unique restriction endonuclease cutting sites at the 5’ and 3’ end extremities. This design enables the generation of transcripts mimicking the core transcripts to be generated by in vitro synthesis. cDNA sequences for each segment (with the exception of segment 3) were cloned into pUC57 (Figure 4.1A), a high copy plasmid as it contains a pMB1 origin of replication. Segment 3 was instead cloned into pJet1.2, a low copy plasmid.

![Figure 4.1 Schematic representation of the constructs used to generate viral mRNA (A) and to express viral proteins (B). A. An exact copy of the full-length AHSV-4 segment 9 was cloned into the pUC57 vector flanked by a T7 promoter at the 5’ end and a restriction endonuclease site (Bsal) at 3’ end in order to create a correct 3’ end of the transcript. B. The full-length AHSV-4 segment 9 was cloned into pCI that contains a CMV promoter and CMV enhancer at 5’ end.](image-url)
4.2.2 In vitro transcription

The quality of ssRNA generated from the plasmid during in vitro transcription is critical for the rescue of an infectious virus. The ssRNA needs to be an exact copy of the AHSV viral genome, as these RNA segments are then used as the templates to synthesize the genomic dsRNA. In order to prepare the genome ssRNA for reverse genetic, plasmids containing the genomic segments of AHSV-4 were cut and linearized with the designed restriction endonuclease and then purified by phenol-chloroform. Viral genome ssRNAs were in vitro transcribed, using purified plasmids as templates, by mMESSAGE mMACHINE T7 Ultra Kit (Ambion), purified initially by phenol-chloroform extraction and then through an Illustra Microspin G25 column.

The length and quality of the synthesized ssRNA was analyzed by a Bioanalyzer Instrument 2100 (Agilent) following the manufacturer’s instructions (Figure 4.2). The output from the Bioanalyzer demonstrated that the ssRNA generated was of a high quality and of the expected size. In each picture, the smaller peak of 25 nt indicated the dye of the ssRNA in the loading buffer, and the following larger peak showed the length of each ssRNA segment. The last lane showed the ladder (Figure 4.2A). The width of each peak suggested the integrity of the ssRNA. Briefly, the sharper the peak, the more integrity of each ssRNA segment. The width of segment 1 peak suggested that some of the ssRNA molecules were degraded, however, most of the ssRNA segments were intact. In order to provide a better view of each RNA segment, the Bioanalyzer also performs simulated gel electrophoresis run in the presence of a ladder (Figure 4.2B). This mimics the ssRNA migration in 1% TAE agarose gel electrophoresis. Samples were also run in a 1.5% TAE agarose gel electrophoresis, and the result was the same as the simulated migration results (data not shown).
4.2.3 Rescue of AHSV-4 by reverse genetics.

The ssRNA was of sufficient quality to be used to test a two-step AHSV RG system (Matsuo, Celma et al. 2010). However, the initial experiments using this protocol either failed or the rescue efficiency was very low. Thus, the method described by Kaname et al. and Van de Water et al. (Kaname, Celma et al. 2013, Van de Water, van Gennip et al. 2015) was subsequently followed. BSR cells were initially transfected with expression plasmids encoding AHSV-4 VP1, VP3, VP4, VP6, NS1 and NS2 (100 ng each). For this scope cDNAs of AHSV4 segment 1, 3, 4, 5, 8 and 9 were cloned into the pCI plasmid, which contains the CMV promoter (Figure 4.1B). The transfection of these plasmids enabled the expression of the transcription complex.
and the formation of inclusion bodies prior to transfection of $10^{11}$ molecules of ssRNA corresponding to each of the AHSV-4 10 genomic segments (see Figure 4.3 for a diagram of the method). To ensure that the protocol was optimized for the recovery of orbiviruses, BTV-1 was used as positive control throughout the RG process (Ratinier, Caporale et al. 2011). Negative controls, included cells transfected with ssRNA corresponding to 9 of the 10 AHSV-4 genomic segments and mock-transfected cells. Cytopathic effect in BSR cells was visible after 72 hours in cells transfected with AHSV-4 10 ssRNAs, but not in the negative controls. dsRNA was then extracted from the BSR cells were AHSV-4 was rescued (termed from now on “rgAHSV-4” for RG AHSV-4) and from BSR cells infected with AHSV-4 used as control. Results showed all 10 segments of rgAHSV-4 were present and the migration pattern was the same as the AHSV-4 reference strain (Figure 4.4).

![Diagram](image)

Figure 4.3 Schematic representation of the 2-step reverse genetic protocol for the recovery of AHSV. Plasmids expressing VP1, VP3, VP4, VP6, NS1 and NS2 were transfected into BSR cells. Eighteen 18 h post-transfection, $10^{11}$ molecules of each of the 10 AHSV T7-generated ssRNA were further transfected. Cells were monitored for CPE or morphological changes for a minimum of 48–72 h.
Figure 4.4 Migration pattern of genomic dsRNA extracted from rescued AHSV-4 in comparison to dsRNA extracted from the AHSV-4 reference strain. The migration of the segments was the same between the rescued and reference strain AHSV-4 viruses. Rescued BTV-1 dsRNA was used as a control.

4.2.4 Recovery of AHSV segment 9 reassortants and NS4 deletion mutants.

Next, we generated AHSV-4 reassortants with segment 9 from either AHSV-7 (JQ742012), AHSV-1 (AM883170) or AHSV-6 segment 9 (AHU33000) in order to have viruses within the same genetic background expressing NS4-IIα, β and γ respectively. rgAHSV-4 instead represented a virus expressing NS4-I. The reassortants are named from now on rgAHSV4-NS4-I, -NS4-IIα, -NS4-IIβ and -NS4-IIγ. In addition, we
generated for both the “wild type” rgAHSV-4 and the segment 9 reassortants described above, NS4 deletion mutants. To generate AHSV NS4 deletion mutants, extra care was taken to ensure that coding region of the overlapping VP6 reading frame was not disrupted. The start codon of the NS4 ORF and following downstream ATG codons were mutated and several stop codons were introduced inside the NS4 ORF without changing any aa residue of the AHSV VP6 protein (Figure 4.5). The sequences of all the viruses rescued by reverse genetics were verified by Sanger sequencing. Note that the aa similarity among the VP6 proteins encoded by the AHSV strains encoding NS4-II proteins is between 93.8% to 96.5%. While the VP6 of AHSV-4 (expressing NS-I) is approximately 63% similar to the type II proteins.

4.2.5 NS4 is not essential for AHSV-4 replication

The validation of an AHSV RG system using wt virus and the generation of constructs that would enable recovered viruses to express the different forms of NS4 and their respective deletion mutants allow us to test whether NS4 was essential for virus replication. Previous studies showed that the BTV NS4 was not required for the efficient rescue of BTVs (Ratinier, Caporale et al. 2011). Indeed, wt AHSV-4 expressing NS4-I, NS4-IIα, -IIβ or -IIγ, and the corresponding ∆NS4 viruses were all
successfully rescued. These data indicated that similarly to BTV, AHSV NS4 is not essential for replication, at least in BSR cells.

Next we assessed the replication kinetics of all the rescued viruses and their ability to induce plaques in BSR cells. We also analyzed the genomic dsRNA profile and protein expression. All rescued viruses were passaged once in BSR cells and then plaque assays were carried out also in BSR cells. Plaque size and morphology was examined 72 h p.i. by staining the monolayer with crystal violet. All recovered viruses formed plaques in BSR cells (Figure 4.6) and there was no obvious difference in the plaques regardless of whether it was the wt or corresponding ΔNS4 recovered virus.

![Figure 4.6. Plaque morphology of AHSV expressing NS4-I, NS4-IIα, NS4-IIβ or NS4-IIγ and the respective NS4 deletion viruses. BSR cells were infected and overlaid with 1.5 % low melting point agarose for 72 h when monolayers were fixed and stained with crystal violet. The top panel shows the plaques formed by AHSV-4 NS4-I, -IIα, -IIβ or -IIγ, whereas in the bottom panel are the plaques from the corresponding ΔNS4 viruses. The shape and size of the plaques are comparable between wt and ΔNS4 viruses.](image)

In addition, analysis of dsRNA from all the reassortants and NS4 deletion mutants did not reveal any difference with wt AHSV. (Figure 4.7). In addition, to ensure that the segment 9 mutations had been maintained after rescue, we amplified the VP6 ORF (which includes also the NS4 ORF) by RT-PCR from all reassortants and sequenced the resulting products. Analysis of the sequence chromatograms showed that all the
mutations had been maintained after virus recovery. Importantly, no additional mutations were introduced into the coding sequence of VP6 and NS4 ORF.

Figure 4.7 Migration pattern of dsRNA of rescued AHSV viruses. The migration pattern of the genomic segments was the same between the rescued wt and the ΔNS4 AHSV-4 viruses.

Next, BSR cells were infected and after 24 h cell lysates were harvested and analyzed by western blotting in order to ensure either NS4 expression (or lack of) from wt and corresponding NS4 deletion mutants. For all of the ΔNS4 viruses, no NS4 expression was detected (Figure 4.8). This indicated that the mutations introduced into segment 9 stopped NS4 expression as expected. On the other hand, AHSV-4 wt and the segment 9 reassortant viruses expressed the various types of NS4 (Figure 4.8, top panel). Of note, the relative expression of NS4-IIα appeared to be higher than that of the other either types of NS4. The NS4-I polyclonal antisera detected both NS4-I and NS4-IIα, likely because of conserved epitopes between NS4-I and NS4-II (discussed in chapter 3) and the relatively high levels of expression. The monospecific polyclonal NS4-II antisera, as expected, did not detect NS4-I expression.
by western blot. VP7 expression was also assessed in order to control that cells had been infected with replicating viruses (Figure 4.8; third panel) while α-tubulin was used as a loading control.

![Western immunoblot to examine expression of NS4 in wt and mutant viruses. BSR cells were infected at MOI ~0.01 and cell lysates harvested 24 h post infection. The samples were probed with antibodies against NS4-I, NS4-II, VP7 or α-tubulin. Single expression of either AHSV-4 NS4-I, -IIα, -IIβ or -IIγ was detected in the wt viruses but not in the corresponding mutant viruses. Interestingly, NS4-IIα was detected by the anti-NS4-I antibody. Anti-VP7 and α-tubulin were used as controls of viral replication and loading respectively.](image)

4.2.6 Replication kinetics of NS4 reassortants and ΔNS4 viruses in BSR cells.

Thus far, we showed that segment 9 from different strains of AHSV-4 easily reassorted into the AHSV-4 backbone. Furthermore, the successful rescue of the ΔNS4 mutants clearly indicated that NS4 is dispensable for AHSV replication in BSR cells (Kawai, Matsumoto et al. 1975).
Next, we assessed the replication kinetics of segment 9 reassortants and NS4 deletion mutants. BSR cells were infected at an MOI of 0.01, cell-free viruses were harvested at 24, 48 and 72 h p.i. and titrated by standard endpoint dilution assays. In all cases, the presence of NS4 did not affect the replication kinetics of AHSV (Figure 4.9). We noticed however the viruses with segment 9 from AHSV-4 and AHSV-7 reached higher titres than reassortants with segment 9 from AHSV-1 and AHSV-6. These patterns were similar however, in the absence of NS4 this can be attributed likely to differences in the VP6 between different proteins.

![Virus growth kinetics in BSR cells. BSR cells were infected with AHSV-4 NS4-I, NS4-IIα, β, or γ and the corresponding ΔNS4 viruses at MOI 0.01. Cell-free virus was harvested at the indicated time points and the titre was determined by limiting dilution and expressed as TCID\(_{50}\)/ml. The growth kinetics of ΔNS4 viruses (blue) were similar to the related wt viruses (red). Each experiment was performed independently three times using two independently generated virus stocks.](image-url)
4.3 Discussion

When developing the RG system for AHSV, there were a number of different approaches that could have been undertaken. A helper virus could have been used to recover infectious particles in a similar manner as the first RG system developed for the Reoviridae (Roner, Sutphin et al. 1990, Roner and Joklik 2001) or a plasmid-based system (Kobayashi, Antar et al. 2007). We discarded this option as this method may provide elements of confusion in distinguishing the helper virus from the rescued one. Instead, I based our approach on the existing systems that had been published for both BTV and AHSV. This initially involved a two-step transfection of ssRNA (Matsuo and Roy 2009). Briefly, in the first step, ssRNAs of the segments that encode the transcription complex (VP1, VP3, VP4, VP6) and two non-structural proteins (NS1 and NS2) are transfected into BSR cells. After 18 h, cells are transfected again with all the 10 ssRNAs. This method was shown to work for both BTV and AHSV (Ratinier, Caporale et al. 2011) (Matsuo, Celma et al. 2010) but in our hands the rescue efficiency was very low.

We then used a modification of this protocol with the first step using expression plasmids for the polymerase complex, NS1 and NS2, instead of ssRNA. The CMV promoter in the pCI expression plasmid likely allow high levels of protein expression, which may be required to create an optimal "cellular environment" for the rescue of the 10 ssRNAs. The expression plasmids used in this study were an exact cDNA copy of the viral genomic segment and included the UTR rather than just the coding regions. The UTR of these segments were maintained in the constructs based on the published study showing that BTV NS1 preferentially up-regulates viral protein expression over host cell proteins by binding the UTR of the viral genome segments (Boyce, Celma et al. 2012). Also, unlike the method used by Van de Water et al. the expression of VP7 was excluded from the first round transfection. The data presented in this chapter supports the notion that the transcription complex is critical for AHSV rescue and that a two-step protocol using DNA, followed by ssRNA, increased the efficiency of the method.

Since developing this method, a further RG system was developed for AHSV solely based on plasmids and using a T7-expressing cell line. Although this approach would
be a cheaper and quicker alternative to the synthesis and purification of ssRNA, in
our hands we were not able to clone some of the segments in some expression
plasmids as already described in other systems (Casais, Thiel et al. 2001, Thiel,

Like BTV, AHSV can readily reassort (Meiring, Huismans et al. 2009, Weyer, Grewar
et al. 2016). In this chapter, it was demonstrated that AHSV-4 could reassort with
segment 9 from two different clades. Indeed, the two different clades of VP6 (see
chapter 3) share 63% similarity at the aa level (AHSV VP6 clade I Vs II), indicating
that there must be conservation of structural domains that confer functional
homology between this essential protein. Deletion of VP6 has been shown to prevent
rescue of the virus (Matsuo, Celma et al. 2010). AHSV VP6, like BTV VP6, has been
reported to be an RNA binding protein and acting as a helicase to unwind viral
genomic RNA (De Waal and Huismans 2005).

To date, the role of AHSV NS4 in virus replication has not been studied. Initial studies
by Zwart. et al. (Zwart, Potgieter et al. 2015) had used recombinant protein
technology to investigate the role and functions of AHSV NS4. These authors
demonstrated that NS4 has a dsDNA binding activity but due to the lack of a reverse
genetic system, they were unable to determine its importance during virus
replication.

In this chapter, we successfully rescued AHSV-4 reassortants expressing the 4
different forms of the NS4 protein and the corresponding NS4 deletion mutants.

We established that NS4 was not essential for replication in BSR cells. Furthermore,
the viral replication kinetics of wt and ΔNS4 viruses was similar between the
respective viruses. However, there appeared to be differences in the replication
kinetics among the wt viruses expressing different VP6. The ability of the AHSVΔNS4
viruses to replicate in BSR cells was similar to what had been already reported for
BTVΔNS4 mutants (Ratinier, Caporale et al. 2011). These data so far suggest that
there may be some functional homology between the NS4 proteins of these two
different orbiviruses. In the next Chapter we will focus on trying to understand the
role of the NS4 in AHSV biology.
Chapter 5. The AHSV NS4 modulates the host IFN
5. The AHSV NS4 modulates the host IFN response

5.1 Introduction

In Chapter 4, a reverse genetics system for AHSV-4 was established and viruses that expressed the four types of NS4 identified in this thesis were rescued. Furthermore, viruses that did not express NS4 were also rescued. AHSV-4, segment 9 AHSV reassortants and NS4 deletion mutants induced similar plaque morphology and possessed similar replication kinetics in BSR cells. These data clearly indicated that the NS4 is not essential for virus replication similarly to what previously observed with BTV (Ratinier, Caporale et al. 2011). However, the BTV NS4 provides a replicative advantage to the virus in IFN competent cells and is a determinant of virus virulence in vivo in sheep (Ratinier, Shaw et al. 2016). As BSR cells are IFN incompetent, which do not produce IFN and respond to IFN from extracellular matrix, it is possible that the AHSV NS4, similarly to the BTV NS4, modulate the IFN response of the host.

The type I IFN response is one of the immediate barriers to viral infection deployed by the host innate immune system. Viruses have evolved a variety of mechanisms to counteract the antiviral immune responses of the host. Viral proteins expressed by several viruses directly hamper IFN induction or response and antagonize the host immune system. Often these viral proteins are non-structural and are not essential for virus replication but they are absolutely necessary to subvert the innate immune response that hinder replication. Well-studied examples of these IFN antagonists include for example the influenza virus NS1 (Talon, Horvath et al. 2000), the orthobunyaviruses NSs that counteracts IFN induction (Weber, Bridgen et al. 2002) and the HIV-1 Vpu or VIF proteins (Van Damme, Goff et al. 2008).

The induction of interferon in response to AHSV infection and virus modulation of this response has not been studied in detail to date. The only data examining the type I IFN response for AHSV comes from studies in mice (de la Grandière, Dal Pozzo et al. 2014) indirectly demonstrating that IFN has a role in controlling infection. Indeed, wild type mice are resistant to AHSV infection but knock-out mice for the type-I IFN receptor (IFNAR−/−) are instead killed by the same virus. The interaction
of BTV with the host IFN response has been instead better characterised. At least two of the viral non-structural proteins of BTV have been shown to modulate the IFN response. NS3/NS3A was highlighted as a potential IFN antagonist by its ability to shutdown host cell protein synthesis and reducing the levels of IFN-β mRNA transcripts (Chauveau, Doceul et al. 2013, Vitour, Doceul et al. 2014). As mentioned before, also the BTV NS4 modulated the IFN response (Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016) with a mechanism that has not been clarified completely. NS4 appears however to act at transcriptional level inhibiting gene expression of a variety of promoters. Our hypothesis is that AHSV shares functional homology in its ability to antagonise the IFN response to a similar extent to BTV NS4, even though they localise to different regions of the cells. In this chapter therefore, we aim to examine the role of the AHSV NS4 in virus replication in vitro and pathogenesis in an experimental model of the disease.
5.2 Results

5.2.1 NS4 is not required for AHSV replication in culicoides cells

AHSV is an arbovirus transmitted by culicoides midges. Hence, the virus is adapted to grow both in mammalian and insect cells. Hence, we addressed whether the AHSV NS4 had a role in virus replication in insect cells, using a cell line (Kc) derived from *Culicoides sonorensis* (Wechsler, McHolland et al. 1991).

Kc cells were infected with either AHSV-4, the AHSV segment 9 reassortants or the corresponding deletion mutants at an MOI equivalent of 0.01 (in BSR cells). Supernatants were collected at 24, 48 and 72 h p.i. and titrated by endpoint dilution assays in in BSR cells. No differences were observed between the replication kinetics of the viruses possessing the NS4 as opposed to the corresponding NS4 deletion mutants (Figure 5.1A). These data indicate that NS4 does not play a significant role in AHSV replication in culicoides cells.

5.2.2 NS4-I, -IIα and -IIγ provide a replicative advantage to AHS in E. Derm cells.

We next investigated the role of NS4 in AHSV replication in horse cells. E. Derm is an IFN competent cell line derived from the dermis of a 4 year old female horse (ATCC). E. derm were infected with either AHSV-4, or the various reassortants described above and the NS4 deletion mutants, and supernatants collected and titrated as described above for Kc cells. We observed that the titre of the virus expressing NS4-I was ten folds higher than the corresponding ΔNS4-I mutant (Figure 5.1B) at 48, 72 and 96h p.i. Similar differences were also observed for NS4-IIα and IIγ and the corresponding ΔNS4-II viruses (Figure 5.1B). In stark contrast, the replication kinetics of the reassortant expressing NS4-IIβ and the corresponding ΔNS4 mutant did not show any difference. Our results indicate AHSV NS4-I, NS4-IIα and IIγ provide a replication advantage to AHSV in equine cells.
Figure 5.1 Virus growth kinetics in Kc cells (A) and E. Derm (B) cells. Cells were infected at a MOI ~0.01 and cell-free virus was harvested at the time points indicated. Titres were determined by limiting dilution and expressed as TCID\(_{50}\)/ml. In KC cells, the growth kinetics of all the ΔNS4 viruses (blue) were similar to that of related wt viruses (red). In contrast, in E. Derm cells, wt viruses grew to higher titres than NS4 deleted viruses, except for NS4-IIβ wt and mutant viruses.
Each experiment was performed three times independently using two independently generated virus stocks.

5.2.3 Type I IFN hampers AHSV replication

Next, we investigated whether NS4 could facilitate AHSV replication in cells in which an antiviral state was induced by pre-treatment with IFN.

E. Derm cells treated with 1000U of uIFN for 24 h or mock-treated were infected with AHSV-4, AHSV segment 9 reassortants and NS4 deletion mutants as per previous experiments and supernatants titrated in BSRC cells at different time points p.i. As expected, titers of all viruses used in the experiment were lower at all time points in cells pre-treated with IFN (Figure 5.2). For example the titres of the AHSV NS4-I wt and ΔNS4 viruses decreased -1log₁₀ at 24 h p.i. and over -2log₁₀ at 48 h p.i in IFN-treated cells compared to the mock-treated controls. Hence, the presence or absence of NS4 did not affect the sensitivity of AHSV to IFN. Similar results were obtained for viruses expressing NS4-IIα, β and γ and the corresponding NS4 deletion mutants; replication of both sets viruses was restricted in cells were pre-treated with IFN (Figure 5.2). The titres reached by AHSV NS4-I, -IIα and -IIβ in IFN-treated E. Derm cells was about 100 fold lower at 48 h p.i than the titers reached in mock-treated cells. Treatment with IFN reduced the titers reached by AHSV NS4-IIy, only about 10 folds (Figure 5.2).
Figure 5.2 Virus growth kinetics of wt (red) and ΔNS4 (blue) viruses in E. Derm cells pre-treated with IFN. Cells were pre-treated with 1,000 u of uiIFN and infected with a MOI~0.01. Cell-free virus was harvested at the time points indicated and titres were determined by limiting dilution and expressed as TCID₅₀/ml. The dashed lines indicate virus samples harvested from IFN pre-treated cells. IFN pre-treatment significantly decreased the titres of both wt and ΔNS4 viruses at all times points analysed (p<0.0001). Each experiment was performed three times independently using two independently generated virus stocks.

5.2.4 NS4 confers a replicative advantage to AHSV in equine primary cells.

E. Derm is an immortalised cell line. The cells appear to be relatively unstable and their chromosome numbers for example varies widely between 52 and 145 (whereas the horse genome contained 64 chromosomes) (ATCC). Hence, various pathways in E. Derm, including IFN induction and response may vary widely in these cells between different passages. Indeed we observed inconsistent data when we attempted to measure IFN induction in E. Derm as a result of AHSV infection. Therefore, we generated primary endothelial cells from horse aorta to further investigate the function of the AHSV NS4. As expected, these cells were able to produce IFN in response to polyI:C stimulation (data not shown). The viral replication kinetics of the different viruses used in this study in primary horse endothelial cells
was similar to those observed in E. Derm cells. The titers reached by AHSV NS4-I, IIα and -IIγ in primary horse endothelial cells were at least 10 times higher than the corresponding ΔNS4 viruses (Figure 5.3). On the other hand, the replication kinetics of AHSV NS4-IIβ and the corresponding ΔNS4 mutant were essentially identical in these cells (Figure 5.3).

![Figure 5.3 Replication kinetics of wt (red) and ΔNS4 (blue) viruses in horse primary aorta endothelial cells. Cells were infected with a MOI~0.1. Cell-free virus was harvested at the time points indicated and titres were determined by limiting dilution and expressed as TCID₅₀/ml. AHSV wt expressing either NS4-I, NS4-IIβ or Iγ generated higher virus titres at each time point in comparison to the respective ΔNS4 viruses. NS4-IIβ wt and mutant viruses were an exception with similar titres observed at all time points. Each experiment was performed three times independently using two independently generated virus stocks.](image)

5.2.5 Inhibition of the IFN response rescues the replication defect displayed by AHSV NS4 deletion mutants in primary horse cells.

Collectively, the data presented in section 5.2.3 and 5.2.4 suggested that NS4 favours virus replication in IFN competent cells. Hence, we repeated the growth
curve assays described above in cells treated (or mock-treated) with ruxolitinib, in order to rule out that factors other than IFN affect the replication of the AHSV NS4 deletion mutants. Ruxolitinib is an inhibitor of the JAK/STAT pathway, hence cells treated with this chemical are unable to mount an effective IFN response (Delgado-Martín, Meyer et al. 2017). Horse aorta endothelial primary cells were treated with 5µM ruxolitinib for 4 h, and then infected with the different AHSV recombinant viruses used above or the corresponding ΔNS4 viruses. Supernatants were collected at 24, 48, 72 and 96 h.p.i and then titrated in BSR cells. In cells treated with ruxolitinib the NS4 deletion mutants in general replicated as efficiently as the viruses possessing the NS4. Treatment with ruxolitinib increased the replication efficiency of both viruses with and without NS4 (Figure 5.4). This is in contrast to the mock-treated horse aorta endothelial primary cells where viruses expressing NS4-I, IIA and IIγ reached higher titres than the corresponding NS4 deletion mutants. Interestingly, we observed that AHSVΔNS4-IIβ reached higher titres than AHSV-NS4-IIβ virus in ruxolitinib treated cells (Figure 5.4). This is in contrast to the untreated cells where the replication kinetics of these two viruses was essentially the same.
Figure 5.4 Viral replication kinetics in horse primary cells pre-treated with ruxolitinib. Horse aorta endothelial cells were pre-treated with 5 μM ruxolitinib and infected at a MOI 0.1. Cell-free virus was harvested at the time points indicated and titres were determined by limiting dilution and expressed as TCID₅₀/ml. Viruses titres from ruxolitinib-treated cells are depicted as dashed lines while titres from untreated cells are depicted by solid lines. Wild-type viruses are depicted in red and ΔNS4 viruses in blue. A. The average and standard deviations of 3 independent experiments is presented. Both wt and DNS4 viruses grew to similar titres. However, the titres in ruxolitinib-treated cells were higher than in untreated cells at time points. (B) Given that primary cells are intrinsically variable, the data from each of the 3 independent experiments is also shown.

These results indicated that at least NS4-I, IIα and IIγ have a role in modulating the IFN response during AHSV infection.

5.2.6 The AHSV NS4 hampers IFN induction in infected horse cells

Next, we carried out an IFN protection assay in order to further understand the role of AHSV NS4 in modulating the host antiviral responses. Primary horse aorta endothelial cells were infected at high MOI and cell supernatants were harvested to measure the IFN response as described in Materials and Methods.

Initially, it was important to find the best method to quantify type-I IFN production in horse cells. We acquired a commercial ELISA kit (The Equine IFNβ Do-It-Yourself Kit; Kingfisher Biotech Inc) but we were not able to detect IFN produced by horse cells infected by Sendai virus (rich in defective interfering particles; a gift from Prof.
Rick Randall), although the kit was able to detect recombinant equine IFNB up to a concentration of 12.44ng/ml IC₅₀. Hence, we used a biological assay where the detection of IFN in AHSV infected cells is based on the inhibition of an IFN sensitive virus (encephalo myocarditis virus, EMCV) in target cells. In order to select the indicator cell line, a variety of cell lines were treated with commercial equine IFNα1 (eIFNα1) and then infected with EMCV. The results confirmed the findings of (Adolf, Traxler et al. 1990) that neither A549 nor A549-Npro responded to eIFN (DATA NOT SHOWN). On the contrary CPT-Tert cells (an immortalised sheep choroid plexus cell line) were sensitive to and established an antiviral state after treatment with eIFN (Figure 5.5). CPT Tert cells were completely protected when cells were treated with as little as 25 pg/ml of eIFNα1; while 50% of the monolayer was protected when cells were treated with 12.5 pg/ml of eIFNα1. As expected, supernatants from mock-infected cells did not protect CPT-Tert cells against EMCV-induced CPE, suggesting that there was no background IFN secreted from uninfected primary horse cells (Figure 5.5A). Hence, CPT-Tert cells were used as indicator cell line.

Horse primary endothelial cells were infected with the full set of AHSV viruses expressing the different forms of the NS4 protein and the corresponding ΔNS4 viruses (at ~MOI 2). Supernatant was collected at 24 h p.i, UV-treated (to inactivate AHSV) and added to CPT-Tert cells for 24h. Subsequently, media was removed and cells were challenged with EMCV for 48 h before staining with crystal violet in order to assess cytopathic effect. Serial dilutions of eIFNα1 were used as positive control and to calibrate the results obtained with the supernatants of AHSV infected cells. Consistently, we observed that primary aorta endothelial cells produced higher levels of IFN when infected with the ΔNS4 rescued viruses compared to the corresponding viruses expressing NS4 (Figure 5.5A). These results suggest that the AHSV NS4 play a role in modulating the IFN response.
Figure 5.5 Production of IFN in horse endothelial cells in response to infection of AHSV wt and ΔNS4 virus. Horse aorta endothelial primary cells were infected with AHSV wt or ΔNS4 viruses at MOI~2. Supernatants were harvested 24 h post infection and UV-treated to deactivate AHSV. The medium was serially diluted and placed onto CPT-Tert cells. CPT-Tert cells were challenged 24 h later with ECMV for 48 h, fixed and stained with crystal violet. Equine IFNα1 (eIFNα1) was used as a positive control and calibrator. Supernatants were 2-fold serially diluted from the top lane to bottom lane. A. Crystal violet-stained CPT-Tert cells from a representative experiment.

B. Equivalent IFN concentration in supernatants infected with the indicated viruses from 3 independent experiments performed in duplicate. Note that differences between the equivalent amounts of IFN released in the supernatants of the viruses expressing NS4 and the corresponding ΔNS4 viruses were the following: p=0.2891 (NS4-I vs ΔNS4-I); p= 0.0161 (NS4-IIα vs ΔNS4-IIα); p=0.0056 (NS4-IIβ vs ΔNS4-IIβ); p=0.068 (NS4-IIγ vs ΔNS4-IIγ). The lack of statistical support in two of the series can be explained by two factors. The first one is that in one of the experiments we obtained higher levels of IFN released by cells infected with NS4-I and NS4-IIγ. Secondly, and more importantly, samples were not diluted enough to calculate accurately the amount of IFN released by ΔNS4 cells. This means that the amount of IFN released by cells infected with the ΔNS4 viruses is likely higher than what has been calculated.

AHSV expressing NS4-IIβ appeared to be the most efficient in counteracting the IFN response of the cell in comparison to the viruses expressing the other types of NS4. AHSV NS4-I, NS4-IIα and γ were able to suppress the production of IFN in comparison to their respective mutants to varying degrees (Figure 5.5). NS4-IIα was the next most efficient at reducing IFN production with ~133 pg/ml, followed by NS4-I and NS4-IIγ that both had an average of 300 pg/ml (Figure 5.5B). There was quite large variation in the amount of IFN secreted in to the cell culture supernatants by these viruses and this may be due to the different batches of primary cells used in these
experiments (see appendix II). Despite these batches difference, there was a difference in the ability of the AHSV viruses expressing NS4 to decrease IFN production in comparison to the ΔNS4 viruses (Figure 5.5B). These results confirmed our hypothesis that NS4 has role in limiting the IFN response to infection.

5.2.7 AHSV NS4 is a determinant of virus virulence in experimentally infected mice

Next, we investigated whether NS4 could be identified as a determinant of virus virulence in vivo in experimentally infected mice. NIH Swiss new-born mice were inoculated intracerebrally with 200 pfu of AHSV expressing the different types of NS4 or the corresponding deletion mutants (1 litter for each virus) (animal inoculations and monitoring were carried out by Marco Caporale at the IZS Teramo, Italy). Each of the viruses used killed 100% of the infected animals. However, all animals inoculated with ΔNS4 viruses survived longer than those infected with viruses expressing NS4 (NS4-I, p=0.0048; NS4-IIα, p<0.0001; NS4-IIβ, p<0.0001; NS4-IIγ, p=0.0002) (Figure 5.6). Indeed 50% of the mice inoculated with NS4 expressing viruses died by day 4 p.i. and 100% by day 6 p.i. (Figure 5.6). Conversely, mice inoculated with the ΔNS4 viruses did not start dying until day 5 and all (but one) died by day 8. These results therefore suggest that NS4 is a determinant of viral pathogenesis.
Figure 5.6 AHV NS4 is a determinant of virulence. Kaplan-Meier plots displaying survival of new-born NIH-Swiss mice inoculated intracranially with 200 PFU of the indicated viruses. Survival rate was monitored for 14 days.
5.3 Discussion

In this chapter we aimed to understand the biological function of the AHSV NS4. Previous studies on the phylogenetically related BTV have found that its NS4 protein is an IFN antagonist (Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016). Hence, in this Chapter we concentrated our efforts on determining whether also the AHSV NS4 modulates the antiviral response of the host. Experiments in this thesis showed that the NS4 does not provide any replication advantage to AHSV in the IFN-incompetent BSR cells or in the culicoides Kc cells. These results suggest that NS4 does not play any major role in virus replication per se.

However, AHSV NS deletion mutants (with the exception of AHSV-NS4II-β) replicated less efficiently than the corresponding viruses expressing NS4 in IFN competent cells (E. Derm cells and horse primary endothelial cells). Importantly, the AHSVΔNS4 mutants replicated as efficiently as the corresponding NS4 expressing viruses when the same cells were treated with an inhibitor of the JAK/STAT pathway, and therefore inhibiting the IFN response. Furthermore, by IFN protection assays we established that high levels of IFN were detected in the supernatants of primary horse cells infected with the ΔNS4 viruses but not with the corresponding “wild type” viruses expressing NS4.

Hence, the NS4 is at least one of the tools used by AHSV to modulate the IFN response. In vivo, we found the AHSV NS4 is a determinant of virulence at least in an experimental mouse model of AHS. Interestingly, the NS4 did not provide a replication advantage in cells which were already in an antiviral state as a result of IFN stimulation. Collectively, the data obtained suggest therefore that the AHSV NS4 blocks IFN induction.

We obtained seemingly contradictory results with AHSV-NS4II-β. Similarly to the other three types of NS4, NS4II-β was also proven to modulate the IFN response in vitro and be a determinant of virulence in vivo. However, AHSV-ΔNS4II replicated as efficiently as AHSV-NS4II in both E. Derm and primary horse endothelial cells. Hence, NSII behaves as an IFN antagonist but does not provide a replication advantage to AHSV in IFN competent cells. Interestingly, in ruxolitinib-treated cells, AHSVΔNS4IIβ
replicated even more efficiently than AHSV-NS4IIB. It appears therefore that NS4IIB has additional features that distinguish it from the other types of AHSV NS4 and more studies will be required to understand these differences.

The results obtained in this chapter demonstrate that both the orbiviruses BTV and AHSV utilise their NS4 proteins to modulate the antiviral responses of the host. However, it is interesting to note that there are important differences in the mechanisms followed by these two proteins to modulate the IFN response of the host. First of all, the BTV NS4 has a strong nucleolar localisation as opposed to the cytoplasmic localisation of the AHSV NS4 (Ratinier, Caporale et al. 2011). In addition, the BTV NS4 is able to provide a replication advantage to BTV in cells already in an antiviral state as a result of IFN pre-treatment. The BTV NS4 has been suggested to act at the level of cellular transcription. The AHSV NS4 seems to act before the transcription of IFN, hence at either PAMP detection, downstream signalling or transcription. More studies will be necessary to dissect how exactly the AHSV NS4 inhibits the IFN response.
Chapter 6. General Discussion
6. General Discussion

The major objective of this project was to investigate the functions of AHSV NS4 in viral infection and replication in mammalian cells, and to investigate if this protein played any roles in counteracting the host IFN response. The origins of this project were inspired by the detection of an overlapping ORF in orbivirus segment 9 (Firth 2008), including BTV, AHSV, PALV and PHSV/YUOV and the role that BTV NS4 has in subverting the IFN response (Ratinier, Caporale et al. 2011, Ratinier, Shaw et al. 2016). During this research project, research published by others including the development of a RG system for AHSV (Kaname, Celma et al. 2013) (Van de Water, van Gennip et al. 2015) (Conradie, Stassen et al. 2016) and the identification of AHSV NS4 in infected cells were reported (Zwart, Potgieter et al. 2015). Although these papers were published, a question remained to be answered: what is the role of AHSV NS4 during virus replication?

The bioinformatics analysis presented in this thesis supported the finding of Zwart et al. and not Firth confirming that there were at least two forms of NS4 (NS4-I and NS4-II). NS4-I and NS4-II share between 45.6% to 59.5% similarity and cluster into two phylogenetically distinct clades. Segment 9 VP6 and NS4 ORFs are not the only AHSV segments that cluster into distinct clades with high variation observed at the amino acid level. Segment 10 clusters into 3 clades (Quan, Van Vuuren et al. 2008) (Martin, Meyer et al. 1998) (Van Niekerk, Freeman et al. 2003) and Segment 1 into two distinct clades (see Chapter 3 Figure 3.3). Our analysis of NS4, VP1 and VP3 supported that the segment reassortment of AHSV genome was not linked to virus serotypes and that there appeared to be no obvious factors influencing genome reassortment. In the field, reassortment appears to be associated with increased viral fitness with virus derived from the live attenuated vaccine strains associated with virulent outbreaks (Weyer, Grewar et al. 2016). Traditionally virulence has been associated with reassortment of segments 2 and 5 and 10 (Quan, Van Vuuren et al. 2008). Weyer et al. suggested that along with VP2 and VP5, VP6 also has a role in virulence (Weyer, Grewar et al. 2016). We did not have any of these changes in the VP6 sequences used in our backbone viruses, indicating that the differences in
virulence and replication observed between the rescued viruses are most likely due to NS4.

Moreover, here we showed that there are three different forms of NS4-II, which we refer to as NS4-IIα, β and γ based on N- and C-terminal truncations, which had not been identified by either Firth or Zwart et al (Firth 2008) (Zwart, Potgieter et al. 2015). We identified other smaller truncated forms but these all derived from highly passaged cell culture adapted-viruses and we hypothesized that these forms of NS4 do not exist in the field. The different forms of AHSV NS4: NS4-I, NS4-IIα, β and γ, comprised 26.1%, 4.3%, 67.9% and 1.7% respectively of the circulating field strains with the NS4-II forms being the most common.

When comparing the percentage of NS4 populations in the field isolates with the prevalence of the different forms of disease, one could speculate that the percentages of different disease outcomes is roughly similar. Among the four presentations of AHS, the mixed form is the most common form whereas the horse sickness fever is the least. Thus, we propose a very bold hypothesis that the four types of NS4 are associated with the different clinical presentations of AHS. Until data linking disease outcome to sequence information of whole isolates is available this will not be validated. Although, we inoculated IFN competent mice with the RG engineered-viruses expressing the different NS4 proteins, there were no statistical significant differences in the survival rates among the different groups. Mice may not be the best model to study AHSV pathogenesis and this type of studies may be better suited for ponies or horses.

In order to investigate the role of AHSV NS4, comparative analysis was made with BTV NS4 due to the position and conservation in the genome. Although AHSV NS4 is almost twice the size of BTV NS4 (143 to 169 aa compared to 77 to 79 aa) and AHSV NS4 has more than one form, there are similarities. The BTV NS4 C-terminal part contains a conserved sequence where leucine residues are present at positions 49, 56, 63 and 70 (Ratinier, Caporale et al. 2011). In this study, I identified a putative leucine zipper in the AHSV NS4 consensus sequence (NS4-I and NS4-II see chapter 3 Figure 3.2) with leucine residues spaced every 7-8 residues (aa positions 92, 100, 108 and 115). This is a typical feature of leucine zipper domains (that leucine residue
occurs every 7 aa). Functionally leucine zipper domains have a role in binding protein to DNA, but would not preclude the proteins ability to bind ssRNA and/or dsRNA. The DNA binding function of AHSV was eluded by Zwart et al (Zwart, Potgieter et al. 2015), who undertook a DNAse and RNAse protection assay. However, this property for NS4 needs to be further validated through either mutational studies, electrophoretic mobility shift assay (EMSA) and sequence analysis of the fragments of nucleic acid the protein is interacting with to determine if it is a specific effect.

Although, there is this putative leucine zipper and data suggesting DNA binding ability, the localisation studies of NS4 presented here revealed that all forms of the proteins did not localise in cell nucleus but were diffuse throughout the cytoplasm (Chapter 3 Figure 3.6, Figure 3.7). There was no specific localisation to any of the cellular structures or organelles. This contrasts with BTV NS4, which localises to the nucleolus and the data presented by Zwart et al (Zwart, Potgieter et al. 2015) that indicated that AHSV NS4 localises to both the nucleus and the cytoplasm. The diffuse localisation of the proteins did not provide insights or direction to follow up the functional analysis.

In order to control the host cell response against viral infection, virus including BTV (Ratinier, Shaw et al. 2016) and Schmallenberg virus (SBV), (Varela, Pinto et al. 2016) have been known to shutdown the host protein synthesis. For SBV, this function was attributed to the non-structural protein NSs and the glycoprotein (Gc). For BTV, 3 non-structural proteins have been implicated including NS3, S10-ORF2 and NS4 (Chauveau, Doceul et al. 2013) (Ratinier, Shaw et al. 2016) (Stewart, Hardy et al. 2015) (Varela, Pinto et al. 2016). Therefore, the role of AHSV NS4 in host protein shutdown was assessed using a firefly luciferase expression assay. The data revealed that all the forms of NS4 could decrease firefly expression but there were differences the degrees that Firefly luciferase expression was turned off. NS4-IIβ had the strongest effect on firefly luciferase expression (~77% decrease) while NS4-IIγ was the weakest (~10%). NS4-I and NS4-IIα acted similarly with 25% decrease in comparison to the control. A major difference was associated with truncation in the NS4-IIβ and IIγ in comparison to the full length of NS4-IIα and NS4-I. The removal of either the N-terminal from NS4-IIβ or the C-terminal from NS4-IIγ changed the ability of the proteins to affect host cell protein synthesis (NS4-IIβ 75%
decrease and NS4-IIγ 10%). This data would indicate that the ability to shutdown was in the core region of the protein and the N- and C-terminals suppress or enhance this ability, respectively. Other form of AHSV NS4s, NS4-IIγ lacks 26 aa at C-terminal, therefore the C-terminal of AHSV NS4 should play the critical role in host protein shutdown. Although the experiments were not undertaken here, to further validate this hypothesis, the NS4-IIα sequence should be truncated at the N- and C-terminal to generate either NSI-IIβ or γ and the assay repeated to examine whether the phenotypes could be recapitulated.

Studying a protein outside of the context of virus replication only provides limited information and overstates or understates the role of the protein. In order to investigate the role of NS4 in virus replication, a reverse RG had to be established. During the time that the RG was developed in the laboratory, three research groups published an AHSV RG system (Kaname, Celma et al. 2013, Van de Water, van Gennip et al. 2015, Conradie, Stassen et al. 2016). The RG system developed in this thesis was similar to Kaname and Van de Water using a two-step transfection protocol (Kaname, Celma et al. 2013, Van de Water, van Gennip et al. 2015). This enable the successful rescue of AHSV wt and ΔNS4 viruses for the four forms of NS4 and provided opportunities to investigate the functions of this protein during virus replication. Similar to BTV NS4 and AHSV NS3 and VP2, AHSV NS4 was not required for the successful rescue of the reassortant viruses and that its function like other non-structural and accessory proteins maybe cell type dependent.

This assumption was inferred by the replication kinetics data of both AHSV wt and ΔNS4 viruses in an interferon-incompetent mammalian cell line (BSR) and in an insect cell line (Kc) where no significant or observable differences were found at any of the time points investigated. The size and shape of plaques in BSR cells did not display any difference and the dsRNA genome was not affected by lack of NS4 expression. This ruled out the role of AHSV NS4 in the dsRNA genome synthesis, virus assembly, egress of the particle and cell-to-cell spread to the virus.

As BTV NS4 had a similar phenotype in both BSR (interferon incompetent) and the vector host cell line, it was a natural progression to investigate if AHSV had a role in antagonising the IFN response. To date the IFN response to AHSV has not be studied
Unlike BTV where there were studies in 1969 measuring the production of IFN *in vitro* and *in vivo* in response to infection (Huismans 1969) and the role of the cytokine storm and IFN in controlling infection (DeMaula, Jutila et al. 2001, DeMaula, Leutenegger et al. 2002) (Ratinier, Shaw et al. 2016). There has only been one study (de la Grandière, Dal Pozzo et al. 2014) that has eluded to the role that IFN plays in controlling the infection in IFN-competent mice in comparison to IFNAR(−/−) mice. In this study, we have reported that IFN influences AHSV replication and that AHSV NS4 acts as an antagonist of IFN induction. The replication kinetics of AHSV wt viruses in an equine-derived cell line (E. Derm cells and horse primary endothelial cells) was significantly higher than that of the corresponding ΔNS4 viruses except for NS4-IIβ. Regardless of whether NS4-IIβ was present or not, there was no difference in the replication kinetics of the virus.

The role of AHSV NS4-I, NS4-IIα -IIβ and -IIγ as IFN antagonists was further supported when the JAK/STAT pathway was blocked by ruxolitinib, which stops the induction of IFN. The viral titres at all time points for the AHSV ΔNS4 viruses increased, including AHSV NS4-IIβ ΔNS4 virus. When ruxolitinib was added to the cells, the ΔNS4-IIβ virus grew to higher titres than wt virus, which was unexpected. It could be speculated from these results that NS4-IIβ is an IFN antagonist but its ability to shutdown host cell proteins to the extent that it does has a detrimental effect on the viruses i.e., it comes at the expense of virus fitness. Pre-treatment of E. Derm cells with IFN caused a similar restriction on both the wt and ΔNS4 viruses growth. We expected that the growth of the ΔNS4 viruses would have been more retarded than the wt, but as E. Derm cells respond to and secret IFN this may have confounded the experiment. It would be important to use a cell line that responds to IFN to induce an antiviral state but does not have the capacity to produce IFN or keep IFN in the media to study these phenomena in greater depth.

To monitor the effect of IFN production in response to AHSV, we set up a biological assay that enables equine IFN to be measured; this was the first time such a study has been performed. Infection of horse primary endothelial cells with AHSV regardless of which type of NS4 was expressed, did not induce the release of very much IFN into the supernatant. This was particularly obvious in the supernatant of AHSV NS4-IIβ wt-infected cells, with the IFN concentration below the detectable
limits of the assay. In contrast, high concentrations of IFN were secreted in the supernatant from cells infected with AHSV ΔNS4 viruses, including ΔNS4-IIβ. This data supported the assumption that NS4 acts as an antagonist of IFN.

It was not surprising that AHSV infection stimulated an IFN response and encodes a potential IFN antagonist. Like BTV, the AHSV genome dsRNA is also a strong PAMP for host cell IFN response and the outer capsid proteins have been shown to induce apoptosis (Mortola, Noad et al. 2004, Stassen, Huismans et al. 2012, Vermaak and Theron 2015). Host cell sensors, including RIG-I and MDA5 are upregulated and activated in response to AHSV. The activation of sensors results in the production and secretion of IFN. Viral non-structural proteins play a role in counteracting host interferon response in diverse ways. For example, the Influenza A viral protein NS1 prevents RIG-I to recognise viral genome as PAMP (Guo, Chen et al. 2007) and the NSs protein of Schmallenberg virus inhibits the host cell transcriptional machinery to block the IFN response (Barry, Varela et al. 2014). As AHSV NS4 shuts down the host cell protein synthesis, with NS4-IIβ being the most efficient one, it is hypothesised that AHSV NS4 may regulate host cell translation. Furthermore, the arginine rich motif (RXRRQRL) identified in this study in the C-terminal of AHSV NS4 may play a role in host cell mRNA binding in order to block the translation. However, this needs to be verified using mutational and ssRNA:protein interaction studies.

We had postulated that not only was AHSV NS4 a potential IFN antagonist but also may have a role in virulence based on the NS4 variability and disease prevalence (i.e. NS4-IIβ was the most frequent and the mixed form of disease is the most common presentation). Therefore, we tested the virulence of the viruses in IFN competent 4-day-old NIH-Swiss new-born mice. The wt viruses were all more virulent that the NS4-deletion viruses but were not significantly different to each other. Furthermore, the lack of NS4 did not prevent death, but significantly increased the time of survival compared to animals infected with wt AHS. Although, this would suggest that AHSV NS4 plays roles in blocking the host innate immune system and a determinant of virulence, it would be critical to find a better model to examine virulence such as the horse.
6.1 Future work

There were drawbacks in this project. In order to study NS4-IIα, β and γ, the AHSV-4 genome was reassorted with the whole segment 9 from AHSV-7, -1 and -6 to generate the AHSV NS4-IIα, β and γ respectively. However, this resulted in the expression of different VP6 proteins, although the amino acid sequences were similar, they were not the same, which may also impact on the viral replication. VP6 has been implicated in virulence but the amino acid residues responsible for this are not in the sequences used in the viruses rescued here. Thus, for the future work to study the potential functions of the C-terminal and N-terminal regions of AHSV NS4-II, it is important to introduce the mutations on the same sequences.

To further analyse the role of AHSV NS4 interacting with the host interferon response, a protein-protein interaction assay is needed to reveal the binding target either protein in the IFN induction pathway or specific ISG or the functional site of AHSV NS4 in counteracting the IFN pathway. This could be performed using an overexpression assay linked to a firefly reporter assay and co-immunoprecipitation such as the one used for Ukanemi virus (Rezelj, Li et al. 2017).

AHSV NS4 nucleic acid binding activity has been shown (Zwart, Potgieter et al. 2015), however the data needs further validation. As a putative leucine zipper domain and a putative arginine-rich domain have been identified in this work further mutational and nucleic acid:protein interaction studies are required.

It would be also interesting to explore the global effect of AHSV NS4 manipulation of host transcription and/or translation. This could be achieved by using RNA-seq technique as described for BTV (Ratinier, Shaw et al. 2016). It would be important to undertake this study in primary horse cells that are IFN competent rather than a cell line like A549 (AHSV cannot replicate in these cells). This would allow us to compare the upregulation of ISGs mRNA levels of wt and ΔNS4 virus-infected cells and could define the key genes of the host innate system in response to AHSV infection.
6.2 Concluding statement

This study provides an overview of the role of the NS4 protein. NS4 has a clear role in antagonising the IFN response and the mice studies highlight that it may have a role in the virulence *in vivo*. Indeed, it is easy to speculate that this protein maybe a diagnostic indicator of the clinical outcome of disease. However, the details of the exact function and mechanisms of action of the protein in the context of viral replication are unclear and it would be important to undertake follow up studies to investigate this, particularly for NS4-IIβ which is functionally different.
Bibliography


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Appendix I

standard solution

Phosphate buffered saline (PBS)  
10 mM Na$_2$HPO$_4$  
150 mM NaCl  
pH 7.4

Luria Bertani (LB) Medium  
1% (w/v) Bacto-tryptone  
0.5% (w/v) Yeast extract  
0.5% (w/v) NaCl  
in deionized water

LB plates  
LB Medium  
1.5 % (w/v) agar

PBS-tween  
10 mM Na$_2$HPO$_4$  
150 mM NaCl  
0.1% (v/v) Tween-20  
pH 7.4

6× DNA/dsRNA loading dye  
10 mM Tris·HCl  
60 mM EDTA  
0.03% (w/v) bromophenol blue  
60% (v/v) glycerol

4× protein loading dye  
200 mM Tris·HCl (pH 6.8)  
30% (v/v) glycerol  
10% (v/v) β-mercaptoethanol  
0.03% (w/v) bromophenol blue  
8% (w/v) sodium dodecyl sulfate
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate-EDTA buffer</td>
<td>40 mM Tris</td>
</tr>
<tr>
<td></td>
<td>20 mM acetic acid</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>pH 8.0</td>
</tr>
<tr>
<td>Tris-Glycine transfer buffer</td>
<td>120 mM glycine</td>
</tr>
<tr>
<td></td>
<td>25 mM Tris</td>
</tr>
<tr>
<td></td>
<td>20% (v/v) methanol</td>
</tr>
<tr>
<td></td>
<td>pH 8.3</td>
</tr>
<tr>
<td>4% formaldehyde</td>
<td>4% in PBS, pH 7.4-8.0</td>
</tr>
</tbody>
</table>
Appendix II

Production of IFN in horse endothelial cells in response to infection of AHSV wt and ΔNS4 virus. Horse aorta endothelial primary cells were infected with AHSV wt or ΔNS4 viruses at MOI~2. Supernatants were harvested 24 h post infection and UV-treated to deactivate AHSV. The medium was serially diluted and placed onto CPT-Tert cells. CPT-Tert cells were challenged 24 h later with ECMV for 48 h, fixed and stained with crystal violet. Equine IFNα1 (eIFNα1) was used as a positive control and calibrator. Supernatants were 2-fold serially diluted from the top lane to bottom lane. The experiment has been carried out by triplicate due to the different batches of primary cells used in these experiments. A. The second repeat of the experiment. Horse endothelial cells interferon production was inhibited when infected with AHSV wt type compared with the corresponding ΔNS4 viruses. B. The third repeat of the experiment. AHSV NS4-IIβ inhibited the primary cells interferon production completely, while AHSV NS4-IIα slightly inhibited the production. However, we did not identify the difference of the capability of host cell inhibition between AHSV NS4-I, ANSV NS4-IIy and the corresponding ΔNS4 viruses in this experiment.