CHAPTER 5: CONCLUDING REMARKS

The primary aim of this study was to further characterise the role that the AHSV NS3 protein plays in the AHSV life cycle and host cell interactions. In the first part of this study, the involvement of NS3 in several parameters during AHSV infection of mammalian cells, including virus yield, virus release, cytopathicity and membrane permeability, was examined. The strategy used to achieve this was based on *in vitro* reassortment of the genome segment 10 encoding NS3 between parental virus strains that showed quantitative differences in these parameters. The parental virus strains used during reassortment encoded NS3 proteins representative of each of the three NS3 phylogenetic clades (α, β and γ). Two parameters of these parental viruses, the amount of virus released and the degree of membrane permeabilisation induced, were found to be associated with the genome segment encoding NS3. The induction of cytopathic effects was not associated with NS3 alone and the results showed a possible involvement of NS3 together with NS1. Differences in the parental viruses such as their effect on cell viability and protein synthesis within cells could not be related to their encoded NS3 proteins. Each of these aspects, and their implications for future research, will be discussed here in the context of what is currently known about AHSV and the prototype orbivirus, BTV.

The finding that the amount of virus released was associated with NS3 is significant, as this influences the spread of the virus to other target cells within or between organs and so may have an impact on pathogenesis. This also agrees with a previous study by Martin *et al.* (1998) in which it was shown that viruses encoding NS3 from the β and γ clades differed in the timing of virus release. The role played by NS3 in the release mechanism of AHSV remains to be elucidated although it is likely to be similar to that proposed for BTV (Roy, 2008). AHSV may be released from mammalian cells by both a lytic or non-lytic budding mechanism (Stoltz *et al.*, 1996). It would be of interest to investigate whether the ratio of lytic to non-lytic release in these cells is affected by the different NS3 proteins. Another intriguing question in orbivirus research is the differences in release in mammalian and insect cells. In BTV-infected mammalian cells mature virions remain cell-associated, as has been commonly observed with reoviruses and, in the majority, are released by cell lysis. This may explain the high levels of CPE observed in BTV-infected mammalian cells. However, there is also support for the egress of virions as enveloped particles by budding at the cell membrane (Hyatt *et al.*, 1989). In insect cells virus release occurs mainly by budding in both AHSV and BTV, and although persistent infections are established no apparent CPE is observed (Eaton *et al.*, 1990; Martin *et al.*, 1998). This may be due to a number of factors, including the mechanism of virus release. It would be interesting to compare the mode of release of the parental and reassortant strains in mammalian and insect cells. This could be achieved by ultrastructural electron microscopic investigations of infected cells.

Vero cells infected with viruses encoding the NS3 proteins from the α, β and γ clades also showed distinct differences in membrane permeability. Using reassortants these differences

could clearly be associated with NS3. This lends support to the classification of NS3 as a viroporin. Membrane permeabilisation may impact both the virus and the infected cell as reviewed in chapter 1. Several factors may also influence viroporin permeabilisation activity including cell type, other functions of the protein and interactions with other viral or host proteins. Many viroporins are multifunctional and the other activities or functions of the protein may affect their permeabilisation activity. For example, picornavirus 3A blocks ER-to-Golgi transport of cellular proteins and may therefore prevent targeting of 3A itself to plasma membrane. Other viral proteins may also directly or indirectly affect viroporin activity in a variety of ways such as being necessary for the localisation of the viroporin to the plasma membrane, or having anti- or pro-apoptotic functions themselves (Madan *et al.*, 2008). The activity of viroporins may therefore be weakened or enhanced in the context of viral infection. Differences observed in the membrane permeabilising effects of the α, β and γ NS3 proteins may then be directly due to differences in their membrane permeabilisation activities and/or due to differences in their interactions with other viral or host proteins that affect their membrane permeabilising properties.

The cytopathic effect on Vero cells of the reassortant viruses generated here could not be associated with NS3 alone, but possibly with both NS3 and NS1. In BTV it is thought that NS1 could be a major determinant of cytopathogenesis, and that modifying the ratio of NS1 to NS3 can shift the mechanism of viral release from a lytic process to one of non-lytic budding from the cell surface (Owens *et al.,* 2004). The possible involvement of NS3 and NS1 in AHSV cytopathology, and the relative levels of expression of AHSV NS1 and NS3 in the different strains, should be investigated. NS3 is probably a component of a complex system influencing cytopathology involving virus tropism, the velocity of virus replication and release. Also affecting this would be host cell factors, in a recent study it was shown that 250 host factors or proteins are required during HIV-1 infection (Brass *et al.*, 2008). The cell innate defence responses such as apoptosis, the interferon response and RNA interference (De Vries *et al.*, 2008) may additionally be involved.

Although genomic reassortment studies have provided important information for many segmented viruses, this approach does have some drawbacks. This includes the necessity to screen large numbers of progeny viruses from crosses to identify reassortants and the unexplained preferential selection of certain genome segments. The recent development of a reverse genetics system for BTV (Boyce *et al.*, 2008) should makes it feasible to in future design and recover viruses containing specific mutations, or reassortants with desired combinations of gene segments. The use of RNA interference techniques should also prove useful in providing key information, as has been shown for rotavirus (Zambrano *et al.*, 2008). For studies as to the specific role played by each of the viral proteins in the viral life cycle it would also be more appropriate to use equine endothelial and *Culicoides* cell lines.

In chapter 3 the AHSV-2 (γ) NS3 protein was expressed as a recombinant in an inducible prokaryotic system that has been found to be ideally suited to the identification and analysis of virally-encoded membrane active proteins (Studier, 1991; Browne *et al.*, 2000). The results showed that NS3 was cytolytic to these cells and this activity could be related to membrane permeabilisation induced by the protein. A series of truncated AHSV-2 NS3 mutants were additionally expressed and analysed in this same system, and the results indicated that the HDs within the protein are responsible for this activity and that the presence of both HDs is critical to this. The full length AHSV-2 NS3 protein was also compared to BTV and EEV NS3 in this system. Differences in the cytolytic and membrane permeabilising activities were observed that, although cannot be related to the activities of these proteins in host cells, imply potential differences in structural stability and association with membranes of specific cell types, which in turn may impact on cytotoxicity

In chapter 4 the NS3 proteins from AHSV-2, AHSV-3 and AHSV-4 (i.e. from the γ, β and α NS3 clades) were expressed in the BAC-TO-BAC expression system as both wild-type proteins and C-terminal eGFP (enhanced green fluorescent protein) fusions. The potential impact of the sequence variation between these NS3 proteins on structure and function was then examined by comparing properties such as membrane permeabilisation, cytotoxicity, membrane association and localisation. As an initial comparison, the membrane permeabilising effect of the baculovirus expressed wild-type NS3 on Vero cells following exogenous addition was monitored. The α, β and γ NS3 proteins differed in their permeabilisation effect on Vero cells in a manner that could linked to that induced by the parental AHSV strains encoding these proteins. To further explore these apparent differences in permeabilising activity of the NS3 proteins, the effect on insect cell viability following *in vivo* recombinant expression was compared. Trypan blue viability assays showed that, in this system, all three proteins were equivalently cytotoxic. Membrane association assays additionally revealed that all three proteins associated with the membranous components of these cells. Future experiments to compare these proteins could be carried out in a mammalian cells through the use of an inducible recombinant expression system. The association of NS3 with the permeabilising properties of the parental and reassortant virus generated in this study could also be analysed by investigating the effect of siRNA knockdown of NS3 expression during AHSV infection of Vero cells. This technique had been used with great success by Zambrano and coworkers (2008) to examine the role of NSP4, and other viral proteins, in rotavirus induced permeabilisation of cells. Additionally the targeting of NS3 to the outer membrane in AHSV infected cells could be abrogated through the use of the membrane traffic inhibitor, Brefeldin A, and the effect on membrane permeabilisation monitored. In this case indirect immunofluorescent labelling of NS3 with monospecific antibodies could be used to

confirm blocking of the outer membrane targeting of NS3. This type of approach is described by Ruiz *et al.* (2005).

In this study the subcellular localisation of the AHSV-2, AHSV-3 and AHSV-4 NS3 proteins was examined through recombinant expression as C-terminal eGFP fusions in the baculovirus expression system. Live cell confocal imaging of infected insect cells showed that NS3 localised to the plasma membrane, and as distinct punctuate foci in the perinuclear region. This suggests localisation to the internal membrane systems of cells and would require further investigation. This could be achieved by co-localisation studies via immunfluorescent labelling of NS3 and specific cellular markers in AHSV infected cells. As outlined in the discussion in chapter 4, localisation to the ER, ERGIC and/or Golgi may have important implications for the function of AHSV NS3 during the viral life cycle. In a study by Bansal and coworkers (1998) BTV NS3 was was also found to be localised to the ER and Golgi.

In summary, NS3 plays a role in virus release, membrane permeabilisation and cytopathology making it a multifunctional pleiotropic protein in the virus life cycle. A greater understanding of the factors and events that contribute to the virulence and pathogenesis of AHSV are vital in developing strategies that may prevent, or lesser the impact of, African horsesickness disease.

Parts of the results presented in this thesis have been published:

Meiring, T.L., Huismans, H. and van Staden, V. (2009). Genome segment reassortment identifies non-structural protein NS3 as a key protein in African horsesickness virus release and alteration of membrane permeability. *Arch Virol* **154**, 263-271.

Parts of the results presented in this thesis are in preparation for publication:

Meiring, T.L., Teixeira, L., Fick, W.C., Huismans, H. and van Staden, V. Membrane destabilising activity of the orbivirus non-structural protein NS3 is mediated by the transmembrane domains. To be submitted.

Non-peer reviewed publication:

Huismans, H., van Staden, V., Fick, W.C., van Niekerk, M., Meiring, T.L. 2004. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Vet Ital* **40**, 417-425

Parts of the results in thesis have been presented at scientific meetings:

NATIONAL CONFERENCES:

Van de Merwe, E., R. van der Sluis, T.L. Meiring, A.N. Hall, H. Huismans, V. van Staden. 2008. Conserved residues in non-structural protein NS3 of African horsesickness virus influence subcellular localisation. Microscopy Society of Southern Africa (MSSA), July 2008, Gabarone, Botswana (Presentation)

Meiring, T.L., Huismans, H., van Staden, V. 2008. Using genetic reassortants of African horsesickness virus (AHSV) to study the role of non-structural protein NS3 in viral phenotypic properties in Vero cells. 20th Congress of the South African Genetics Society (SAGS), April 2008, Pretoria, RSA. (Presentation)

Meiring, T.L, van Staden, V., Huismans, H. 2006. Correlating the cytolytic effect of viral infection and single viral protein expression for three orbiviruses. 19th Congress of the SAGS, April 2006, Bloemfontein, RSA. (Presentation)

Meiring, T.L, Teixeira, L., Fick, W.C., van Staden, V., Huismans, H. 2005. Identification of regions of non-structural protein NS3 involved in membrane destabilisation in different orbiviruses. South African Society for Biochemistry and Molecular Biology XIX th Conference, January 2005, Stellenbosch, RSA. (Poster)

Meiring, T.L, Teixeira, L., Fick, W.C., van Staden, V., Huismans, H. 2004. Membrane destabilisation activity of non-structural protein 3 (NS3) of different orbiviruses. Combined South African Society of Microbiology and 18th SAGS congress, April 2004, Stellenbosch, RSA. (Poster)

INTERNATIONAL CONFERENCES:

Tracy Meiring, Henk Huismans and Vida van Staden 2008. Using African horse sickness virus reassortants to study the role of non-structural protein NS3 in several phenotypic properties of the virus. XIV International Virology Congress, International Union of Microbiological Sciences, August 2008, Istanbul, Turkey. (Presentation)

Tracy Meiring, Henk Huismans and Vida van Staden 2006. Membrane topology of African horsesickness virus non-structural protein NS3. 9th dsRNA Virus Symposium, October 2006, Cape Town, RSA. (Poster)

Vida van Staden, Tracy Meiring, Wilma Fick and Henk Huismans 2005. Cytotoxicity and membrane permeabilising activity of non-structural protein NS3 of different orbiviruses. Virology Africa, November 2005, Cape Town, RSA. (Presentation)

Vida van Staden, Tracy Meiring, Luisa Teixeira, Wilma Fick and Henk Huismans 2005. Membrane destabilization activity of non-structural protein NS3 of different orbiviruses. XIII International Virology Congress, International Union of Microbiological Sciences, July 2005, San Francisco, USA. (Poster)

Vida van Staden, Michelle van Niekerk, Tracy Meiring and Henk Huismans 2003. The effect of sequence variation in AHSV non-structural protein NS3 on viral and protein phenotypic properties. 3rd International Symposium on Bluetongue, 26 to 29 October 2003, Taormina, Italy. (Presentation)

Van Staden, V., van Niekerk, M, Meiring, T.L., Huismans, H. 2003. An investigation into the effect of sequence variation in AHSV non-structural protein NS3 on viral and protein phenotypic properties. 8th International Symposium on Double-Stranded RNA viruses, September 2003, Lucca, Italy. (Presentation)

Huismans, H., van Staden, V., Fick, W.C., van Niekerk, M., Meiring, T.L. 2003. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. Bluetongue virus Workshop 2003, Sardinia, Italy. (Presentation)