A comparison of different orbivirus proteins that could affect virulence and pathogenesis

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Summary

The factors that determine the virulence and pathogenesis characteristics of bluetongue virus (BTV), African horse sickness virus (AHSV) and other orbiviruses are not well known. With respect to the viral proteins that are expected to play a role it may be assumed that proteins, such as the outer capsid proteins VP2 and VP5, that are involved in the attachment of virus particles to target cells and influence replication efficiency are particularly important. Equally important are viral proteins such as non-structural protein NS3 that influence the release of virus particles from a target host or vector cell. The authors compare the amino acid sequence variation, structural motifs and some phenotypic characteristics of proteins VP2, VP5 and NS3 of different orbiviruses, such as AHSV, BTV and equine encephalosis virus (EEV). The most variable protein is VP2 and a pair-wise alignment of VP2 sequences of different serotypes of both BTV and AHSV indicated variation of between 48% to 64% and 46% to 52% for most isolates, respectively. Several regions of high variability can be identified. VP5 of BTV is much less variable than VP2 but still more so than the cognate AHSV VP5. In contrast, the NS3 protein of AHSV is much more variable than its BTV or EEV counterpart with maximum levels of NS3 variation up to 36% as compared to 10% for BTV. The AHSV NS3 variation is clustered into three discreet phylogenetic groups. All orbivirus NS3/NS3A proteins share a number of highly conserved structural features that include two hydrophobic domains (HD1 and HD2) that are involved in the interaction with the membrane. Most of the NS3 variation is located in HD1 and the adjacent variable region between HD1 and HD2. In the case of AHSV this region only has 13% identity compared to 64% in the case of BTV. NS3 of AHSV is also a highly toxic protein and mutation analysis has indicated that the toxicity is associated with the two hydrophobic domains. Expression of NS3 deletion mutants in bacterial cells has shown that both HD1 and HD2 are necessary for cytotoxicity and that removal of the adjacent N-terminal domains increases cytotoxicity. Preliminary results with different AHSV strains and the corresponding NS3 equivalent have indicated that the membrane permeabilisation effect of the individual NS3 proteins correlate with the permeabilisation effect of the corresponding viruses. These results would suggest that characterisation of the NS3 protein by itself might predict some phenotypic characteristics and potential membrane destabilisation effect of the corresponding virus.

Keywords

African horse sickness virus – Bluetongue virus – Cytotoxic protein – Hydrophobic domains – Membrane permeability – Non-structural proteins – Orbiviruses – Pair-wise alignment – Pathogenesis – Viroporin – Virulence.

Introduction

Although the pathogenesis and the clinical aspects related to the disease caused by orbiviruses such as bluetongue virus (BTV), African horse sickness virus (AHSV) and equine encephalosis virus (EEV) have been well characterised (7, 10, 24, 38), very little is known about the molecular basis of virulence and pathogenesis of these viruses (22). Most molecular studies have been focused on BTV, the orbivirus prototype, but in recent years an increasing number of the other orbivirus proteins have been studied in greater detail. The increase in sequence data within a serogroup has also enabled more comparative and functional analysis of the cognate proteins in different serogroups (43). The authors report on the

sequence variation in a few of the orbivirus proteins that are presumed to be associated with virulence or pathogenesis. The possible involvement of an orbivirus protein in the disruption of the cellular membrane is also addressed.

The factors involved in the pathogenesis and virulence of orbiviruses are complex and multi-factorial (22, 29, 37, 50). All the different steps in the viral replication cycle are potentially involved. The first of these is the interaction of the outer capsid proteins with cellular receptors resulting in viral entry and penetration into the cell (17). Also important in pathogenesis are the steps associated with removal of the outer capsid layer, the release of core particles in the cytoplasm (16) and the general velocity of viral replication. Equally important are possible differences in the cytopathogenicity of different virus strains and the budding and release of viruses from a cell (37). The latter may have a major impact on pathogenesis as it determines the spread of infection within and between organs. The different steps of viral replication are in turn influenced by the intracellular milieu, induced cellular functions and the capacity of the host to develop a proper immune response (37).

Pathogenesis may therefore be determined by any or a selected combination of the orbivirus viral proteins and host factors. Some viral proteins are nevertheless more likely than others to be important in determining pathogenicity. These are the proteins involved in the interaction with cellular receptors as well as those involved in uncoating and the spread of virus particles in and between cells. Proteins that affect membrane destabilisation are also of particular importance since these proteins often induce cytopathogenicity that could play a role in disease symptoms. In orbiviruses, this focuses the attention on the two outer capsid proteins involved in virus entry, as well as on the non-structural protein involved in the release of virus particles from a cell.

Orbivirus particles are icosahedral structures composed of a core particle or inner capsid surrounded by an outer capsid layer (20, 34, 35, 48, 49). The core is composed of two major structural proteins (VP7 and VP3) and three minor structural proteins (VP1, VP4 and VP6) that enclose a genome of ten dsRNA segments. The minor proteins have different enzymatic activities such as RNA polymerase (VP1), RNA capping (VP4) and helicase activity (VP6) that all support the role of the core proteins in viral replication and transcription (30, 31, 39). Outer capsid protein VP2 is primarily involved in cell attachment and virus penetration (17). VP2 is also the major determinant of serotype specificity (9, 17, 19) and involved in the induction of a neutralisation-specific antibody immune response. After entry of the virion into the cell, the virus is enclosed in endocytotic vesicles in which the outer capsid is removed, resulting in the release of transcriptionally active core particles into the cytoplasm. The other outer capsid protein, VP5, appears to play a major role in the destabilisation of the membrane of endocytosed vesicles (16).

In addition to the structural proteins, the viral genome encodes four non-structural proteins, NS1, NS2, NS3 and NS3A (2, 34, 35). NS3 and NS3A are encoded from two in-phase overlapping reading frames from the smallest of the ten genome segments (44). BTV NS3 is proposed to play a role in the final stages of BTV morphogenesis and release of virions from the cell (21). Baculovirus expressed AHSV NS3 is membrane-associated and cytotoxic in insect cells (41, 46). It has been suggested that it plays a role in virulence and influences the timing of virus release from infected cells (25, 29).

The orbivirus proteins that are assumed to play an important role in virulence and pathogenesis are therefore the major cellular attachment protein VP2 and the membrane destabilising proteins VP5 and NS3. In this paper, the sequence variation in these proteins is compared, identifying some of the conserved and variable amino acid sequence patterns and motifs on these cognate proteins. A unique feature of AHSV NS3 proteins is that it is highly variable amongst the different AHSV serotypes (42, 43). We have also compared different AHSV NS3's and NS3 deletion mutants with respect to their cytotoxic effect on bacterial and eukaryotic cells.

Percentage variation in the VP2, VP5 and NS3 proteins of different African horse sickness virus, bluetongue virus and equine encephalosis virus isolates

The VP2, VP5 and NS3 amino acid sequences of BTV, AHSV and EEV used in the analysis were obtained from GenBank. The origins of the various sequences are summarised in Table I and the proteins compared by means of pairwise alignment (PAUP version 4.0b8). The results are displayed in Figure 1. VP2 is the most variable of the proteins, with more than 80% of all BTV isolates showing a variation of between 48% to 64% between different serotypes. The minimum level of variation between serotypes is about 28%. The AHSV VP2 proteins are less variable, with almost 90% of all isolates varying between 46% to 52% with a minimum of 28% variation between different serotypes.

Table I
Number of sequences used in pair-wise comparison for each of the cognate proteins

Serogroup	Number of different virus isolates compared for each of the cognate proteins[a]		
	VP2	VP5	NS3
BTV	10[b]	8[d]	28[f]
AHSV	9[c]	7[e]	22[g]
EEV	–	–	15[h]

a) Details on virus serotypes are indicated in footnotes (b) to (h). In some cases, multiple isolates from the same serotype were included, as indicated by the number in brackets after the serotype number

b) BTV serotypes 23, 17 (2), 13, 11, 10, 3, 1(3)

c) AHSV serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9

d) BTV serotypes 17, 11 (3), 10, 2, 1 (2)

e) AHSV serotypes 4 (4), 6 (2), 9

f) BTV serotypes 1(4), 2 (2), 3 (3), 4 (4), 8 (3), 11 (4), 12, 13, 15, 16, 17 (2), 18 (2)

g) AHSV serotypes 2 (4), 3 (4), 4(1), 5 (1), 6 (4), 7 (3), 8 (4), 9

h) EEV serotypes: 1 (6), 2, 3 (2), 4, 5, 6 (3), 7

VP5 of BTV is also more variable than AHSV VP5, with most of the variation between different BTV serotypes clustered between 25% to 28% as compared to 15% to 19% in the case of AHSV. In contrast to that, AHSV NS3 is much more variable than BTV NS3. BTV NS3 sequence variation is confined to between 1% and 10% whereas the AHSV NS3 sequence variation is clustered in three distinct groups of 33%-36%, 21%-29% and 2%-12%. This result reflects the discreet phylogenetic NS3 clusters of the nine different AHSV serotypes, α, β and γ (25, 36, 42, 51). In EEV, the level of variation is confined to two groups showing 2%-5% and 13%-17% variation respectively, that also reflect the corresponding phylogenetic clusters (43).

Variable and conserved domains of VP2, VP5 and NS3

The observed variation in the different sequences is not evenly distributed over the total length of the different proteins. As illustrated in Figure 2 in the case of protein VP2, regions of high sequence diversity are flanked by more conserved sequences. The result shows the percentage of identical conserved amino acids in 150 amino sections of the different VP2 proteins of BTV and AHSV for which sequence data is available. The C-terminal region is the most conserved region of VP2, both for AHSV and BTV. In the case of AHSV, the 150 amino acids at the N-terminus are also more conserved. The most variable regions in AHSV are in the region of amino acid 600 to 690, as well as in the region of amino acid 220 to 410. The currently identified neutralisation specific epitopes fall within these variable regions (4, 9, 26, 27, 47).

The most variable BTV VP2 region is also from amino acids 590 to 690 with another highly variable region from amino acid 150 to 290. These regions also include the epitopes that determine serotype specificity (5, 12, 18, 28, 33). The AHSV and BTV proteins differ in length, complicating any comparisons. Furthermore, the very high variability of VP2 makes it very difficult to assign or identify specific VP2 virulence markers.

The results obtained with VP5 (not shown) are very similar, although the proteins are much less variable. A uniquely conserved feature is the presence of the amphipathic helices at the N-terminal ends of both BTV and AHSV. This region is involved in

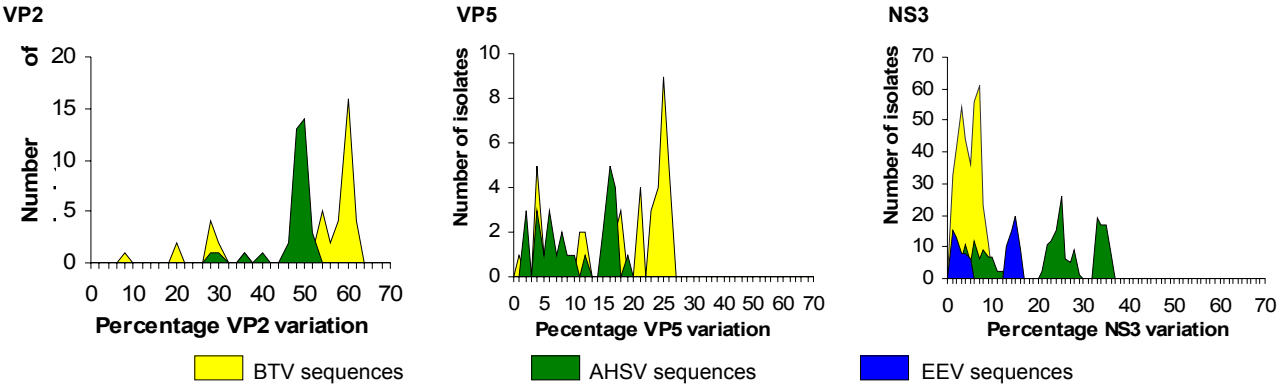

Figure 1
The percentage variation in pair-wise amino acid sequence comparison of the VP2, VP5 and NS3 proteins from bluetongue virus, African horse sickness virus and equine encephalosis virus
The number of isolates and the serotypes included in comparisons are as indicated in Table I
Comparison by PAUP analysis (PAUP version 4.0b8)

membrane destabilisation (16) and therefore potentially important in determining virulence characteristics. It is a highly conserved region and the limited number of virulent and avirulent AHSV isolates that were compared did not reveal any mutations in the amino acids that affect the amphipathic helices.

The variable and conserved domains of BTV, AHSV and BTV NS3 have been very well characterised (2, 3, 44, 45) and are shown in Figure 3. These regions include the amphipathic helix at the N terminus, the in-phase NS3/NS3A overlapping reading frames in the NS3 encoding genes, the proline rich region, the conserved domain, the two hydrophobic domains predicted to be transmembrane regions, and the variable region between the two domains. The percentage identity of the domains shown in Figure 3 reflects the result in Figure 1, illustrating that AHSV NS3 is more variable than the cognate EEV and BTV proteins. By far the most variable domains in AHSV NS3 are hydrophobic domain HD1 and the variable region between HD1 and HD2. In the case of BTV and EEV NS3 the sequences of HD1 are 73% and 64% conserved, respectively, whereas the AHSV HD1 domain shows no more than 35% identity. Even more variable is the region situated between the two hydrophobic domains, with only 13% identity in the case of AHSV compared to 64% and 61% in the corresponding BTV and EEV regions. It is not yet known if and how proteins such as NS3 contribute to the overall pathogenesis of the disease. If it does play a role, the sequence variation in hydrophobic

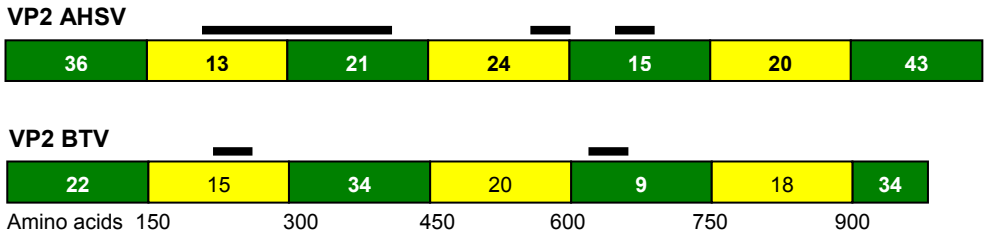

Figure 2
Variation in the VP2 amino acid sequence of bluetongue virus and African horse sickness virus
The numbers shown in the blocks are sequence identity based on the percentage of aligned identical amino acids in 150 amino acid sections of the 9 AHSV and the 10 BTV VP2 sequences indicated in footnotes (b) and (c) in Table I
The dark bars indicate the broad regions where neutralisation specific epitopes have been located in both viruses
Numbers below indicate amino acid residues

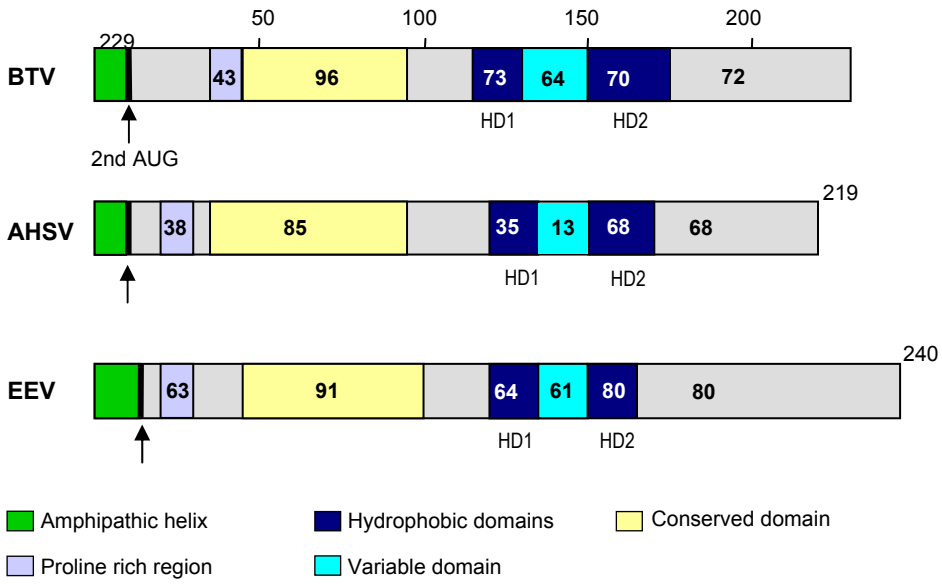

Figure 3
Comparison of the conserved amino acid sequence elements of the NS3 proteins of different orbiviruses
Numbers within the sections shown the percentage sequence identity for that region, based on the percentage of aligned identical amino acid sequences of the NS3 proteins indicated in footnotes (f), (g) and (h) in Table I

domains and the intermediate region could reveal strain differences that could be important in determining phenotypic characteristics of the virus associated with virulence and disease.

Possible relevance of NS3 and NS3 variation in disease

NS3 has a cytotoxic effect on its host cells when expressed in a baculovirus system (45), resulting in the death of approximately 90% of an infected cell population at 48 h post infection. Site-specific mutations in either of the two hydrophobic domains, predicted to be transmembrane regions, abrogated this cytotoxicity. However, modifications to other regions did not affect the detrimental effect of NS3 on its host cells. As illustrated in Figure 4, this includes deletion of the N-terminal 13 amino acids of NS3 that are predicted to form an amphipathic α-helix and mediate interaction with a cellular membrane trafficking protein in BTV (3). Therefore the cytotoxicity of AHSV NS3 is dependent on its membrane topography, and this involves both hydrophobic domains HD1 and HD2 (41).

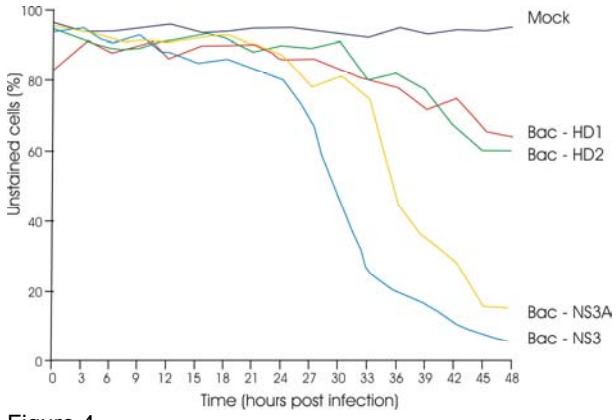

Figure 4
Effect of recombinant baculoviruses expressing African horse sickness virus NS3 or NS3 mutants on viability of Sf9 insect cells
Cells were infected with recombinant baculoviruses expressing AHSV-3 NS3 (Bac-NS3), a truncated NS3 lacking the 11 N-terminal amino acids (Bac-NS3A), NS3 with 4 non-polar amino acids substituted with charged amino acids in the first hydrophobic domain (Bac-HD1) or in the second hydrophobic domain (Bac-HD2)
Mock refers to uninfected Sf9 cells
Cell viability was determined by staining aliquots of cells at three-hourly intervals over a 48-h period with 0.2% trypan blue and counting stained (non-viable) cells

The mechanism whereby NS3 causes cell death is not known, however it shares many structural properties with a class of molecules termed viroporins. Viroporins are small viral proteins that interact with membranes, thereby modifying cellular membrane permeability (1, 6, 11, 13). This leads to changes in the metabolism and morphology of the cell, and promotes the release of viral particles (6, 13). This raises the questions of whether NS3 causes cell death by modification of the membrane permeability of the host cell and whether the high level of sequence variation reflects on any phenotypic, virulence or disease related properties of either the NS3 protein or the parental virus. One NS3 protein representing each of the three discreet phylogenetic clusters α, β and γ were selected, namely AHSV-4 (α), AHSV-3 (β) and AHSV-2 (γ) and investigated for its effect on mammalian cell membrane permeability.

The NS3 genes encoding the relevant proteins were cloned and expressed in Sf9 insect cells using the baculovirus expression system. Crude cell extracts from Sf9 cells expressing the three different NS3 proteins were prepared, and approximately equimolar amounts added externally to Vero cells. The permeabilisation of the Vero cell membranes was investigated using the hygromycin B (Hyg B) translation inhibition assay (23, 32). Only permeabilised cells allow the entry of the translation inhibitor Hyg B to inhibit the [^{35}S]-methionine uptake of cellular proteins. Using this method the percentage of permeabilised Vero cells could be calculated from the ^{35}S incorporation in the presence of Hyg B divided by the control ^{35}S incorporation in the absence of Hyg B for each sample. The results are shown in Figure 5. There were distinct differences in the degree of membrane permeabilisation caused by the different NS3 proteins. The addition of lysates containing AHSV-2 NS3 resulted in 72% permeabilisation of cells, AHSV-3 caused 62% permeabilisation and

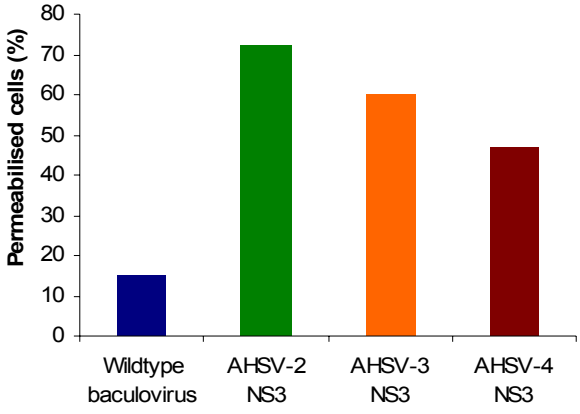

Figure 5
Membrane permeabilisation of Vero cells 180 min after addition of Sf9 cell lysates without NS3 or with NS3 from three different African horse sickness strains, measured using the protein synthesis inhibitor hygromycin B
The percentage [^{35}S]-methionine incorporated into the permeabilised cells was calculated as follows:
% permeabilisation = [S^{35} incorporation in the presence of Hyg B/S^{35} incorporation in the absence of Hyg B] ×100

AHSV-4NS3 caused 47%. NS3-free lysates from wildtype baculoviruses caused only a slight 15% increase in membrane permeabilisation after 3 h.

It was subsequently investigated whether the effect of the different NS3 proteins correlated with the effect of AHSV infection. The corresponding AHSV-2, AHSV-3 and AHSV-4 virus strains were used to infect Vero cells, and the Hyg B assay described above used to monitor the membrane permeabilisation at different times post infection (Fig. 6). Infection with AHSV-2, AHSV-3 and AHSV-4 resulted at 24 h post infection in 49%, 29% and 9% membrane permeabilisation, respectively, compared to the 2% in the case of mock-infected cells. The two results are diagrammatically compared in Figure 7. In both cases, AHSV-2 (whole virus or AHSV-2 NS3) has the most drastic effect on cell membrane permeability, followed by AHSV-3 with an intermediate effect and AHSV-4 with the least severe effect. Although permeability as the result of virus infection could be the result of other viral proteins and the interaction of multiple factors, and does not necessarily relate directly to pathogenesis, it is tempting to speculate that NS3 acts as a viroporin, and is a key determinant of the cellular cytopathogenicity of AHSV.

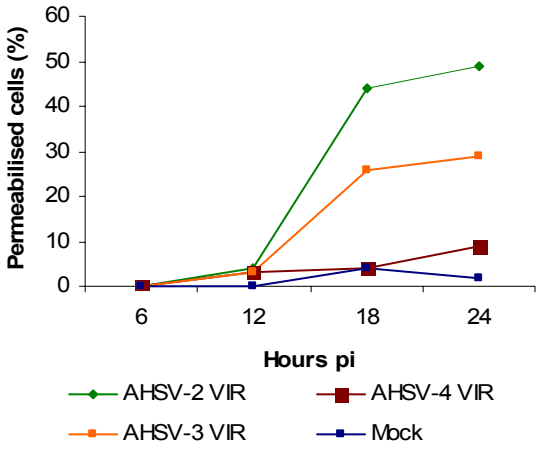

Figure 6
Membrane permeabilisation of Vero cells following infection with AHSV-2, AHSV-3 or AHSV-4 compared to mock infected control cells
Membrane permeabilisation assayed as in Figure 5

The effect of NS3 on bacterial cells

The cytotoxic nature of NS3 was further investigated in bacterial cells, using the pET vector expression system (40). This tightly regulated inducible system is particularly amenable for the synthesis of toxic proteins and has been used for analysing the cytotoxic proteins of a number of viruses. This includes the p10 protein of avian reovirus (6), NSP4 of rotavirus (8), Vpu and gp41 of HIV-1 (14) and the M2 protein of influenza virus (15).

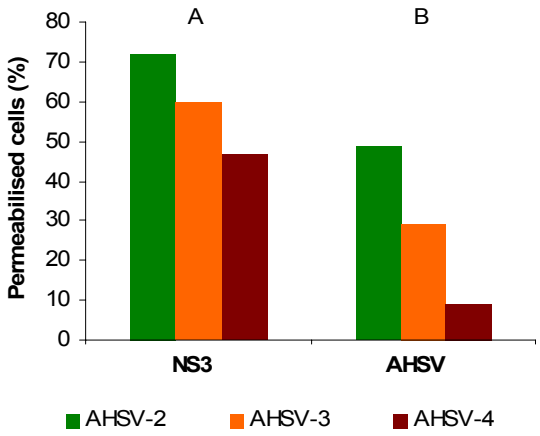

Figure 7
Comparison of the effect of externally added NS3 proteins (A) versus infection with the African horse sickness virus strains harbouring the corresponding NS3 genes (B) to Vero cell membrane permeability

The full-length NS3 gene and a series of NS3 truncated mutants (M1, M2, M3, M4, M5 and M6) were prepared and cloned in pET41c vectors (Fig. 8). The mutants represent different N-terminal and C-terminal truncations, including deletions of either one or both hydrophobic domains HD1 and HD2 (see diagrammatic representation in Fig. 8). The wild-type and mutant proteins were expressed in *Escherichia coli* cells and the effect on cell growth monitored at various times after induction by measuring the optical density at 600 nm. A growth curve indicating the percentage increase compared to a non-cytotoxic control was constructed for each of the mutants (results not shown). The results clearly indicated that the full-length NS3 protein was detrimental to *E. coli* growth. The results at 4 h post induction are summarised in Figure 8. The growth of cells expressing full-length NS3 was inhibited by 35% compared to the control. The expression of the C-terminal truncated M1 and M2 mutants that did not contain either the HD1 or HD2 hydrophobic domains did not inhibit bacterial growth in any way. The expression of the M3 and M4 mutants that each contained only one of the two hydrophobic domains had very little effect on cell growth, inhibiting growth by 10% and 5%, respectively. However, a dramatic inhibitory effect was seen in the case of the M5 and M6 mutants that each contained both hydrophobic domains. As early as 1 h after induction, cell density was already noticeably affected and by 4 h post induction, growth in the M5 and M6 mutants were inhibited by as much as 92% and 70%, respectively. Truncation of the regions adjacent to the hydrophobic domains therefore appears to

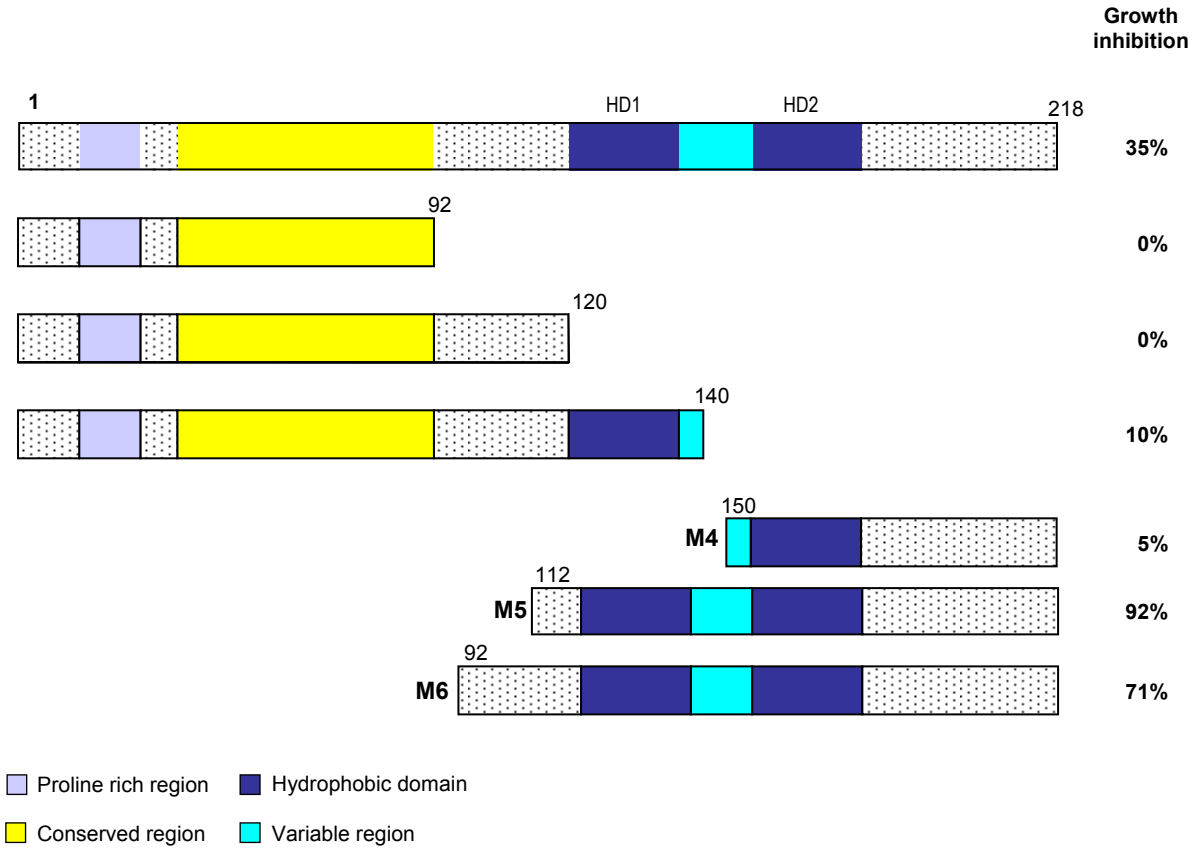

Figure 8
The cytotoxic effect of expressing different NS3 truncation mutants in *Escherichia coli* cells
A diagrammatic representation of the different mutants M1 to M6 is shown
The mutants were prepared and cloned in pET41c vectors
The percentage inhibition in cell growth after expressing the wild-type NS3 or the respective mutants M1 to M6 is given on the right
Cell growth was measured by optical density readings at 600 nm, 4 h post induction

enhance cytotoxicity compared to the wild-type NS3 control. The results also suggest that NS3 cytotoxicity requires both the hydrophobic domains.

Conclusion

The comparison between the variability of different orbivirus proteins that could be involved in virulence or pathogenicity indicated that VP2 and VP5 of BTV are more variable proteins than the cognate AHSV proteins. Distinct regions of high variability were identified in the internal sequences of VP2 of both viruses. In contrast, the NS3 protein of AHSV is much more variable than its BTV counterpart. AHSV NS3 is also a highly toxic protein and the expression of NS3 in bacterial cells appears to mimic the cytotoxic effect observed in mammalian cells. Mutation analysis indicated that cytotoxicity is associated with the presence of both the hydrophobic domains. Preliminary results have also suggested that the membrane permeabilisation effect of an individual NS3 protein correlates with the permeabilisation effect of the corresponding virus. These results would suggest that characterisation of an NS3 protein by itself might predict some phenotypic characteristics and potential membrane destabilisation effect of the corresponding virus. If the NS3 cytotoxicity can be linked to membrane permeabilisation and disease in future studies, the NS3 sequence might be able to predict some of the disease characteristics of the corresponding virus.

Acknowledgements

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