

IMMUNOGENICITY OF AHSV-4 VP7 CRYSTALS AND THEIR POTENTIAL USE AS A VACCINE DELIVERY PLATFORM

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Abstract

African horse sickness (AHS) is a devastating disease of many equids and in the absence of effective treatment, vaccination plays an important role. Concerns of the commercially available live attenuated vaccine have stimulated the development of novel recombinant vaccines. The viral protein VP7 of AHSV induces high levels of antibody in infected horses. Both viral replication, and expressed VP7 in insect cells, results in the generation of hexagonal crystalline structures composed entirely of VP7 (~5µm in diameter). The aim of this thesis was to explore the immunogenicity of VP7 crystals and their potential as a novel vaccine delivery platform.

Firstly, successful expression of AHSV-4 VP7 was characterised *in vitro* using recombinant baculovirus and live vaccinia vectored systems. These provided the necessary tools to explore VP7 crystal immunogenicity. Pilot VP7 vaccination studies were conducted in the equine and mouse animal model.

Secondly, insertions of AHSV-4 VP2a peptide into VP7 at amino acid position 200 did not compromise the ability of the modified VP7 constructs to form crystals and allowed investigations of the immunogenicity of VP7/VP2a200 in the mouse animal model.

This project demonstrated that VP7 crystals were poor immunogens in the absence of adjuvant and multiple vaccinations. However, immunogenicity was improved when crystals were partially solubilised with urea, indicating that soluble VP7 was more immunogenic than VP7 crystals. Hence, crystal generation may provide a mechanism for immune

evasion in AHSV infection. Nevertheless, both VP7/VP2a200 untreated crystals and urea treated VP7/VP2a200 crystals induced insert-specific antibodies. Importantly, VP2a peptide antibodies raised against urea treated crystals induced low levels of AHSV-4 neutralisation *in vitro*.

Future work should include exploring the immunological and protective potential of soluble VP7 and further investigating the use of VP7 as a vaccine platform.

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Chapter 1: Introduction

1.1. Scope of the project

African horse sickness (AHS) is a lethal disease of equids that has significant welfare, social and economic implications. In the absence of treatment, control relies heavily on vaccination. Concerns associated with the use of the commercially available live attenuated vaccine (particularly in countries free of disease) have promoted the development of new generation vaccines that do not inherit the same concerns. This project focuses on AHSV antigen VP7 which is considered to be a highly immunogenic antigen during AHSV infection. The project studies the structural qualities of AHSV VP7 crystals in the context of immunogenicity and investigates the use of VP7 crystals as a vaccine delivery platform to display immunologically important epitopes/peptides to the immune system.

1.2. General introduction

African horse sickness is an arthropod-borne disease of equids caused by an orbivirus belonging to the family *Reoviridae* (Calisher & Mertens, 1998). There are nine serotypes of African horse sickness virus (AHSV) which are transmitted by biting haematophagous midges of the genus *Culicoides*. AHS occurs regularly in most countries of sub-Saharan Africa and is regarded as one of the major scourges of the continent (Mellor, 1993; Mellor & Boorman, 1995).

AHS has devastating welfare, social and economic implications when the disease is introduced into a population of naïve equids, particularly horses, which are the most susceptible

equine species. In the UK alone the equine industry produces an estimated turnover of £7 billion pounds per annum (Allison, 2009). An AHS outbreak would therefore have severe economic consequences particularly for the horse racing and competitive horse sport sectors. The financial loss to the UK in the event of an AHS outbreak is estimated at over £3.5 billion in direct costs (Allison, 2009).

In the event of an outbreak, strategies are in place to minimize the extent of disease spread. These include vaccination programmes, restrictions in animal movement and quarantine, slaughter of infected animals and control of insect vectors. In light of the severity of the disease and the capacity for rapid expansion AHS is classified as an OIE listed disease (OIE, 2012).

1.3. Epidemiology and transmission

Horses are most susceptible to AHS and in a naïve horse population mortality rates can exceed 95% (Mellor & Hamblin, 2004). Donkeys and mules are considered to be less susceptible and although zebra can be infected they are asymptomatic and are resistant to the disease itself but are considered to be the main wildlife reservoir of AHSV in Africa (Mellor & Hamblin, 2004). Despite AHS being a disease of equids, AHSV can also infect certain carnivorous species via the oral route (e.g. ingestion of infected meat) (Alexander *et al.*, 1995). Antibodies against AHSV have been detected in African carnivores, elephants and dogs (Binepal *et al.*, 1992), although their epidemiological significance is unknown and is usually considered minimal.

Although AHS is currently endemic only in sub-Saharan Africa it has the capacity for rapid expansion over great distances with little prior warning (Mellor, 2008) and on several

occasions the disease has expanded to areas beyond these boundaries. Outbreaks in Northern Africa, Middle East and the Iberian Peninsula (Lubroth *et al.*, 2007; Portas *et al.*, 1999; Rodriguez *et al.*, 1992) demonstrate the existence of competent vector species of *Culicoides* in the regions, and all resulted in severe economic losses to the local equine industries. To date, AHS has not yet become endemic during any of these excursions although it did persist in western Mediterranean countries for 5 years, between 1987 and 1992.

Multiple AHSV serotypes have been detected at the northern limits of the virus' range (Aklilu *et al.*, 2012; Wilson *et al.*, 2009). The ability of *Culicoides* midges to travel on the wind for up to 100km and an increase in the international travel of equids suggest potential mechanisms for the further spread of AHSV into Northern Africa and Europe. Climate change, the availability of susceptible hosts and the presence of competent vector species in southern Europe (Calvete *et al.*, 2008) provides an opportunity for AHS to be maintained in the event of a future incursion.

AHSV is transmitted between susceptible equid hosts by the bite of adult female haematophagous *Culicoides* midges. The major vector of AHSV in Africa is *C. imicola* (Mellor, 1994). However, the distribution of *C. imicola* is not limited to sub-Saharan Africa and members of this midge species can be found in North Africa and southern Asia. It is also present and is spreading in southern Europe and is thought to be the principal vector species responsible for the epizootic outbreaks of AHS in Spain and Portugal (1987-1993) (Wittmann *et al.*, 2001). *C.imicola* is recognised as a major vector species for bluetongue virus (BTV - another closely related *Orbivirus* species). However, it is evident that BTV can also be

transmitted by several other *Culicoides* species, including members of the *C. obsoletus* and *C. pulicaris* complexes that are abundant throughout Europe. It is considered likely that certain other species of *Culicoides* will also be able to transmit AHSV.

Other vector species that have been recorded as being able to transmit AHSV include mosquitoes of the *Aedes*, *Anopheles* and *Culex* species, although none of these species have yet been shown to have a significant involvement in the transmission of disease under natural conditions (Maclachlan & Guthrie, 2010). The recent spread of bluetongue virus (BTV, which is also transmitted by *Culicoides* midges) in northern Europe (Carpenter *et al.*, 2009), the recent arrival of BTV-8 (Maan *et al.*, 2008) and Schmallenberg virus in the region (Gibbens, 2012), suggests that the whole of Europe may be at risk of further incursions of AHSV.

1.4. Molecular organisation and the structure of AHSV

1.4.1. Aetiology

Fifteen genera comprise the family *Reoviridae*, the largest being the genus *Orbivirus* with >29 species and 5 unassigned viruses (Calisher & Mertens, 1998; Mertens, 2000; Mertens, 2006; Roy *et al.*, 1994). Within the genus *Orbivirus*, the three most economically important viruses are BTV, AHSV and epizootic haemorrhagic disease virus (EHDV). BTV is the type species of the genus and serves as a paradigm for the other orbiviruses. AHSV shares many similarities with BTV and other members of the genus, e.g. equine encephalosis virus (EEV) and EHDV, in terms of their physicochemical characteristics, structure and antigenic properties (Maree, 2000; Walton *et al.*,

1992). Nine distinct serotypes of AHSV have been identified (Calisher & Mertens, 1998).

1.4.2. The viral genome

A triple-layered icosahedral protein capsid encapsulates the AHSV genome which consists of 10 linear double stranded RNA segments (Gouet *et al.*, 1999; Roy *et al.*, 1994). Each segment codes for at least one of the seven structural (VP1-7) or the four non-structural proteins (NS1-NS4) (Belhouchet *et al.*, 2011; Roy *et al.*, 1994) (Table 1.1). The AHSV genome segments are numbered 1 to 10 (Seg-1 to Seg-10) in order of decreasing molecular weight showing a 3-3-3-1 size distribution pattern during agarose gel electrophoresis. They have sometimes also been allocated into groups (large, medium and small) according to their migration during polyacrylamide gel electrophoresis (Bremer *et al.*, 1990; Roy *et al.*, 1994). The size of the ten AHSV genome segments range from 3965-756 bp with a total molecular weight of 13.2×10^6 Dalton (Vreede & Huismans, 1998). The total genome size of AHSV is approximately 19.5 kbp but can show minor variations according to virus strain and serotype (Bremer *et al.*, 1990).

Table.1.1: Summary of the genome segments and proteins of African horse sickness virus

Genome Segment and size (bp)	Protein nomenclature	Protein location	Protein structure and function
1 (3965)	VP1	Within the sub-core	RNA-dependent RNA polymerase, highly conserved
2 (3205)	VP2	Outer capsid	Highly variable, trimer, serotype specific, target for virus neutralising antibodies
3 (2792)	VP3	Sub-core capsid layer	T=2 symmetry, interacts with minor structural proteins, controls size and organization of capsid structure, highly conserved, RNA binding
4 (1978)	VP4	Within the sub-core	Core associated dimer, capping enzyme, Guanylyl-transferase, transmethylase-1, transmethylase-2 activity
5 (1748)	NS1	Cytoplasm	Conserved, forms tubular structures of unknown function in the cytoplasm
6 (1566)	VP5	Outer capsid	Variable, trimer, causes membrane fusion indicating a role in membrane penetration
7 (1169)	VP6	Within the sub-core	Binds single stranded and double stranded RNA, helicase
	NS4	-	Nuclear localization, role in antiviral host responses
8 (1167)	VP7	Outer capsid	Immuno-dominant major serogroup specific antigen, role in cell entry, T=13 symmetry, forms flat hexagonal crystals made up of layer of hexameric rings of trimers.
9 (1166)	NS2	Cytoplasm	Phosphorylated protein, associated with virus inclusion bodies, single stranded RNA binding
10 (756)	NS3 and NS3a	Cell membrane	Glycosylated membrane protein, involved in virus release, cytotoxic, highly variable
	NS5	-	Unknown

(Mertens, 2006)

1.4.3. The AHS Virion

BTV and AHSV are comparable in molecular structure and composition (Basak *et al.*, 1996). AHSV virions are spherical in appearance, non-enveloped, 60-80 nm in diameter and organized into three concentric protein layers that display icosahedral symmetry (Figure 1.1) (Gouet *et al.*, 1999; Roy *et al.*, 1994).

The innermost capsid layer of AHSV is composed of 120 copies of VP3, arranged with T=2 quasi-equivalent icosahedral symmetry. This 'sub-core' layer surrounds the ten segments of dsRNA that make up the AHSV genome as well as the minor, enzymatically active structural proteins VP1, VP4 and VP6 that form the polymerase complexes (Basak *et al.*, 1996; Diprose *et al.*, 2001; Grimes *et al.*, 1998; Roy *et al.*, 1994). The VP3 layer (encoded by genome segment 3) provides a 'scaffold' for these polymerase complexes, which are attached to the inner surface, at the fivefold vertices of the sub-core shell (Diprose *et al.*, 2001; Grubman & Lewis, 1992). It also determines the overall size of the particle and the organisation of the core-surface and outer-capsid layers and therefore is under very significant function constraints reflected by the highly conserved nature of its amino acid sequence. VP7, encoded by AHSV genome segment 8, constitutes the 'outer' capsid layer of the virus-core, assembling into 260 trimeric units with T=13L icosahedral symmetry (Grimes *et al.*, 1998). The trimers of VP7 combine to generate a lattice of 5 and 6 membered rings that make up a 'bridge' between the inner and outer capsid layers (Maree, 2000; Roy *et al.*, 1994). The outer capsid layer is composed of major proteins VP2 and VP5 (Roy *et al.*, 1994) encoded by genome segments 2 and 6 respectively. VP2 is the most variable of the AHSV proteins and is the principle serotype-specific antigen containing the

main determinants for virus neutralization (Burrage *et al.*, 1993; Martinez-Torrecuadrada *et al.*, 2001; Roy *et al.*, 1994). VP5 also contains neutralizing epitopes (Martinez-Torrecuadrada *et al.*, 1999) but is considered to play a less important role in the determination of virus serotype (Guthrie *et al.*, 2009).

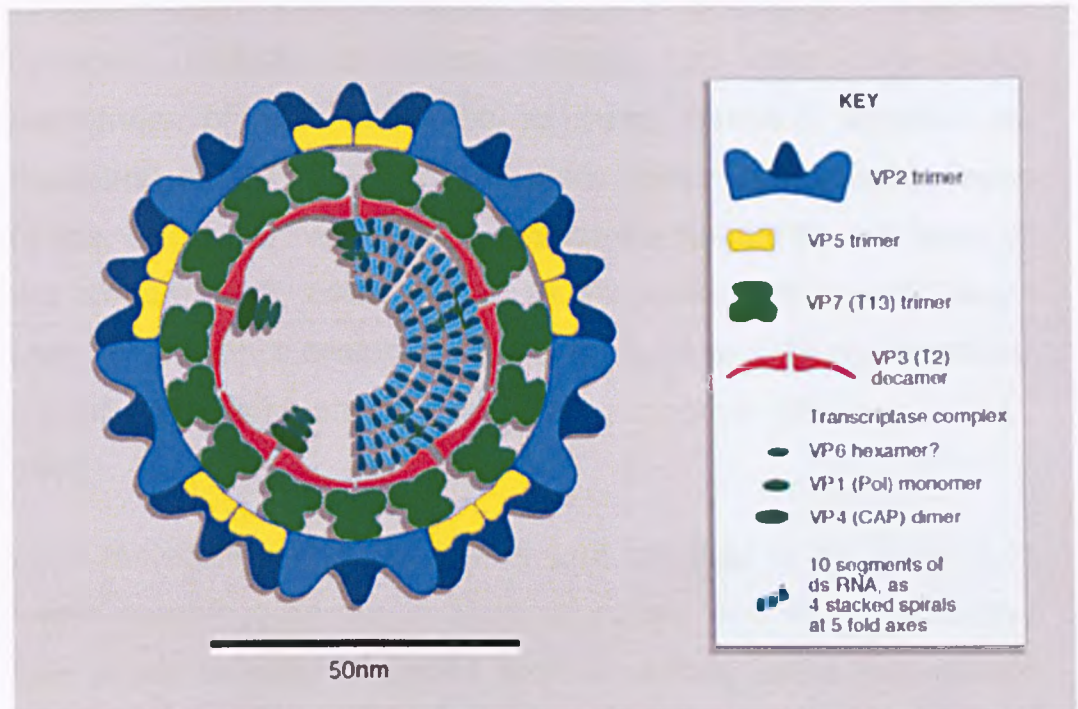


Figure 1.1: Diagram of the orbivirus structure.

Figure published in Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses, Mertens P.P.C., Attoui H., Duncan R., Dermody T.S. (Eds.), Reoviridae, pp. 447–454 (Reproduced with permission).

1.4.4. VP7 and the atomic structure of VP7 trimers

Structural protein VP7 is encoded by segment-8 and is approximately 350 amino acid residues in length and 37.8kDa is size. VP7 is a highly immunodominant protein, is highly conserved and is a primary determinant of the *Orbivirus* serogroup (Grimes *et al.*, 1998). It is often used as the basis for the serological diagnosis and identification of infections by

the members of different *Orbivirus* species/ serogroups. Greater than 98% amino acid identity has been described for VP7 between AHSV serotypes (Bachanek-Bankowska, 2012; Williams *et al.*, 1998). The AHSV and BTV VP7 amino acid sequence shares ~43% identity (Basak *et al.*, 1996). However, VP7 of BTV and EHDV is considered more closely related than VP7 of AHSV and BTV (Williams *et al.*, 1998).

Unsurprisingly, the structure of VP7 is highly conserved between related orbiviruses. Within the virus core three monomers of VP7 assemble to form spiralled trimers. As described earlier, 260 trimeric units comprise the outer layer of the virus core, whereby the relatively flat bottomed base of the trimeric unit associate on to the surface of the VP3 sub-core in 5 distinct orientations (P, Q, R, S and T) representing 13 different positions for the VP7 monomer (Grimes *et al.*, 1998).

Each molecule within the trimer unit consists of an upper and lower domain differing in their structure and hydrophobicity. The upper domain of AHSV VP7 is slightly more hydrophilic than the lower domain and contains one third of the polypeptide chain exhibiting an antiparallel beta sandwich and jelly roll topology (Basak *et al.*, 1996)(Figures 1.2 and 1.3). VP7 is largely hydrophobic with an amino acid sequence rich in alanines, methionines and prolines (Basak *et al.*, 1996). Because VP7 is insoluble, only the upper domain of AHSV VP7 has been resolved at an atomic level by X-ray crystallography (Basak *et al.*, 1996). The lower domain is predicted to be composed of nine alpha helices with long extended loops and contains both the 'N' and the 'C' termini of the protein (Figure 1.3), similar in structure to that of BTV VP7, which has been

resolved to atomic resolution by X-ray crystallography (Basak *et al.*, 1996; Grimes *et al.*, 1998).

Unique to AHSV, replication in cell culture or over expression of AHSV VP7 in insect cells results in the formation of two-dimensional hexagonal VP7 crystals (Burroughs *et al.*, 1994). VP7 crystals have never been observed during infection with any other orbivirus. The crystals are approximately 5µm in diameter but crystal size can range up to 25µm (Chuma *et al.*, 1992; Maree & Paweska, 2005). The crystal structures are comprised of VP7 trimers arranged in 5-6 membered rings mimicking the organisation displayed by VP7 on the outer core surface (Burroughs *et al.*, 1994; Chuma *et al.*, 1992). The role of the VP7 crystals during AHSV replication remains unknown.

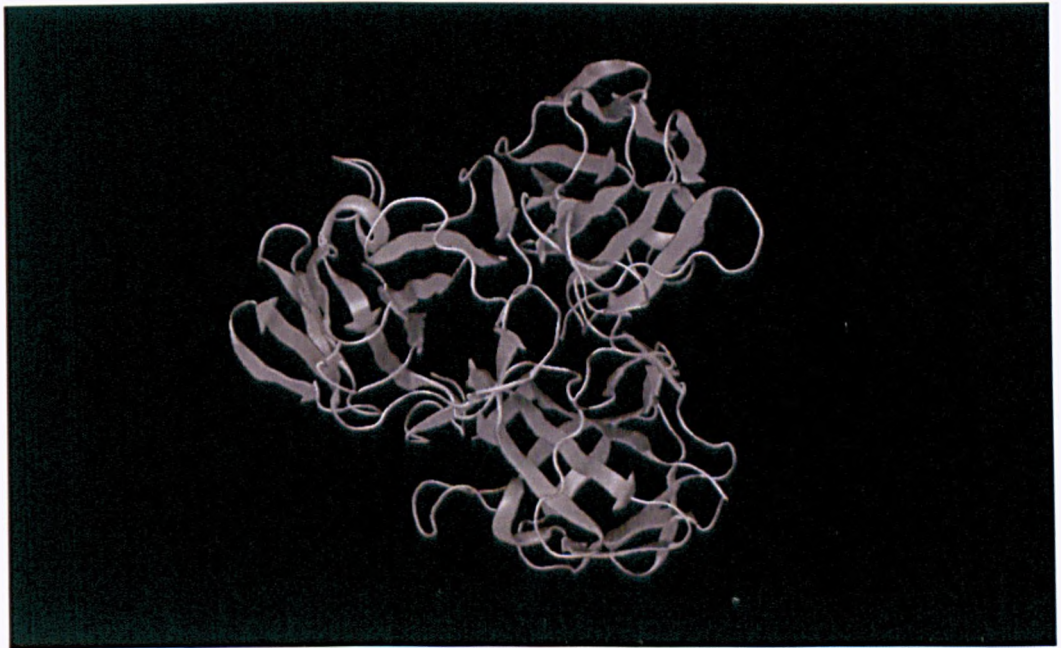


Figure 1.2: The atomic structure of the top domain of an African horse sickness VP7 trimer (top view).

Generated by Protean 3D imaging software, using the VP7 sequence obtained from Basak *et al.* 1996.

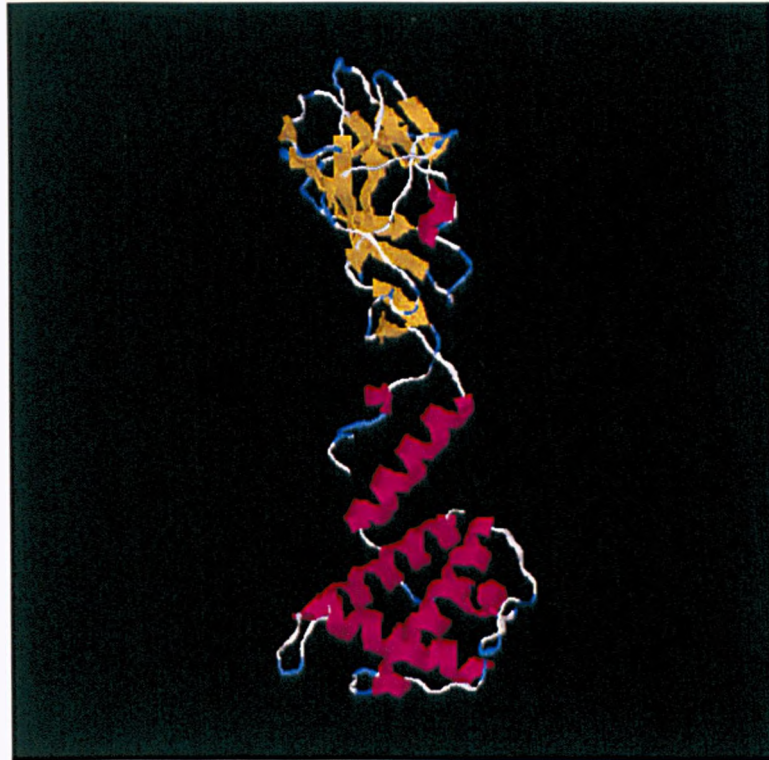


Figure 1.3: The predicted full atomic structure of a VP7 monomer.

Generated by I-Tasser software using the VP7 sequence obtained from Basak *et al.* 1996.

1.5. Orbivirus replication in the cell

Very little is known specifically about AHSV replication in the cell. However, it is considered likely that the underlying mechanisms are similar to that of the prototype orbivirus BTV.

BTV-10 is reported to enter cells by receptor mediated endocytosis (Forzan, 2007). In contrast BTV-1 has been shown to enter cells via a clathrin-independent macropinocytosis-like mechanism (Gold *et al.*, 2010). In both cases the initial attachment process is mediated via outer capsid protein VP2 that also controls the virus serotype. It is therefore possible that different serotypes of these viruses may attach to and enter cells via distinct mechanisms. During entry of BTV-10 the virus attaches to the cell, mediated by VP2, and is then absorbed and internalised by formation of a clathrin-coated

vesicle (Hassan & Roy, 1999) that subsequently fuses with the early endosome. A low pH environment within the endosome prompts removal of VP2 and VP5 from the virus core and allows VP5 fusion with the endosomal membrane, penetrating the membrane and releasing the intact core particle into the host cell cytoplasm (Mertens, 2003).

As the outer capsid proteins are lost the core becomes transcriptionally active. The dsRNA genome segments, the mRNA synthesising enzymes, RNA polymerase (VP1), RNA capping enzyme (VP4) and RNA helicase (VP6) are contained within the core thus avoiding exposure of dsRNA to the host cell's cytoplasm and activation of antiviral responses. Newly synthesised mRNA is released into the cytoplasm via open pores at the fivefold axis in the VP3 sub-core shell ready for translation into viral protein (Mertens, 2003).

Viral proteins are transported to virus inclusion bodies (VIB), the sites of virus assembly and replication in the cytoplasm. The VIBs contain all of the newly synthesised viral proteins, particularly NS2, and viral RNAs. The VIB matrix protein NS2 can be phosphorylated by a ubiquitous cytoplasmic 'protein kinase 2' leading to its aggregation and the formation of VIB. The interaction of NS2 with viral ssRNAs and other viral proteins and the formation of VIB within the cytoplasm of infected cells, are early steps leading to the assembly of progeny core particles within the VIB. VP2 and VP5 are added to the progeny core particles as they are released from the VIB surface (Mertens, 2003). Transport to the cell surface is mediated by microtubules (involving VP2 and vimentin interactions) within the cytoplasm (Bhattacharya *et al.*, 2007). Virus release occurs by budding, by direct penetration through the cell membrane, or by cell lysis (Martin *et al.*, 1998). NS3 is

thought to be involved in at least some of these processes (van Niekerk *et al.*, 2003).

1.6. Pathogenesis

In vivo, primary AHSV replication is thought to occur in the regional lymph nodes, although current data for BTV suggests that primary replication may occur in skin fibroblasts and dendritic cells, prior to transport of the virus to the lymph nodes (Schwartz-Cornil *et al.*, 2008). The virus then disseminates around the host's body associated with the cellular fraction of the blood. AHSV has been shown to infect and replicate in equine peripheral blood mononuclear cells (PBMCs) (Burrage & Laegreid, 1994b). AHSV infects target organs such as the lungs, spleen and lymphoid tissue (Clift & Penrith, 2010) leading to further virus replication at these sites and giving rise to a secondary viraemia (Coetzer, 1994) within less than 9 days. The duration of viraemia is species dependent. In horses, it typically does not exceed $10^{5.0}$ TCID₅₀/ml and has been reported to last only 4 to 8 days. In zebra and donkeys viraemia may last as long as 4 weeks although the level of viraemia is much lower (10^3 TCID₅₀/ml) (Hamblin *et al.*, 1998; Mellor & Hamblin, 2004).

Similar to observations of BTV infection, AHSV also shows endothelial tropism (Burrage & Laegreid, 1994a; Laegreid *et al.*, 1992), which results in severe microvascular injury particularly in the heart and lungs. AHSV replication has been described in the endothelial cells of the lungs (Laegreid *et al.*, 1992). However, AHSV replication in the lungs has been documented at very low levels and therefore cannot be solely responsible for the extent of damage and severe injury observed. This indicates that AHSV pathogenicity is

multifactorial (Laegreid *et al.*, 1992) and both direct (e.g. viral replication) and indirect injury (e.g. release of cytokines from affected cells and monocyte –macrophages) may influence the extent of injury (Clift & Penrith, 2010).

AHS is a highly lethal disease of equids that can exhibit a number of different clinical forms (Burrage & Laegreid, 1994b; Coetzer, 1994; House, 1993; Mellor & Hamblin, 2004). It has been suggested that several factors contribute to the severity, pathogenicity and the 'form' that the disease manifests and consequently the overall outcome of AHS infection. These include both host-specific factors (such as species, sex, age, immune status and genetics) and virus-specific factors (strain-variation/virulence phenotype, dose and route of infection).

There is strong evidence to suggest that host genetics plays an important role (Burrage & Laegreid, 1994b; Oura *et al.*, 2012). The differences in AHS susceptibility and outcome between equid species indicate a genetic influence. Horses are highly susceptible and frequently succumb to the severe forms of AHS infection. Donkeys are considered to be much less susceptible, whilst zebra are considered the reservoir host and may only develop a relatively mild fever. African donkeys are also less susceptible than European donkeys (Abu Elzein E.M.E., 1989; al-Afalet *et al.*, 1998). Indigenous horse populations also show a higher degree of resistance to AHS compared to highly susceptible imported 'exotic' horse breeds (Burrage & Laegreid, 1994b; Doutre, 1962; Oura *et al.*, 2012).

1.6.1. Clinical disease

Traditionally, AHS is categorized into four clinical presentations each associated with different mortality rates and pathogenicity (Burrage & Laegreid, 1994b; Erasmus, 1973). However, the mechanisms underlying the clinical

manifestations of AHS are not yet fully understood. Clinical disease varies from fever to impairment of cardiac and respiratory functions and death, according to the form of disease (Mellor & Hamblin, 2004). The four forms of AHS are: the peracute pulmonary form, the mixed form, the sub-acute cardiac form and the febrile form (Burrage & Laegreid, 1994b).

The 'pulmonary' peracute form ('Dunkop' 'thin head') of AHS is typically observed in completely susceptible horses that have been infected with virulent virus strains. The incubation period is relatively short (3-5 days). Fever precedes the onset of rapid respiratory distress where the disease is characterised by severe dyspnoea, coughing spasms, profuse sweating and frothy fluid from the nares (Coetzer, 1994; House, 1993; Mellor & Hamblin, 2004). Recovery is rare and the outcome grave with a mortality rate greater than 95%.

The sub-acute cardiac form ('Dikkop' 'thick head') is usually observed in horses that are infected with low virulence virus strains or exhibit pre-existing immunity but are subsequently infected with a heterologous virus (Mellor & Hamblin, 2004). The incubation period is longer than the pulmonary form of disease (7-14 days) and the mortality rate lower (at around 50%). Disease is typically characterised by oedematous swelling of the neck and head including characteristic bulging of the supraorbital fossae (Coetzer, 1994; Erasmus, 1973; Mellor & Hamblin, 2004). Petechial haemorrhages may be observed in the conjunctivae and the mouth and tongue (Coetzer, 1994).

The mixed form is considered the most common form of disease and is a mixture of the pulmonary and the cardiac

syndromes. Mortality rate is approximately 70% in susceptible populations (Coetzer, 1994; Mellor & Hamblin, 2004).

AHS fever is the mildest form of the disease and is observed in less susceptible species such as the zebra, African donkey, in resistant horse breeds from historically endemic countries and in horses that are at least partially protected by vaccination (Coetzer, 1994; House, 1993; Mellor & Hamblin, 2004). Animals may suffer intermittent pyrexia. The incubation period varies from 5 to 14 days. Typically no mortality is observed (Coetzer, 1994; Mellor & Hamblin, 2004; OIE, 2012).

1.6.2. Pathology

Gross pathology

When an animal exhibits the pulmonary form of AHS, the most prominent observation is severe pulmonary oedema and hydrothorax (Gomez-Villamandos *et al.*, 1999). Gelatinous exudate invades sub-pleural and interlobular tissues and the bronchial tree and lungs are filled with frothy fluid (Maurer & McCully, 1963). This can lead to the animal being unable to breathe and in effect it can drown in the froth. Ascites can occur in abdominal and thoracic cavities and the mucosal lining of the stomach may be hyperaemic and oedematous (Mellor & Hamblin, 2004).

When animals exhibit the cardiac form of AHS, the most prominent observation is that subcutaneous intramuscular tissues and lymph nodes are filled with gelatinous exudate. Hydropericardium is also observed in the pericardial cavity and haemorrhage can be observed on the epicardial or endocardial surfaces of the heart (Clift & Penrith, 2010; Gomez-Villamandos *et al.*, 1999). Petechial haemorrhages may also be observed on membranes of the caecum, colon and rectum

(Coetzer, 1994; Gomez-Villamandos *et al.*, 1999; Mellor & Hamblin, 2004).

In the mixed form of AHS, lesions common to both the pulmonary and cardiac forms of the disease are observed.

Histopathology

The histopathological changes observed during AHSV infection are a direct result of the virus' endothelial tropism whereby damage to endothelial cells result in the impairment of circulation and increased vascular permeability (Clift & Penrith, 2010). In the lungs, protein-rich serous fluid enters the alveoli and interstitial tissue where fibrin masses can fill the alveolar spaces (Coetzer, 1994; Mellor & Hamblin, 2004). The high protein composition of the fluid leads to the production of protein-stabilised froth which can block airways and prevent gas exchange. In addition, macrophages, monocytes, neutrophils infiltrate the alveoli (Carrasco *et al.*, 1999b; Coetzer, 1994). The activation of pulmonary intravascular macrophages is associated with additional changes in the pulmonary microvasculature mediated by the release of chemical inflammatory mediators (Carrasco *et al.*, 1999a).

1.7. Laboratory diagnosis

Rapid diagnosis and prompt virus serotyping are critical for the development of effective control strategies and epidemiological surveillance of AHS. AHS can be confused with other equine diseases, such as equine encephalosis, caused by members of a distinct but related *Orbivirus* species '*Equine encephalosis virus*' that has epidemiological similarities to AHSV including its geographical distribution and host range (Mellor & Hamblin, 2004). Other equine diseases including equine infectious

anaemia, equine morbillivirus pneumonia, equine viral arteritis and babesiosis may also be confused with AHS (OIE, 2012).

Diagnostic methods for AHSV are based on isolation and identification of the virus and detection and identification of viral nucleic acid, virus antigens or specific antibodies (House *et al.*, 1990). Traditionally virus isolation and serological identification were the diagnostic methods of choice but these have largely been superseded by faster more sensitive and more reliable molecular diagnostics, such as conventional reverse transcriptase (RT)-PCR (and sequence analyses) and real-time *q*RT-PCR assays (Bachanek-Bankowska *et al.*, 2014; Coetzer, 1994; Maan *et al.*, 2011b; Maree & Paweska, 2005). RT-PCR is a highly sensitive assay that reverse transcribes the viral RNAs into cDNAs which are then 'amplified' and detected either by gel electrophoresis or (in real time *q*RT-PCR) by a fluorescent signal.

Several agarose based conventional RT-PCR assays have been described for detection of AHSV-specific RNA, targeting viral-genome segments 2, 3, 7 and 8 (Aradaib, 2009; Bremer & Viljoen, 1998; Maan *et al.*, 2011a; OIE, 2012; Stone-Marschat *et al.*, 1994; Zientara *et al.*, 1998). The RT-PCR assays targeting Seg-2 can be used to reliably identify the virus serotype in a single day from a blood sample rather than the weeks required for virus isolation (which may be unsuccessful) and neutralisation tests (VNT). They also generate cDNAs that are suitable for sequencing and phylogenetic analyses which can be used to confirm the initial typing results. Real-time *q*RT-PCR is a quantitative polymerase chain reaction that enables the detection and quantification of the target RNA in 'real time' (within hours). Several real-time *q*RT-PCR assays have also been developed against AHSV- specific RNA based

on viral genome segments 1, 2, 3, 5 and 7 (Bachanek-Bankowska *et al.*, 2014; Fernandez-Pinero *et al.*, 2009; Rodriguez-Sanchez *et al.*, 2008).

Serological diagnostic methods for AHSV infection include complement fixation test (CF), immuno-blotting and VNTs. However, these methods have limitations making them unsuitable for extensive, high throughput epidemiological studies (Williams *et al.*, 1993). The VN procedure is very laborious and time consuming (Maree & Paweska, 2005) and is both insensitive and prone to error. Some zebra and donkey sera are anti-complementary to CF (OIE, 2012). Immuno-blotting is also laborious and time consuming making it suitable only for screening small numbers of serum samples (OIE, 2012).

Competitive and indirect ELISAs are alternative diagnostic methods for detection of AHSV specific antibodies (Williams *et al.*, 1993) that are better suited to high throughput. As described previously, VP7 is an immunodominant antigen that is conserved between serotypes within each *Orbivirus* species (orbivirus-serogroup specific) (Bremer *et al.*, 1990; Gumm & Newman, 1982; Roy, 1991). A 'VP7-specific indirect competitive ELISA', is prescribed by OIE for detection of AHSV-specific antibodies (OIE, 2012) and is commercially available. Several other AHSV VP7 based ELISAs have also been described (Bremer *et al.*, 1994; Wade-Evans *et al.*, 1993) including a VP7 crystal based ELISA (Maree, 2005).

1.8. African horse sickness infection and antiviral immunity

1.8.1. Innate immunity

The immune response is a dynamic process, varying in nature and specificity over time. In the earliest stages of AHSV infection the first line of defence is a rapidly occurring innate response which offers non-specific defence against pathogens and can prime a more specific and long lasting adaptive immune response (Murphy, 2008).

Pathogen-associated molecular patterns (PAMPs) are components of a microorganism (for example microbial nucleic acid) that alert the immune response to an invading pathogen, for example during viral infection. PAMPs are recognised by pattern recognition receptors (PRRs, including Toll-like receptors (Akira *et al.*, 2001)) which initiate a cascade of signals that leads to the up-regulation of co-stimulatory molecules (such as CD40, CD80 and CD86) and the synthesis and secretion of cytokines from virus infected cell and mononuclear cells (Takeuchi, 2009) that shape the immune response to follow.

Interferons (IFNs) are a family of cytokines that constitute an early component of the host's antiviral immune defences. IFN binds to interferon receptors on infected and nearby cells and regulates the expression of antiviral and immunomodulatory genes of the innate and adaptive immune system (Le Page *et al.*, 2000; Samuel, 2001). IFN-induced antiviral gene activation leads to the synthesis of proteins (such as 2'5' oligo A synthase) in the infected cells, which limits viral protein synthesis and ultimately viral replication, for example via the degradation of viral RNA (Le Page *et al.*, 2000; Takeuchi, 2009). IFN also stimulates cellular components of the immune

system (for example macrophages and natural killer cells), induces apoptosis in the target cell and directly or indirectly primes and regulates the adaptive immune response (e.g. by increasing the expression of MHC I and MHC II molecules) (Le Page *et al.*, 2000). The role of the adaptive immune response and MHC molecules will be discussed later in the chapter.

There are two families of interferon, Type I (IFN α , β and ω) and Type II (IFN γ) (Murphy, 2008; Samuel, 2001). All nucleated cells produce Type I IFN, Type II IFN production is mainly secreted by lymphocytes, natural killer cells and macrophages (Le Page *et al.*, 2000). Several studies have demonstrated the particular importance of Type I interferon (IFN α , IFN β) in antiviral immunity (Muller *et al.*, 1994). Mice lacking IFN I (α/β) receptors were observed to be more susceptible to RNA viruses (for example the filoviruses: Ebola and Marburg virus), where interferon plays an important role in controlling infection. Normal BALB/c mice are resistant to filovirus infection, whereas when treated with anti-IFN- α/β antibodies, or as with the IFN- α/β R $^{-/-}$ strain of A129 mice, they are susceptible to lethal infection by a number of filoviruses (Bray, 2001; Muller *et al.*, 1994). Previous studies have indicated that IFN α and IFN β can play an important role in controlling AHSV and BTV infections (Castillo-Olivares *et al.*, 2011; Jameson *et al.*, 1978; O'Hara *et al.*, 1998). The use of interferon knockout mice (IFN α/β $^{-/-}$ R strain A129) as a mouse model for AHSV and BTV has been described (Calvo-Pinilla *et al.*, 2009a; Castillo-Olivares *et al.*, 2011).

Natural killer (NK) cells, a sub-set of lymphocytes which do not have receptors that recognise specific antigens, represents another component of the vertebrate host's innate response. NK cells are granular lymphocytes capable of mediating

antibody dependent cell-mediated cytotoxicity (ADCC) that respond rapidly to other virus-infected cells that are marked by surface interactions with antibodies (Janeway, 2001; Murphy, 2008).

The complement system opsonizes pathogen-infected cells and induces cell death via inflammatory responses that involve a series of plasma protein reactions. Most complement proteins are proteases that are themselves activated by proteolytic cleavages. Complement can be activated through three different pathways; the classical pathway, the mannan-binding lectin pathway and the alternative pathway. Each pathway culminates in the formation of C3 convertase. The complement system ultimately results in the release of complement components that mediate inflammation, opsonizing pathogens and the direct killing of pathogens by the formation of a membrane attack complex (Dunkelberger & Song, 2010; Janeway, 2001; Murphy, 2008).

1.8.2. Adaptive immunity

Unlike the innate response, 'adaptive immunity' generates immunological memory and includes both humoral and cell-mediated immune responses (Janeway, 2001).

The humoral immune response is mediated by antibodies, derived from B-cell lymphocytes. B-cell receptors (membrane bound immunoglobulins) located on the surface of the B-cell lymphocytes bind to and recognise native antigen with high specificity. B-cells can also themselves act as antigen presenting cells (APCs) (Murphy, 2008; Rodriguez-Pinto, 2005). T-helper cells (CD4⁺ lymphocytes) assist and regulate immunological process including the maturation of B-cells and the activation of macrophages and cytotoxic T-cells (cell mediated immunity). Cytotoxic T-cells (CD8⁺ lymphocytes)

mediate cell death by lysis. Unlike B-cells, T-cells can only recognise antigens once they have been processed by APCs and are displayed on major histocompatibility (MHC) molecules (Penn, 2002). MHC I molecules present endogenous proteins stimulating CD8+ T cells, whilst MHC II molecules present exogenous protein fragments stimulating CD4+ T-cell responses (Janeway, 2001; Murphy, 2008).

MHC I molecules are found on all nucleated cell surfaces (Murphy, 2008; Penn, 2002). The MHC I molecules mainly present endogenous peptide antigens to CD8+ T lymphocytes (CTLs). Intracellular peptides derived from proteins in the cytosol are degraded by proteasomes and loaded onto the MHC I molecule within the endoplasmic reticulum. The MHC I-peptide complex is then transported to the cell surface (via the golgi network) to be presented to CD8+ lymphocyte receptors (Murphy, 2008; Penn, 2002; Vyas *et al.*, 2008). In addition, APCs (such as dendritic cells) can also present exogenous protein on MHC I molecules by means of cross-presentation (Bachmann *et al.*, 1996; Brode & MacAry, 2004; Cresswell *et al.*, 2005; Moron *et al.*, 2003; Rock & Shen, 2005).

MHC II molecules are found only on specialised APCs (Penn, 2002). Extracellular exogenous proteins are captured by APCs (for example via endocytosis) and are subsequently degraded by proteases in low pH endosomal compartments. These peptides are bound to MHC II molecules and transported to the cell surface, whereby the antigen is presented to T-lymphocytes (Murphy, 2008; Penn, 2002).

Humoral immunity

As described previously humoral immunity is mediated by antibodies derived from B-lymphocytes. Upon interaction with an antigen (for example upon recognition and binding), naïve

B-cells become activated. Once activated, and in the presence of co-stimulatory responses provided by T- helper cells, the B-cells can respond in two ways 1) to proliferate and the progeny B-cells differentiate into plasma cells secreting antibodies; 2) to proliferate into memory B-cells, primed and ready to respond to repeat exposure to the priming antigen (Janeway, 2001).

Pathogens typically possess structural qualities (e.g. repetitive structures) that are capable of inducing a T-cell independent antibody response by cross-linking BCRs, predominantly eliciting IgM antibodies in the absence of direct T-helper cell co-stimulation (Bachmann & Jennings, 2010; Bachmann & Zinkernagel, 1996; Fehr *et al.*, 1998). The generation of antibodies in this manner occurs earlier and more readily than a T-cell dependent response alone. However, this T-cell independent antibody response is not sufficient to generate complete humoral immunity. T-helper cell assistance is required to induce high affinity isotype switching and the generation of immunological memory (Janeway, 2001; Murphy, 2008).

Antibodies are Y shaped molecules. The arms of an antibody are highly variable binding sites that facilitate specific antigen recognition. The stem is far less variable and determines the antibodies isotype. There are five different antibody isotypes; IgM, IgD, IgG, IgA and IgE (Table 1.2) (Murphy, 2008).

Table 1.2: Antibody classes and their functions

Antibody class	Features
IgM	* First antibody to be produced. * Found predominantly in the blood * Role is the activation of the complement system.
IgG	* Seven IgG subclasses in horses (IgG1-7)(Wagner, 2006). * Principal antibody in the blood and extracellular fluid. * Main roles include virus neutralisation, complement and opsonisation. * Can cross the placenta.
IgA	* Principal antibody in secretions. * Provides barrier mucosal immunity * Actively transferred across epithelial surfaces.
IgE	* Almost always bound to tissue cells for example mast cells * Low levels are found in the blood. * Role in allergies
IgD	* Predominately membrane bound *Function remains unknown.

Adapted from (Janeway, 2001)

IgM, IgG and IgA are important for controlling viral infection (Whitton *et al.*, 2004). The mechanisms by which antibodies can exert antiviral effects on free virus particles and infected cells are by: 1) virus neutralisation; 2) opsonisation or 3) complement activation. Neutralisation plays an important role in antiviral immunity. Antibodies bind specifically to the pathogen surface, blocking the pathogen-cell surface interaction and inhibiting virus entry into the cell and subsequently virus replication (Janeway, 2001; Murphy, 2008).

Cell mediated immunity

CD8⁺ cytotoxic lymphocytes (CTLs) also play an important role in defending against viral infection. As described earlier, CTLs recognise foreign peptides displayed on the MHC I molecule on the cell surface and induce cell lysis and subsequently cell death. The method by which cell death occurs is by granule-mediated killing. Following interaction of CTLs with the infected cell, there is a release of cytotoxic granules (granzymes, perforin and granulysin) into the lymphocyte-target cell immunological synapse. As a result the cell membrane is damaged and this ultimately kills the cells. Dead cells are subsequently ingested by phagocytic cells (Janeway, 2001; Murphy, 2008).

1.8.3. Antigen size and immunity

The properties of a particulate antigen including antigen size, surface charge and hydrophobicity/hydrophilicity can greatly influence the way in which the immune system responds. In particular, the size of a particulate antigen can determine the efficiency of antigen uptake by APCs, the transport to the lymphoid organs and the efficiency of antigen presentation and activation of immune cells.

As described earlier, there is a requirement for antigens to be taken up and processed by APCs in order to induce T-cell responses. APCs can phagocytose particulate antigens much more efficiently than soluble antigens (Vidard *et al.*, 1996) whereby antigens with pathogen-like dimensions (20nm-3µm) are optimally phagocytosed (Bachmann *et al.*, 2010). The optimal particle size for uptake by dendritic cells is 40nm (Foged *et al.*, 2005) whilst macrophages preferably take up particles smaller than 5µm in size (Tabata & Ikada, 1988). However, despite this size preference antibody responses have

been demonstrated against antigens >20µm in size (Hilbert *et al.*, 1999; Katare *et al.*, 2005).

In addition to antigen uptake by APCs, the size of the antigen can also influence the way in which the antigen travels from the peripheral tissue to the secondary lymphoid organs for T- and B-cell activation. Transport to and from the periphery to the lymphoid organs is mediated by the lymphatic drainage system. Antigens greater than 200nm in size are too large to enter the lymph vessels directly and require the assistance of APCs to squeeze through gaps in between the endothelial cells of the lymph vessels (Bachmann & Jennings, 2010; Manolova *et al.*, 2008). In a now cell-associated state, the antigen is transported to the lymphoid organs but may not arrive for at least 24 hours (Bachmann & Jennings, 2010). In contrast, antigens smaller than 200nm can efficiently and directly enter the lymphatic system (Manolova *et al.*, 2008), passing through the endothelial cell junction of the lymph vessels. Smaller antigens can enter lymph drainage freely and can reach the lymph organs within hours (Bachmann & Jennings, 2010; Manolova *et al.*, 2008). There are several advantages relating to the arrival of the antigen in its 'free' state. For example, the antigen will be presented in its native configuration enhancing B-cell-antigen interactions, particularly for conformational epitopes that depend on the secondary and tertiary structure of the molecule. Antigens that possess repetitive epitope organisation can effectively cross link B-cell receptor initiating a strong B-cell activation signal (Bachmann *et al.*, 1995; Fehr *et al.*, 1998; Milich & McLachlan, 1986).

1.8.4. Antigen geometry and immunity

The vertebrate immune system has evolved to respond powerfully to microbial antigens arranged geometrically into

dense repetitive arrays. As described earlier, the repetitive array structure can optimally cross link B-cell receptors stimulating high antibody titres by inducing B-cell proliferation, maturation and the generation of a T-cell independent IgM antibody response in the absence of T-cell help (Bachmann *et al.*, 1995; Fehr *et al.*, 1997; Milich & McLachlan, 1986). The repetitive surface organisation can also induce up regulation of MHC II and T-cell helper co-stimulatory molecules (facilitating T-cell activation) triggering complement which can further aid B-cell stimulation reducing the number of BCRs that are required for B-cell engagement by activating CD19-CD21 complexes (Cherukuri *et al.*, 2001).

However, a repetitive epitope pattern alone is not sufficient to induce an effective immunological response. If the geometrical arrangement is not optimal (i.e. too few hapten molecules) the immune response can be inhibited (Dintzis *et al.*, 1983b). It has been demonstrated that ~20 haptens molecules spaced 5-10nm apart is optimal for B-cell activation (Dintzis *et al.*, 1983a; Vogelstein *et al.*, 1982).

1.8.5. Immunity to AHSV

Humoral immune response

Horses that recover from natural or experimental AHSV infection exhibit long lasting immunity to the homologous AHSV serotype (Theiler, 1921). The humoral nature of immunity is associated with neutralizing antibodies and this was first demonstrated by Alexander (1936) whereby the transfer of sera from AHSV immune horses protected mice against AHSV challenge (Alexander, 1936).

An antibody response against AHSV can be detected between 7 and 14 days post-infection (Martinez-Torrecuadrada *et al.*, 1997). Antibody responses to AHSV proteins VP2, VP3, VP5,

VP6, VP7, NS1, NS2 and NS3 can be observed (Maree & Paweska, 2005; Martinez-Torrecuadrada & Casal, 1995; Martinez-Torrecuadrada *et al.*, 1997). It is well documented that the outer capsid protein VP2 is the major protein responsible for inducing neutralising antibodies against AHSV. Sera obtained from rabbits vaccinated with purified recombinant VP2 were able to elicit antibodies that neutralised homologous AHSV *in vitro* (Martinez-Torrecuadrada & Casal, 1995). Several studies have also demonstrated that horses vaccinated with recombinant VP2 were protected against homologous AHSV challenge (Chiam *et al.*, 2009; Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marschat *et al.*, 1996). Additionally, MVA VP2 vaccinated mice generated neutralising antibodies that conferred protection against AHSV upon passive immunisation (Calvo-Pinilla *et al.*, 2014), highlighting the protective capacity of antibodies raised against AHSV VP2.

The amino acid sequence of VP2 shows a high degree of variation and it is recognised as a serotype specific antigen (Sailleau *et al.*, 2000). However, a significant level of cross-neutralisation is observed between certain AHSV serotypes (AHSV 1- and -2, AHSV-3 and -7, AHSV-5 and 8 and AHSV-6 and -9) (Mellor & Hamblin, 2004). As previously seen for BTV (Maan *et al.*, 2004; Maan *et al.*, 2007), this reflects phylogenetic relationships between the different AHSV serotypes in Seg-2/VP2 (Bachanek-Bankowska, 2012).

Epitope mapping of VP2 of AHSV-4 has identified a strong neutralising domain between amino acids 253 and 413 and polypeptides derived from this and adjacent sub-domains were able to induce virus neutralising antibodies (VNAbs) in mice and rabbits (Martinez-Torrecuadrada *et al.*, 1999). Two additional

major antigenic domains, localized between VP2 amino acid residues 321-339 and 377-400, were identified by PEPSCAN analysis (Martinez-Torrecuadrada *et al.*, 2001).

VP5 is also thought to play a role in virus neutralisation (Martinez-Torrecuadrada *et al.*, 1999). However, there is controversy over the extent of involvement. VP5 appears to preserve the correct conformation of the VP2 molecule and consequently induces an additional subset of neutralising antibodies. Rabbit sera raised against a combination of VP2 and VP5 recognized three extra antigenic sites compared to sera raised against VP2 alone (Martinez-Torrecuadrada *et al.*, 2001).

VP7 is a highly immunogenic antigen, as evidenced by its immuno-dominance during AHSV infections in the natural host (Martinez-Torrecuadrada *et al.*, 1997) and its routine use in antibody based diagnostic ELISAs for AHSV (Bremer *et al.*, 1994; Hamblin *et al.*, 1991; Laviada *et al.*, 1992; Maree, 2005). However, antibodies to VP7 do not neutralise the virus (Mertens *et al.*, 1996; Wade-Evans *et al.*, 1997).

Cell-mediated immune response

Unlike the role of humoral immunity, the role of cell-mediated responses to AHSV infection has not been well documented.

However, there have been instances whereby animals have survived challenged in the absence of neutralising antibodies indicating that potentially cell-mediated responses may play a role in AHSV protection (El Garch *et al.*, 2012; Guthrie *et al.*, 2009; Martinez-Torrecuadrada *et al.*, 1996; Wade-Evans *et al.*, 1997). The role of cell-mediated immunity against BTV has been well documented (Andrew *et al.*, 1995; Jeggo, 1984; Wade-Evans *et al.*, 1996). Multiple CTL epitopes have already

been described for BTV NS1, NS3, VP2, VP5 and VP7 (Andrew *et al.*, 1995; Rojas *et al.*, 2011). For AHSV, significant lymphoproliferative responses of CTLs in horses vaccinated with DNA encoding AHSV-3 VP2 (Romito *et al.*, 1999) and the live attenuated AHSV vaccine (Pretorius *et al.*, 2012) suggest possible cell-mediated involvement during AHSV infection. Additionally, vaccination of mice with AHSV-9 purified VP7 crystals administered with adjuvant provided at least 90% protection after a single inoculation against heterologous AHSV-7 challenge (Wade-Evans *et al.*, 1997). The level of protection did not correlate with the titres of antibodies against VP7 and passive transfer of antibodies to recipient mice failed to provide protection against AHSV challenge (Wade-Evans *et al.*, 1997) indicating that cell mediated immune responses against AHSV VP7 may have accounted for the protection observed.

1.9. Disease prevention and control

In the absence of any known effective treatment, vaccination and restrictions of animal movement play an important role in the control of AHS. However, the only vaccines that are currently available commercially are polyvalent 'modified' or attenuated live vaccines. These are produced by Onderstepoort Biological Products (OBP), South Africa and the monovalent live attenuated AHSV serotype 9 vaccines produced by the National Laboratory, Senegal (Mellor & Hamblin, 2004).

1.9.1. Vaccine classification

1.9.1.1. Live attenuated vaccine

Attenuation of the AHSV was first demonstrated in the 1930's (Alexander, 1936; MacLachlan *et al.*, 2007; Mellor & Hamblin,

2004). Attenuation was achieved by serial intracerebral passage in adult mice whereby large plaque variants were selected as vaccine candidate strains (Coetzer, 1994; Erasmus, 1978). However, as the result of a vaccine plant accident, it was shown that these strains were capable of causing central nervous system infections in humans and they have been 'retired'. Other attenuated strains were generated by multiple passage in BHK cells (Mertens, 2010).

Currently, OBP produces a commercially available live attenuated polyvalent vaccine that is administered in two doses at least 21 days apart (containing either AHSV serotypes -1,-3,-4, or -2,-6,-7). AHSV-5 and AHSV-9 are absent from the inoculum (Mellor & Hamblin, 2004). AHSV- 5 was withdrawn from the vaccine formulation because of adverse effects and its cross protection with AHSV-8, while AHSV-9 was not included because it is a serotype not normally occurring in South Africa and because it cross-protects with AHSV-6.

Vaccination is one of the most effective ways of protecting animals and reducing the spread of disease especially in endemic situations. The live attenuated vaccine induces humoral and cell mediated immune responses and the protective efficiency of the vaccine in the field is known (Pretorius *et al.*, 2012; von Teichman *et al.*, 2010). Crafford *et al.* (2014) reports that there is no enhanced immunity offered by the administration of single monovalent vaccines over the administration of individual attenuated serotypes combined within the polyvalent vaccine (Crafford *et al.*, 2014). However, there are a number of concerns associated with the use of live attenuated vaccines particularly in countries considered free of AHSV. These concerns include the potential for the vaccine

virus to be transmitted by insect vectors (leading to its spread and persistence in the field), reversion to virulence (MacLachlan *et al.*, 2007), an inability to distinguish between infected and vaccinated animals (DIVA) and the likelihood of gene re-assortment between different strains during mixed infections potentially leading to an increase in virulence (House, 1998; MacLachlan *et al.*, 2007; OIE, 2012; Veronesi *et al.*, 2005). For these reasons, the live attenuated vaccines for AHSV are not licenced for use within the European Union. Additionally, there have been several studies that have reported the inefficiency of the live attenuated vaccine. These reports include vaccinated horses succumbing to clinical and sub-clinical infection (Weyer *et al.*, 2013), variation in the neutralising antibody titre in mares who have been repeatedly vaccinated and marked differences in the immunogenicity between AHSV serotypes included in the vaccine (Crafford *et al.*, 2014).

1.9.1.2. Inactivated vaccine

Currently, there are no inactivated AHS vaccines commercially available on the market (Mellor & Hamblin, 2004; OIE, 2012). The inactivated monovalent AHSV-4 vaccine that was previously on the market, 'Equipest®', was based on inactivation of purified virus with formalin. This vaccine was removed from the market soon after the eradication of AHSV from Europe in the 1990s (Mellor & Hamblin, 2004; OIE, 2012).

There are limited data reporting the use and efficacy of inactivated vaccines. However, experimental inactivated vaccines have been reported since the 1960s. The studies that are available for AHSV have demonstrated that animals which received the inactivated vaccine developed immunity and were

protected against homologous AHSV challenge (Mirchamsy & Taslimi, 1968; Ozawa, 1966) for up to 6 months after vaccination (Mirchamsy & Taslimi, 1968). However, these monovalent vaccines induced predominately serotype specific immunity and multiple inoculations were required to achieve a high level of protection. Inactivated vaccines are also very expensive to produce (Laegreid, 1996).

1.9.1.3. Subunit AHSV vaccine

Concerns over the use of the live vaccines and recent advances in DNA technology have prompted the development of novel recombinant subunit vaccines. Several studies have shown the potential of these vaccination strategies, which are based on the expression of immunogenic AHSV proteins, primarily using baculovirus expression systems (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Scanlen *et al.*, 2002). Subunit vaccines have an immediate advantage over conventional vaccines. Since only AHSV genes encoding immunogenic / protective antigen are required, the design of relevant DIVA assays is possible, potentially even using existing serological methods (e.g. those targeting VP7) that are widely available and validated.

Immunogenic proteins expressed in a baculovirus system

Baculoviruses (belonging to the family *Baculoviridae*) are enveloped dsDNA viruses that can infect over 600 insect species. The baculovirus expression vector system (BEVS) offers a robust method for heterologous protein expression in insect cells and for a wide variety of genes from viruses, fungi, plants and animals (Kost *et al.*, 2005). *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the baculovirus most commonly used for biotechnological applications (O'Reilly,

1994). Rod shaped in appearance, the virus is typically 40-50nm in diameter and 200-400nm in length.

The recombinant baculovirus expression system exploits the bi-phasic baculovirus replication cycle. Whilst the 'early' and 'late' phase genes are essential for virus assembly, the 'very late' gene encoding polyhedrin is highly expressed but is non-essential for replication of the virion itself and is therefore dispensable (Hu, 2008; Hu *et al.*, 2008). Recombinant baculovirus generation involves replacing this dispensable gene with a gene of interest (that has been cloned into a suitable transfer vector) by homologous recombination. The foreign gene is therefore under the control of the polyhedrin promoter and is subsequently expressed at a late stage as the virus replicates (Liu *et al.*, 2013).

Using recombinant baculovirus expression, the expression levels of foreign protein are high with similar post-translational modifications to those observed in other eukaryotic cells (including, protein folding, disulphide bond formation, phosphorylation, glycosylation and oligomerization) (Kost *et al.*, 2005). The large baculovirus genome (130kb) also facilitates the insertion of large cDNAs, up to 8kb in size and simultaneous expression of multiple genes (Kost *et al.*, 2005; Roy *et al.*, 1997) e.g. as required for the assembly of orbivirus virus-like particles (VLP) (Noad & Roy, 2003). Additionally, baculoviruses do not replicate in mammalian cells and are therefore easy and safe to work with (Trowitzsch *et al.*, 2010).

Within the plasmid transfer vector the expression cassette contains the gene of interest to be inserted, flanked by baculoviral sequences determining the site of homologous recombination, e.g. baculoviral sequences of the polyhedron

region (Trowitzsch *et al.*, 2010). The plasmid vector is co-transfected into insect cells with infectious baculovirus DNA. Homologous recombination occurs between the baculovirus genome and the homologous baculovirus DNA sequence flanking either side of the foreign gene within the plasmid transfer vector. The gene of interest is thereby inserted into the genome of the recovered virus.

VP2 is a primary target for sub-unit vaccine development against AHSV (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marschat *et al.*, 1996). Vaccination with baculovirus expressed VP2 of AHSV-4 can protect horses against lethal AHSV-4 challenge (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996). However, the level of homologous protection generated by immunisation with baculovirus derived VP2 from other serotypes is variable (Du Plessis, 1998). Neutralising titres have been shown to vary considerably between serotypes (Kanai *et al.*, 2014). This observation could be due to problems associated with protein folding, aggregation and solubility. It has been documented that the quantities of soluble VP2 significantly varies between serotypes (Kanai *et al.*, 2014). Du Plessis *et al.* (1998) reported that the majority of baculovirus-expressed AHSV-5 VP2 was present in the form of insoluble aggregates. Only 10% of the expressed protein was in a soluble form and only immunisation with the soluble form induced neutralising antibodies in guinea pigs (Scanlen *et al.*, 2002). Scanlen *et al.* (2002) further demonstrated the protective capacity of soluble VP2 in the horse. Complications have halted the development of baculovirus-derived VP2 being used in sub unit vaccines for at least serotype 5, because VP2 was predominantly presented as insoluble aggregates (Du Plessis, 1998; Maree, 2000).

Other researchers have investigated the protective role of other AHSV capsid proteins. Wade-Evans *et al.* (1997) demonstrated the protective capacity of AHSV VP7 crystals in a vaccination experiment in mice. The study described the importance of the crystalline structure in its capacity to protect against AHSV challenge since inoculation with denatured VP7 crystals (crystals boiled at 100°C for 30 minutes) provided a much lower level of protection (Wade-Evans *et al.*, 1997). The protection afforded by the crystals was thought to be cell-mediated as passive transfer of sera from the immunized mice failed to protect donor mice from AHSV challenge. The observed protection was therefore unlikely to be due to an antibody-mediated immune response (Wade-Evans *et al.*, 1997).

1.9.1.4. Virus-like particles

Virus-like particles (VLPs) are multi-protein structures that contain viral protein that intrinsically assemble together to form particles (typically 25-100nm in diameter)(Johnson & Chiu, 2000). These structures resemble the authentic virus but lack any genomic material and therefore are not infectious. Viruses are potent activators of the both humoral and cell-mediated immune responses. Virus-like particles inherit the immunogenicity of native viruses because they mimic the properties of the virion (e.g. viral antigen arrangements and size). The effectiveness of VLPs as a powerful immunogen has been well described (Chackerian, 2007b; Grgacic & Anderson, 2006). Several VLP constructs are already on the commercial market for example GlaxoSmithKline's Engerix, a VLP based vaccine for Hepatitis B virus.

Currently there are no published data available for African horse sickness VLPs. There are however experimental data

available for the generation of African horses sickness core-like particles (CLPs). The co-expression of VP3 and VP7 during baculovirus infection results in the generation of AHSV CLPs (Maree *et al.*, 1998b). These structures closely resemble the AHSV core (Maree *et al.*, 1998a; Maree *et al.*, 1998b). Bluetongue VLPs have been described. These structures are comprised of structural proteins VP2, VP5, VP3 and VP7. Purified BTV VLP's have been reported to induce a high level of neutralising antibodies (French *et al.*, 1990; Thuenemann *et al.*, 2013) and afford protection against homologous virus challenge (Roy *et al.*, 1992; Roy *et al.*, 1994).

1.9.1.5. Multimeric antigens as a vaccine delivery platform

Purified sub-unit proteins administered alone can suffer poor immunogenicity owing to the incorrect folding and sub-optimal presentation of the target protein to the immune system. Several studies have described the use of multimeric antigens as delivery platforms to enhance the immunogenicity of target antigens displayed on their surface (Fehr *et al.*, 1998; Kaumaya *et al.*, 1992; Milich & McLachlan, 1986). Many VLPs have been adapted successfully for this purpose for example Hepadnavirus (Whitacre *et al.*, 2009): Hepatitis B core proteins displaying target epitopes for foot and mouth disease (Clarke *et al.*, 1987). The method most commonly used for the incorporation of target antigens / epitopes into the protein structures is by genetic insertion (Brown *et al.*, 1991; Chackerian, 2007b; 2012; Grgacic & Anderson, 2006; von Brunn *et al.*, 1993). Providing insertion does not affect protein folding and assembly, the target antigen will be displayed at the same high densities and conformation as the platform antigen (Chackerian, 2007b; 2012).

The use of multimeric antigens as a vaccine platform is not limited to the display of antigen / epitopes from pathogens (e.g. for prophylactic use); this application is universal. Multimeric antigens can also be used for the delivery of self-antigens to the immune system (Jennings et al, 2008) for therapeutic vaccine development, inducing auto-antibodies against chronic diseases (Chackerian *et al.*, 1999; 2001).

Rutkowska *et al.* (2011) explored the use of soluble AHSV VP7 as an antigen delivery system, inserting different peptides (from Foot and Mouth Virus (FMD) VP1 and AHSV VP2) on the surface of soluble VP7 trimers. These researchers reported that the soluble fraction of VP7 antigens with FMDV VP1 peptides inserted into its structure (VP7-FMDVVP1) induced a neutralizing antibody titre ten times higher than that of the insoluble VP7-FMDVVP1 antigens in guinea pigs (Rutkowska *et al.*, 2011). The insertion of small peptides (FMD VP1: 36aa (aa 129-164), AHSV VP2: 110 aa (aa 298-403), AHSV VP2: 23 aa (aa377-400) into the top domain of the VP7 molecule did not affect trimer formation. Additionally, the chimeric proteins generated exhibited a good expression yield and were able to generate an insert-specific response (Rutkowska *et al.*, 2011).

AHSV VP7 is a highly immunogenic antigen and AHSV VP7 crystals are highly organized structures (within the hexagonal VP7 assemblies) corresponding with other antigens that efficiently cross-link BCR's and can facilitate the induction of a strong T-cell independent antibody response alongside a T-cell dependent response (Fehr *et al.*, 1998; Milich & McLachlan, 1986).

Despite there being limited data available about the immunogenicity of crystalline antigens there are several

advantages to the use of crystal antigens as a potential immunogens/ vaccine platforms; 1) the repetitive nature of crystal structure corresponds with antigens capable of eliciting a T-cell independent and T-cell dependent antibody responses, 2) crystalline structures are by nature considered very stable (Clair *et al.*, 1999), 3) in addition to Wade-Evans *et al.* (1997) demonstrating the potential of AHSV VP7 crystals as an immunogen, Clair *et al.* (1999) describe how cross- linking crystals of human serum albumin (HSA) are significantly more immunogenic than soluble HSA (Clair *et al.*, 1999).

1.9.1.6. Live viral vectors

Recombinant live viruses expressing foreign antigens have been widely used as vaccines for human and veterinary applications to combat infectious diseases and cancer (Brun *et al.*, 2008; Wang *et al.*, 2010).

Poxvirus Vector Vaccines

The *Poxviridae* is a large family of dsDNA viruses that is divided into two subfamilies, *Chordopoxvirinae* and *Entropoxvirinae*. Eight genera comprise the sub-family *Chordopoxviridae* (Strauss J.H., 2007). Members of the genera Orthopoxvirus (e.g. vaccinia virus) and Avipoxvirus (e.g. canarypox and fowlpox) have been successfully engineered to express foreign genes and subsequently have become tools for vaccine development.

Recombinant poxvirus- based vaccines have been described for several diseases of veterinary importance including rabies (Wiktor *et al.*, 1984), canine distemper (Pardo *et al.*, 1997), feline leukaemia (Poulet *et al.*, 2003), equine influenza (Soboll *et al.*, 2010) and Newcastle's disease (Ogawa *et al.*, 1990; Weyer *et al.*, 2009). Recombinant poxvirus vector vaccines induce cell mediated and humoral immune responses (Hanke

et al., 2007; Kundig *et al.*, 1993; Moss, 1996; Ramirez *et al.*, 2000) and offer long last lasting immunity (Kundig *et al.*, 1993). Furthermore, these vaccines are compatible with DIVA strategies and in the case of canarypox and modified vaccinia Ankara virus (MVA) based vaccines have the added benefit of a high safety profile (Moss, 1996).

Vaccinia virus

Vaccinia virus is the prototype of the family *Poxviridae*. The virus is very well characterised and fully sequenced. For this reason, vaccinia has been widely used as a means of generating immune responses against antigenic proteins that are expressed from trans-genes inserted in its genome. As for baculovirus, recombinant vaccinia viruses are generated by means of homologous recombination. The foreign gene of interest is cloned into a suitable transfer plasmid vector. The plasmid vector contains an expression cassette containing a vaccinia promoter (e.g. p7.5.), restriction endonuclease sites for insertion of the foreign gene and flanking vaccinia virus DNA sequences determining the site of homologous recombination. The transfer vector may also include a marker or reporter gene to facilitate the selection of recombinant viruses. The recombination plasmid is transfected into cells previously infected with vaccinia virus, allowing homologous recombination to occur between the vaccinia virus genome and the homologous vaccinia sequences flanking either side of the foreign gene within the plasmid transfer vector (Earl *et al.*, 2001).

Historically there have been safety concerns associated with the use of poxvirus- based vaccines. However, concerns have been diminished since the development of poxviruses that

have lost their capabilities to replicate in human and most mammalian cell lines such as modified vaccinia Ankara (MVA). MVA is now a strain of choice for clinical research (Calvo-Pinilla *et al.*, 2009c; Castillo-Olivares *et al.*, 2011; Chiam *et al.*, 2009; Sutter & Staib, 2003; Sutter *et al.*, 1994).

Modified vaccinia Ankara (MVA)

MVA was derived from the chorioallantois vaccinia Ankara strain of vaccinia virus after being passaged >500 times in primary chicken embryo fibroblasts (CEF). Serial passage has led to six deletions within the parental genome (15%~ 31kb) (Drexler *et al.*, 2004; Meyer *et al.*, 1991; Norder *et al.*, 2010; Sutter & Staib, 2003) of genes involved in host range, virulence and immune-evasion (Blanchard *et al.*, 1998; Meyer *et al.*, 1991). Consequently MVA replication is non-permissive in most non-avian cells lines. MVA has lost its ability to replicate in human and most mammalian cells (Blanchard *et al.*, 1998; Carroll & Moss, 1997; Meyer *et al.*, 1991; Sutter & Staib, 2003). The virus replication in Hela cells is terminated after immature non-infectious virus particles are formed. Despite this restriction of viral replication, the expression of early and late MVA genes is not impaired (Sutter & Moss, 1992) and insertions of a foreign gene into the MVA genome results in a high level of expression of the recombinant protein (Sutter & Moss, 1992).

Several genes of vaccinia virus have been identified as being responsible for immune- evasion and virulence. Vaccinia viruses possess soluble inhibitors for interferon, cytokines and chemokines including receptors for INF- α/β , IFN- γ and TNF. These 'inhibitors' impede the induction of inflammatory and immune responses to infection. It has been described that MVA virus lacks the genes that encode such proteins which

could account for why MVA is immunogenic in the absence of viral replication (Blanchard et al, 1998). Recombinant MVA is immunogenic inducing a humoral and cell mediated response in the absence of adjuvant (Castillo-Olivares et al., 2011; Hebben et al., 2007; Sutter & Moss, 1992; Zanetti et al., 2012), has a great safety profile even in the immunocompromised individuals and can accommodate large insertions of foreign DNA into the genome. It is therefore an attractive choice as a live viral vector vaccine

1.9.1.6.1. Live viral vector vaccines for AHSV

Canarypox and MVA expressing VP2 or co-expressing VP2 and VP5 have shown great promise as viral vector vaccines for AHSV and BTV (Jabbar et al., 2013). Vaccination experiments in horses with a recombinant canarypox virus expressing VP2 and VP5 of AHSV-4 stimulated virus neutralising antibody responses and protection against homologous challenge (Guthrie et al., 2009).

MVA has been successfully used for the expression of AHSV proteins and in subsequent vaccination / challenge trials. MVA has been proven to be a safe vector in the horse capable of stimulating immune responses to foreign antigens (Breathnach et al., 2004). Recently, it has been demonstrated that MVA expressing AHSV VP2 induces serotype-specific virus neutralising antibodies and offers complete protection against homologous AHSV challenge in mice and ponies (Alberca et al., 2014; Castillo-Olivares et al., 2011; Chiam et al., 2009).

1.10. Aims and Objectives

The review of the virology and biology of AHSV above has shown that the AHSV antigen VP7 is immunogenic in horses and highlighted its potential as a platform for rational design

of new vaccines. From this information, two hypotheses can be derived to test:

Hypotheses:

- The structural qualities of AHSV VP7 are at least partially responsible for its highly immunogenic nature during AHS infection.
- AHSV VP7 can serve to enhance the immunogenicity of any antigen/ epitopes displayed in a highly organized manner on the protein/ trimer/ hexagonal surface.

Project aim:

The principal aim of the project is to study the immunogenicity of AHSV VP7 crystals to establish a better understanding of the VP7 specific immune response and investigate the importance of the VP7 crystal structure for its use as a vaccine delivery platform. It is anticipated that the results of the project will have implications in the development of new generation vaccines and provide further insight into the role of VP7 during AHSV infection.

Project objectives:

In order to address the project aim, specific objectives have been identified:

- **Study the immunogenicity of VP7:**
 - Characterise recombinant AHSV VP7 expressed *in vitro* (by recombinant baculovirus and live viral vector MVA)
 - Generate recombinant VP7 based reagents for further characterisation by vaccination and challenge

- Study the immunogenicity of VP7 in the horse (vaccination only) and mouse model systems specifically in relation to the generation of a VP7 specific antibody response.
- **Explore VP7 as a vaccine platform:**
 - Generate modified VP7 gene constructs by inserting characterised peptides/ AHSV VP2 epitopes into the VP7 polypeptide chain at appropriate amino acid positions
 - Generate recombinant baculoviruses and MVAs expressing modified VP7 antigens
 - Characterise modified VP7 antigens specifically determining the ability of the antigens to form crystals
 - Study the immunogenicity of modified VP7 in the mouse animal model specifically in relation to the generation of an insert specific antibody response.

Chapter 2: Materials and Methods

2.1. General methods

All enzymes were used in the supplied reaction buffers, according to manufacturer's instructions. Reagents were supplied from Promega unless stated otherwise.

2.1.1. PCR protocols

2.1.1.1. Generation of inserts: For high fidelity cDNA amplification of nucleotide sequences by PCR, 50 µl reaction mixtures were prepared containing Accuprime™ Taq DNA Polymerase High Fidelity (Invitrogen) and the manufacturer's buffer.

The reaction mixture was prepared as follows:

10x Accuprime™ PCR Buffer I	5µl
Template DNA	Variable
Corresponding primer set (Appendix I, Table A1)	1µl each
Accuprime™ Taq High Fidelity	0.5µl
Nuclease free water	Variable
Final volume	50 µl

The thermal cycling programme was as follows:

Step 1. 94°C	1 minute
Step 2. 94°C	20 seconds
Step 3. 55°C	30 seconds
Step 4. 68°C	1 minute
Step 5. Go to Step 2 (30 cycles)	
4°C	∞

2.1.1.2. Screening of recombinant clones by PCR:

Colonies were picked from the transformation plate (see section 2.1.7.) and suspended in 20µl of nuclease free water

(QIAGEN) and incubated at 96°C for 5 minutes. 2.5µl of this sample was then used as a DNA template for PCR.

Reaction mixtures of 25µl, containing Go Taq® Green Master Mix were prepared according to manufacturer's instructions:

Go Taq® Green Master Mix	12.5µl
Primer set 1 (Appendix I, Table A1)	1µl of each primer
Colony suspended DNA template	2.5 µl
Nuclease Free Water	8µl

The thermal cycling programme was as follows:

Step 1. 94°C	2 minute
Step 2. 94°C	30 seconds
Step 3. 56°C	30 seconds
Step 4. 72°C	45 seconds
Step 5. Go to step 2 (30 cycles)	
Step 6. 72°C	5 minutes
4°C	∞

2.1.2. Restriction endonuclease digestion of DNA

Restriction digests were performed in 30µl according to manufacturer's instructions. Digested products were then analysed by agarose gel electrophoresis (see section 2.1.4.).

2.1.3. Dephosphorylation of plasmids

Restriction digested plasmid vectors were dephosphorylated using thermosensitive alkaline phosphatase (TSAP). TSAP (1µl/µg) was added to 30µl of restriction digest (see section 2.1.2.) and incubated for 15 minutes at 37°C.

2.1.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) was used to analyse DNA. The gels consisted of 1 % agarose (Ultrapure TM Agarose, Invitrogen) in 100mls of 1x TAE buffer with 5µl of edithium bromide (10mg/ml). Samples were run at 120V in 1x TAE for

one hour in a submerged electrophoresis apparatus (SCI-PLAS, UK). The TrackIt™ 1KB Plus DNA Ladder (Invitrogen) was used as molecular weight marker. The gels were visualised under UV light using the Bio-Rad transilluminator (Bio-Rad Gel Doc™ XR⁺) and photographed on thermal paper.

2.1.5. Gel purification of DNA

cDNA inserts generated by PCR or restriction digest were separated by AGE. DNA fragments were excised from the gels and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (Qiagen) according to the manufacturer's instructions.

2.1.6. Ligation of DNA

100ng of linearised plasmid vectors, that had been treated with Thermosensitive Alkaline Phosphatase (TSAP), were ligated with cDNA inserts using the 'LigaFast Rapid DNA Ligation System' at molar ratios of 1:1, 1:3 and 1:6 (3U T4 ligase). These ligation reactions were carried out in 20µl volumes and incubated at room temperature for 3 hours. 1µl of the ligation reaction was used to transform Novablue competent *Escherichia coli* (*E.coli*) cells (Novagen).

2.1.7. Transformation of bacteria

Competent cells (Novablue competent *Escherichia coli* (Novagen)) were thawed on ice. 1µl of the ligation reaction was added to the cells, gently mixed and incubated on ice for 5 minutes. The bacteria were heat shocked in a 42°C water bath for exactly 30 seconds then returned to ice for 2 minutes. 250µl of Super Optimal Broth (S.O.C.) (Novagen) was added to the transformation mix. 30µl of the transformation mixture was grown on Lysogeny broth (LB) and ampicillin agar plates (Microbiological services, Institute for Animal Health, Compton). Plates were then incubated overnight at 37°C.

2.1.8. Bacterial cultures

To start a bacterial culture, positive bacterial colonies were picked and suspended in 5 mls of LB broth (CSU, Institute for Animal Health, Pirbright) containing 5 μ l of ampicillin (100 μ g/ml) (Sigma). The culture was incubated at 37°C in an orbital shaker overnight (12 to 16 hours).

2.1.9. Plasmid preparation from bacterial cultures

QIAGEN® QIAprep® Spin Miniprep Kits were used to isolate plasmids from bacterial cultures. This method was performed according to manufacturer's instruction. The plasmids were eluted in 50 μ l of the EB buffer provided in the kit.

2.1.10. Quantification of DNA

The concentration of DNA (ng/ μ l) was determined using a NanoDrop ND-1000 Spectrophotometer, v 3.5.2, 1 μ l of the appropriate elution buffer was used as blank.

2.1.11. Sequencing

The BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) was used for DNA sequencing. Reaction volumes of 10 μ l were used. The DNA concentration recommended for use in the reaction was dependent on the template length. The manufacturer's directions were followed accordingly:

Template length	Amount of DNA required
100-200 bp	1-3ng
200-500bp	3-10ng
500-1000bp	5-20ng
1000-2000bp	10-40ng
>2000bp	40-100ng

The reaction mixture consisted of:

Big Dye Terminator Sequencing Mix 2.5x	0.5µl
Big Dye 3.1 5x Sequencing Buffer	1.75µl
DNA Template at the appropriate concentration	Variable
Nuclease free water	Variable
Forward/ reverse gene flanking primers (Appendix I, Table A1, primer sets 1, 15, 16 or 17)	1µl
Final volume	10µl

The thermal cycling programme used was:

Step 1. 96°C	1 minute
Step 2. 96°C	10 seconds
Step 3. 50°C	5 seconds
Step 4. 60°C	4 minute
Step 5. Go to step 2	(26 cycles)
4°C	∞

Each 10µl reaction was precipitated with 2.5µl of 125mM EDTA (Sigma-Aldrich) and 30µl of 100% ice-cold ethanol, in the dark for 15 minutes. The reaction mixture was centrifuged at 3000g for 30 minutes at 4°C, to pellet the reaction products. The pellet was then washed twice with 30µl of 70 % ethanol and dried in the dark at room temperature for 1 hour. Finally, the reaction products were dissolved in 20µl of Hi-Di Formamide and loaded into an ABI 3710 DNA Analyzer. Sequence files were assembled using SeqMan II (DNA Star).

2.1.12. Polyacrylamide Gel Electrophoresis (PAGE)**SDS page**

The SDS-page gel (10.5% acrylamide) for protein analysis and electrophoresis under denaturing conditions was prepared as follows:

Resolving gel - per one cassette

30% acrylamide, 2.7% bis (Severn Biotech Ltd)	3.5ml
1.5M Tris, pH8.8 (BioRad Laboratories)	2.5ml
H ₂ O	2.35ml
10% SDS (Sigma)	100µl
10% ammonium persulfate (Santa Cruz Biotechnology)	60µl
TEMED (VWR)	6µl

Stacking gel - per one cassette

30% acrylamide, 2.7% bis	0.6ml
0.5M Tris, pH 6.8	1ml
H ₂ O	2.35ml
10% SDS	100µl
10% ammonium persulfate	30µl
TEMED	6µl

The resolving gel preparation was added to the electrophoresis cassette (1.0mm thick) (Novex, Life technologies) followed by an overlay of distilled water. This gel was left to polymerise at room temperature for 30-45 minutes. The water was removed, the stacking gel was added and a plastic comb was inserted. The gel was then left to polymerise for a further 45 minutes before the comb was removed and 'samples' were loaded.

Sample preparation for SDS-page protein analysis and electrophoresis

Samples were prepared by heating at 85°C for 2 minutes in 15µl final volume:

Volume of original sample	Variable
Tris-Glycine SDS Sample Buffer (2x) (Novex, Life technologies)	7.5µl
NuPAGE ®Reducing Agent (10x) (Novex, Life Technologies)	1.5µl
Distilled water	Variable

The gel was placed in an electrophoresis tank with 1x Tris-Glycine SDS running buffer (Novex, Invitrogen). The samples were loaded onto the gel alongside a molecular weight marker (Novex®Sharp Pre-Stained Protein Standard, Life

Technologies) and run according to manufacturer's instructions (125V for 105 minutes).

2.1.13. Cell lysate preparation for use in western blotting

Cells (DF-1, CEF or Sf9) were seeded in a 6 well plate (0.8×10^6 per well) and the monolayer infected at high MOI (multiplicity of infection) (MOI 1) of virus inoculum. At desired time points the cell monolayer was scraped from each well and centrifuged at 13,000g for 5 minutes. The supernatant was recovered and the cell pellet re-suspended in 60µl of lysis buffer (0.05M Tris/HCl pH 8, 150mM NaCl, 0.25% NP40), vortexed for 15 seconds and placed on ice for 30 minutes. Prior to use for SDS-page protein analysis and electrophoresis, the sample was prepared according to section 2.1.12.

2.1.14. Staining protein bands

Following electrophoresis, the gel was removed from its gel case and added to 30mls of Simply Blue Safe Stain (Invitrogen) and incubated for 1 to 2 hours on a rotary shaker. For de-staining, the gel was placed in deionized water until the bands could be visualized.

2.1.15. Western blotting

The iBOT® dry blotting system was used which consisted of the iBlot® Gel Transfer Device and associated iBlot® Gel Transfer Stacks (Invitrogen, Life Technologies). The pre-programmed voltage program 2 was the program of choice (23V at 6 minutes).

Following the transfer of proteins from the gel to a nitrocellulose membrane, the membrane was placed in a plastic dish and incubated with 10ml of blocking solution 1 (5% w/v skimmed milk, in 1x PBS) on a rotary shaker (gentle agitation). After 30 minutes, blocking solution 1 was removed

and the membrane was incubated at room temperature on the rotary shaker overnight, with 10 ml of the primary antibody (Appendix III, Table A3.2) diluted in blocking solution 2 (2% w/v skimmed milk, in 1x PBS).

The next day, the membrane was washed three times, for 5 minutes each time, with 20ml of prepared 1x Antibody Wash (provided in the Western breeze chromogenic kit, Invitrogen, Life technologies). The membrane was subsequently incubated for 30 minutes with 10ml of the pre-made secondary antibody dilution (provided in the Western Breeze chromogenic kit, Invitrogen, Life Technologies), washed three times (for 5 minutes each time) with 20 ml of 1x Antibody Wash, then washed twice with water, for 2 minutes each time. Chromogenic substrate (5ml) (provided in the Western breeze chromogenic kit, Invitrogen, Life Technologies) was then added directly to the membrane until purple bands developed. Positive detection of the protein was indicated by a band corresponding in size to the target antigen. After development the membrane was rinsed twice with 20mls of water before being left to air dry.

2.1.16. Immunofluorescence

Cells (BHK, DF-1, CEF or Sf9) were seeded (0.2×10^6 per well) onto a glass coverslip in a 24-well plate to form a monolayer, after 12-16 hours, the cell monolayer was infected with virus inoculum at a high MOI (MOI 0.01-1).

At desired time points, the infection media was removed and the cell monolayer washed with 1xPBS (CSU, Pirbright Institute, Pirbright) then fixed by the addition of 4% paraformaldehyde (SantaCruz) for 30 minutes at room temperature. The cell layer was then washed three times with 1xPBS. To permeabilise the cells, 0.4% triton in PBS was

added to each well for 10 minutes at room temperature. Each coverslip was washed 3 times with 1xPBS and then blocked with PBS-FCS 10% for 30 minutes. The coverslips were then incubated with primary antibody (Appendix III, Table A3.2) diluted in PBS-FCS 2% for 2 hours, followed by a five times wash (five minutes per wash) with PBS -FCS 2%. The coverslips were then incubated with secondary antibody conjugated to FITc (Appendix III, Table A3, No.1), diluted in PBS-FCS 2%, for 30 minutes. The coverslips were individually washed with distilled water then incubated with Dapi (1µg/ml) (New England Biolabs) for 5 minutes. Each slip was mounted on a slide for confocal microscopy analysis with mounting media (Vecta-Shield) and sealed with clear nail varnish. The slides were then imaged by confocal microscopy.

2.1.17. Quantification of total protein

The Pierce®BCA Protein Assay Kit was used to quantify total protein of purified VP7 crystal preparations. Diluted Albumin (BSA) standards were prepared in 0.2M Tris buffer and the protocol followed according to manufacturer's instructions. The working reagent (2mls) was added to 100µl of each of the standard and test samples. The preparations were mixed and incubated at 37°C for 30 minutes. The spectrophotometer was set to 562nm, the instrument was blanked with the buffer, and the absorbance was measured for all the samples.

2.1.18. Quantification of VP7 specific protein

Samples of purified VP7 crystals were analysed by SDS-PAGE according to methods described in section 2.1.12, together with known quantity of molecular mass standards (BSA) (Invitrogen). The amount of VP7 present in each sample was estimated using BSA standard comparative analysis of Coomassie blue-stained gels (as described in section 2.1.14).

2.2. Generation of DNA constructs of modified AHSV-VP7 genes, by PCR

2.2.1. Primer design

Primers were designed based on the coding sequence of the pVL1393 AHSV-4 VP7 gene construct (Appendix II, Figure A2.2) received by Dr Javier Castillo-Olivares (Chiam *et al.*, 2009), a biotin acceptor peptide (Tanaka *et al.*, 2010) and two AHSV-4 VP2 epitopes (a) and (b). Epitope (a) spans AHSV-4 VP2 amino acid residue positions 318 to 342 and epitope (b) spans VP2 amino acid residues 374 to 403. Both sites have been previously defined (Martinez-Torrecedrada *et al.*, 2001). The combined coding sequences were visualised using Vector NTI software and the primers designed accordingly. The primer sequences are shown in Appendix I, Table A1.

2.2.2. Site specific mutagenesis using splice-overlap extension polymerase chain reaction

Splice overlap extension (SOE) was used to insert 'foreign' DNA coding sequences into the VP7 coding sequence. This method generates overlapping gene segments that are then used as DNA templates in a final PCR reaction, whereby the gene segments are extended to generate a full length product (Heckman & Pease, 2007).

The method used a pVL1393 AHSV-4 VP7 template and two different sets of primers. The first primer set included two 'external' primers 1f and 1r (Appendix I, Table A1, primer set 1) that were complementary to the pVL1393 plasmid vector. The second sets of primers were two 'internal' primers 2f and 2r (Appendix I, Table A1, primer set 2-14), these primers breached the VP7 sequence while incorporating the sequence of the insert.

The method involves three separate amplification reactions. The first reaction (A) incorporated primers 1f and 2r and was used to amplify cDNA containing the desired mutation at its downstream end, together with the upstream VP7 sequence. The second reaction (B) incorporated primers 1r and 2f and was used to amplify DNA that contains the mutation at its upstream end, together with the downstream VP7 sequence. The mutation is therefore located in the region of overlap between the two cDNA products (Figure 2.1).

The 50 µl reaction mixtures were as follows:

10x Accuprime™ PCR Buffer I	5µl
AHSV VP7 DNA template	1µl
Primers 1f/2f (forward)	1µl
Primers 1r/2r (reverse)	1µl
(Appendix I, Table A1)	
Accuprime enzyme	0.5µl
Nuclease Free Water	41.5µl

The final reaction incorporated the PCR products from reactions 1 (A) and 2 (B) at 100 ng and primer set 1. The reaction mixture was as follows:

10x Accuprime™ PCR Buffer I	5µl
Product A @100ng	Variable
Product B@ 100ng	Variable
Primer 1f (primer set 1)	1µl
Primer 1r (primer set 1)	1µl
(Appendix I, Table A1)	
Nuclease free water	Variable
Final volume	50µl

The same thermal cycle was used for all three reactions:

94°C	1 minute
94°C	20 seconds
55°C	30 seconds
68°C	1 minute
(30 cycles)	
4°C	∞

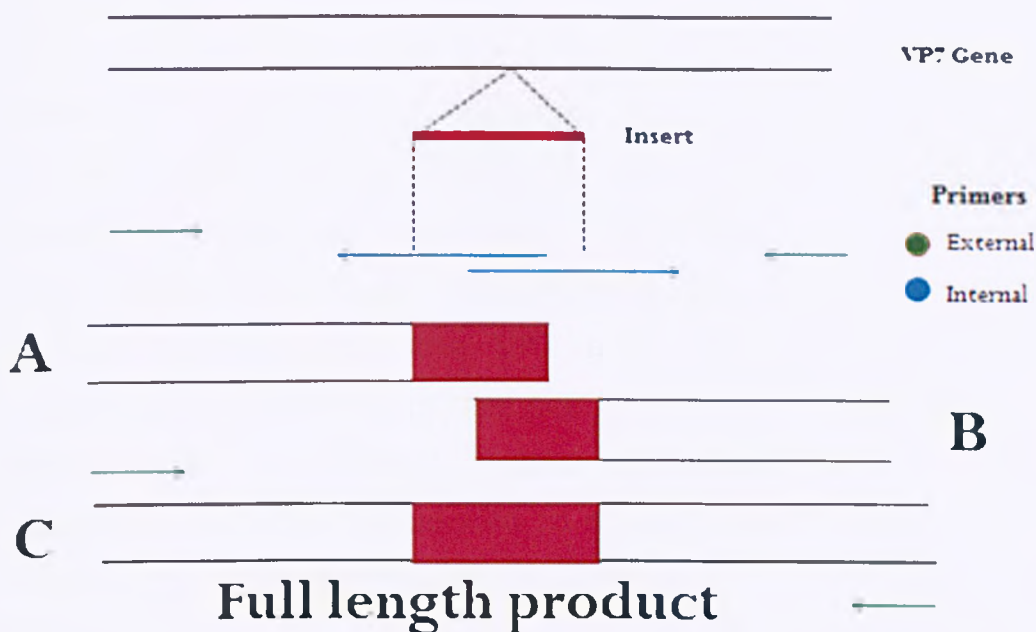


Figure 2.1: Overview of the splice overlap extension technique.

This technique involved three separate PCR reactions. The first and second reaction used a pVL1393 AHSV-4-VP7 template and two different primer sets * to generate PCR products (A) and (B). These internal primers contain the required mutations/insertions, which are therefore also present in the resulting cDNA products, together with the upstream and downstream VP7 sequences respectively. In the final PCR reaction the two overlapping gene fragments were used as templates, with the external primers to generate a full length modified cDNA product (C).

*Green arrows indicate external primers complementary to the pVL1393 plasmid vector. Blue arrows indicate internal primers breaching the VP7 gene, and a portion of the foreign gene sequence to be inserted.

2.2.3. Generation of modified AHSV-VP7 plasmid constructs

Subsequent to DNA purification (section 2.1.5.), the VP7 DNA constructs were ligated (section 2.1.6.) directly with the pGEM-T plasmid vector (pGEM-T Easy Vector System I). Novablue competent cells were transformed (section 2.1.7) with the ligation mixture. Colonies were then screened by PCR (section 2.1.1) using the VP7 gene flanking primer set (Appendix I, Table A1, primer set 17). Plasmid preparations were made from positive colonies (section 2.1.9). The plasmids were then sequenced to confirm that the AHSV VP7 constructs were present (section 2.1.11).

These plasmid VP7 constructs served as a template for the generation of DNA constructs with required 'restriction ends' to facilitate cloning into different vector plasmids. Primers were designed accordingly (Appendix I, Table A1). Restriction sites were chosen according to what transfer vector the constructs were going to be cloned into (Table 2.1). The VP7 constructs were generated by PCR (section 2.1.1.1.), using the appropriate flanking primers (Table 2.1), then analysed by AGE, excised and purified (section 2.1.5.). The constructs were then digested with the appropriate restriction enzyme (Table 2.1) (section 2.1.2.) and ligated (section 2.1.6.) with the corresponding transfer vector, which had already been digested and dephosphorylated (section 2.1.3.). Novablue competent cells were transformed (section 2.1.7) with the ligation mixture. Colonies were then screened by PCR (section 2.1.1.2.) using primer set 17 (Appendix I, Table A1). Plasmid preparations were made from the positive colonies (section 2.1.9.). The plasmids were then sequenced to confirm that the VP7 construct was present and in the correct orientation (section 2.1.11).

Table 2.1: The primer sets and incorporated restriction site sequences for the corresponding plasmid vector plasmids.

Plasmid transfer vector	Restriction site (site for gene insertion)	Primer set (Appendix I, Table A1)
pLV1393	NotI: GCGGCCGC	1
PSC11	XmaI: CCCGGG	15

2.3. Cell culture

All cell culture was performed using aseptic techniques in a Class II safety cabinet. All media was supplemented with penicillin streptomycin (100U penicillin/ml and 100µg streptomycin/ml (Sigma-Aldrich)) and 10% heat inactivated (56°C) foetal bovine serum (FBS), unless stated otherwise.

2.3.1. Insect cell culture

Spodoptera frugiperda Sf9 insect cells (Oxford Expression Technologies) were maintained in Sf-900 II serum free media (Gibco, Invitrogen) at 28°C in 175cm³ flasks and sub-cultured prior to use in VP7 protein expression studies. When confluent, the cells were dislodged from the flask using cell scrapers and the cell-suspension was split at a ratio of 1 in 3 into new 175cm³ flasks with fresh media. Cells were incubated at 28°C, unless stated otherwise.

2.3.2. Mammalian cell culture

Vero and baby kidney hamster (BHK 21) cells (Microbiological services, Institute for Animal Health, Compton/Pirbright)) were maintained in Dulbecco modified minimum essential media (DMEM) at 37°C in 175cm³ flasks and sub-cultured prior to infection. When confluent, the cells were dislodged from the flask using trypsin (Invitrogen), the cell suspension was normally split at a ratio of 1 in 6 into new 175cm³ flasks with

fresh media. Cells were incubated at 37°C, 5% CO₂ unless stated otherwise.

2.3.3. Avian cell culture

Chicken embryo fibroblasts (CEF) and DF-1 chicken embryo fibroblast (DF-1) cells (Microbiological services, Institute for Animal Health, Compton/Pirbright) were maintained in Dulbecco modified minimum essential media (DMEM) at 37°C in 175cm³ flasks and sub-cultured prior to infection. When confluent, the cells were dislodged from the flask using trypsin (Invitrogen), the cell suspension was normally split at a ratio of 1 in 6 into new 175cm³ flasks with fresh media. Cells were incubated at 37°C, 5% CO₂ unless stated otherwise.

2.3.4. Cell density

Cell culture plates were seeded as followed, unless stated otherwise

Cell Culture Plates	Seeding density per ml	Volume per well
6-well	0.8×10^6	2ml
12-well	0.4×10^6	1ml
24-well	0.2×10^6	0.5ml
96-well	0.05×10^6	100µl

2.4. Baculovirus protein-expression

2.4.1. Recombinant-baculovirus generation

2.4.1.1. Co-transfection of Sf9 cells to generate recombinant-baculoviruses

A 6-well plate was seeded (0.8×10^6 per well) with Sf9 insect cells and incubated at room temperature to allow the cells to settle before use. The co-transfection mixture was prepared according to the flashBAC instructions (Oxford Expression

Technologies, Oxford, UK). Eight microliters of Cellfectin (Invitrogen) was added to 1ml of serum free Sf-900 II media (Gibco, Invitrogen) and mixed. Five microliters of flashBAC (Oxford Expression Technologies, Oxford, UK) and 500ng of transfer vector DNA (pVL1393, containing either the wild-type or modified AHSV VP7 gene) was added and mixed. The mixture was incubated at room temperature for 30 minutes. The media was then removed from the Sf9 cells and the transfection mix was added drop by drop to the cells. As a negative control, cells were also incubated with a mixture without the DNA. The transfected cells were incubated at 28°C overnight. A further 1 ml of supplemented culture media was added to the cells before incubation for 5 days at 28°C. The recombinant baculovirus seed stock was harvested then and stored at -80°C.

2.4.1.2. Amplification of recombinant baculovirus

A 6-well plate was seeded (0.8×10^6 per well) with Sf9 cells (as described above) and infected with 100 μ l of the virus seed stock. The plate was incubated for a further 5 days. After which the culture medium containing the P1 virus stock was harvested, centrifuged at 1430g and stored at -80°C.

2.4.1.3. Titration of baculovirus by plaque assay

A 12 well plate was seeded (0.4×10^6 per well) with Sf9 insect cells. The virus was diluted in tenfold steps from $\times 10^{-1}$ to $\times 10^{-12}$ in culture media. The cell culture media was removed from the cells and 1ml of virus inoculum was added to each well. The plate was then incubated for 1 hour at 28°C, before the inoculum was removed and 2.5ml of agarose overlay (1% low melting point agarose (Invitrogen)) was added. The low melting agarose had been previously melted and diluted 1/5 with cell culture media. The overlay was left to set at room

temperature. The plate was then incubated for 3-4 days at 28°C until plaques were visible in the monolayer. Neutral red (Invitrogen) (1ml, 0.33%/well) was added to each well and incubated for 3-4 hours at 28°C. The neural red solution was removed, the plate inverted and the clear plaques visualised. The plaques were then counted and used to calculate the virus titre (PFU/ml).

2.4.2. Expression of VP7 crystals

The Sf9 cells were infected with recombinant baculoviruses encoding AHSV-4 VP7 constructs. A 175cm³ flask of Sf9 cell culture (sub-confluent) was infected with recombinant baculovirus at high MOI (MOI 3-5) and incubated for 3-5 days at 28°C until VP7 crystal expression was observed by light microscopy. The crystals appeared as rod-like structures inside the infected insect cells.

2.4.3. Purification of VP7 crystals from Sf9 cells

This method is based on the purification method described by Maree *et al.* (2005) with modifications. Briefly, infected insect cells were collected by centrifugation at 450g for 8 minutes at 4°C and then rinsed in one-tenth of the original culture volume with 10mM Tris (pH 8). The cells were then recovered by centrifugation (450g for 8 minutes at 4°C) and re-suspended in lysis buffer (0.05M Tris/HCl pH 8, 150mM NaCl, 0.25% NP40) to a final density of $\sim 2 \times 10^7$. The cell suspension was placed on ice for 30 minutes then homogenised by several strokes using a 1 ml syringe and 25G needle. The suspension was fractionated by centrifugation 1500g (for 30 minutes, 4°C). The supernatant was removed very carefully and the pellet re-suspended in 3ml of 0.2M Tris/HCl buffer, pH 8.0 and then frozen at -80°C for a freeze thaw cycle. Upon slowly thawing, the preparation including the aggregated cell debris

'clump' was added to a 40µm cell strainer (BD Falcon) whereby the supernatant (containing the VP7 crystals) passed through the gauze filter whilst the 'clump' was retained. The strainer was washed with a further 3mls of 0.2M Tris/HCl buffer, pH 8.0 to remove any residual crystals. The strainer was discarded and the crystal preparation was stored at -80°C.

2.4.4. Sonication of VP7 crystals

The purified VP7 crystals were sonicated using a micro-tip sonicating probe (30 watts) (VibraCell, Sonics and Materials) at setting 2 (Output control). A 1ml crystal preparation in a 1.5ml sterile plastic Eppendorf was subjected to nine 30 second pulses for a total of 4 minutes 30 seconds; the tube was placed on ice for two minutes after each pulse. After sonication, the preparation was visually inspected under a light microscope and compared to intact non-sonicated crystals.

2.4.5. Digital light scattering

The size of the sonicated crystal material was estimated by DLS analysis using The Malvern Zetasizer. The machine had been pre-calibrated. Sonicated and intact crystals were diluted 1/10 in PBS whereby 100µl was added to a UV micro cuvette (Brandtech scientific, inc). The wet preparation was analysed immediately at 5 reads (40 seconds each read).

2.4.6. Solubilisation of VP7 crystals

Solubilisation of purified VP7 crystals using urea (2, 4, 8M) was carried out by initially pelleting the crystals by centrifugation (10,000g for 8 minutes). The supernatant was very carefully removed and the pellet re-suspended in solubilising buffer (urea in 0.05M Tris/HCl pH 8, 150mM NaCl). The preparation was incubated at room temperature for one

hour. After which soluble VP7 was separated from aggregated crystalline VP7 by centrifugation (10,000g for 10 minutes).

Solubilisation of VP7 crystals using N-lauroylsarcosine (sarkosyl) (Merck) (0.2, 0.5, 1%) was carried out by initially pelleting the crystals by centrifugation (10,000g for 8 minutes). The supernatant was very carefully removed and the pellet re-suspended in solubilising buffer (sarkosyl in 0.05M Tris/HCl pH 8, 150mM NaCl). The preparation was incubated at 25°C for up to 3 hours. After which soluble VP7 was separated from aggregated crystalline VP7 by centrifugation (10,000g for 10 minutes).

2.5. Live viral vector expression

2.5.1. Generation of recombinant MVA

2.5.1.1. Co-transfection of pSC11/modified-VP7-gene in MVA infected cells

Six well tissue culture plates were seeded (0.8×10^6 per well) with DF-1/CEF cells and infected with wild-type MVA at a MOI of 0.1. After 1 to 2 hour of infection, the cells were washed with serum free (SF) DMEM without antibiotics. A 'transfection mix' was generated by mixing 4µg of the recombinant pSC11 plasmid diluted in 250µl of SF DMEM (no antibiotics), supplemented with 20µl of Lipofectamin 2000 (Invitrogen) diluted in 250µl SF DMEM (no antibiotics). The transfection mix was incubated at room temperature for 20 minutes before being added drop wise onto the cell monolayer. After four hours the transfection mix was removed and replaced with fresh DMEM. After two days the infected cells were scraped from the wells and the cell suspension stored at -80°C. Three freeze thaw cycles were used to release the MVA virus particles from cells.

2.5.1.2. Selection of recombinant viruses by plaque purification

Wild-type MVA was separated from recombinant MVA (rMVA) by plaque assay. Several rounds of plaque assays were conducted until no white plaques were visually present in the cell monolayer after X-Gal staining. Six well plates were seeded (0.8×10^6 per well) with DF-1/CEF cells. A transfection / co-transfection harvest (parental rMVA or individual plaque picks) from a plaque assay believed to contain recombinant MVA viruses, were used to infect the cell monolayer. The MVA sample was freeze thawed three times and passed through a 1ml syringe and a 25G needle several times. Dilutions of the inoculum (1/20, 1/40, 1/100 and 1/200) were made and added to the cells.

The infected cell culture was incubated for 2 hours at 37°C. The inoculum was then removed and 2-2.5ml of 1% low melting point agarose (Invitrogen) was added to each well. The low melting agarose has been previously melted and diluted 1/5 with DMEM. The plate was left at room temperature for 10 minutes until the agarose was set. The infection was allowed to progress until plaques were visible in the monolayer (typically 1-2 days). A second agarose layer was added (diluted 1/5 with DMEM), containing 400µg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Invitrogen). After overnight incubation, the cell monolayer was examined for the presence of blue plaques, indicating the presence of recombinant MVA viruses.

Screening for rMVA is based on the co-insertion of the *E.coli* lacZ gene along with the gene of interest. In the presence of recombinant MVA, plaques will be blue in medium containing Xgal. Individual blue plaques present at the lowest dilution

(that showed plaques) were picked using a Pasteur pipette. The pipette tip penetrated the agarose layers, lightly scratching the plaque in the monolayer. The agarose plug was removed by suction. The plug was added to 1ml of DMEM. The virus was released from the cells in three freeze thaw cycles.

2.5.1.3. Generation of recombinant MVA stocks

Primary virus stocks were prepared for recombinant MVAs expressing VP7/VP2a200, and VP7/VP2a238, by infecting DF-1 cells (0.8×10^6 cells per well) in a 6-well plate. After the cells showed more than 70% CPE they were scraped from the wells and the cell suspension freeze thawed three times before needle (25G) homogenisation.

A larger virus stock was generated in 175cm³ flasks containing an 80% confluent DF-1 monolayer. The media was removed and the cell monolayer was infected with 100µl of the primary virus stock in 5 mls of complete DMEM media for 1 hour at 37°C. The inoculum was removed and replaced with 50mls of complete DMEM media. The flasks were incubated at (37°C) until 80-100% CPE was observed (usually 3-4 days). The cell monolayer was then scraped from the flask, pelleted by centrifugation (3,000g for 10 minutes) and re-suspended in 1 ml of complete DMEM media then freeze thawed three times. The titres of recombinant MVA viruses were determined by plaque assay.

2.5.1.4. Titration of MVA by plaque assay

Twelve well plates were seeded (0.4×10^6 per well) with CEF/DF-1 cells. The virus was diluted in tenfold steps from $\times 10^{-1}$ to $\times 10^{-8}$ in DMEM. The cell culture media was removed from the cells and 1ml of virus inoculum was added to each well. The plate was then incubated for 1 hour at 37°C before the inoculum was removed and 2.5ml of agarose overlay (1% low

melting point agarose (Invitrogen) was added. The low melting agarose had been previously melted and diluted 1/5 with DMEM. The overlay was left to set at room temperature. The plate was then incubated for 2 days at 37°C. A second agarose layer was added (diluted 1/5 with DMEM), containing 400µg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Invitrogen). After overnight incubation at 37°C the cell monolayer was examined for the presence of blue plaques. The plaques were then counted and used to calculate the virus titre (PFU/ml).

2.5.1.5. RNA/ DNA extraction from MVA virus

RNA and DNA were extracted from the infected cell monolayer using the Qiagen RNeasy mini kit following the 'animal cells spin' protocol. Six well plates were seeded with CEF/DF-1 cells (0.8×10^6 cells per well). The next day, the cells were infected with recombinant MVA virus stocks at MOI 1. At 24 hour post infection the supernatant was removed and discarded. The cells were then lysed with 350µl of Buffer RTL (provided in the kit), supplemented with β-ME (10µl per ml), collected into a 1.5ml Eppendorf tube and mixed by vortex for 1 minute. Three hundred and fifty microliters of 70% ethanol was added to the lysate and mixed by pipetting before being added to the spin column, provided in the kit. The columns were centrifuged at 13,000g for 30 seconds and the flow through discarded. The membrane was washed using the kit provided RW1 buffer (700µl), centrifuged at 13,000g for 30 seconds then washed twice with the RPE buffer (500µl) that was provided in the kit and centrifuged at 13,000g for 30 seconds. The RNA / DNA were eluted in RNase free water by centrifugation at 13,000g for 1 minute. For RT-PCR applications, the eluted RNA preparation was used immediately. For DNA based applications, the eluted preparation was stored at -20°C.

2.5.1.6. PCR to confirm the presence of the VP7 gene in the MVA genomic DNA

The presence of the VP7 / modified VP7 gene in the MVA genome was verified by PCR. After DNA extraction (section 2.5.1.5.), 6µl of DNA was used as the template in a 25µl PCR reaction. The reaction mixture, containing Go Taq® Green Master Mix, was prepared according to manufacturer's instructions:

Go Taq® Green Master Mix	12.5µl
Primer set 17 (Appendix I, Table A1)	1µl of each
DNA template	6µl
Nuclease free water	4.5µl
Final volume	25µl

The thermal cycling programme was as follows:

Step 1. 94°C	2 minute
Step 2. 94°C	30 seconds
Step 3. 56°C	30 seconds
Step 4. 72° C	45 seconds
Step 5. Go to step 2 (30 cycles)	
Step 6. 72°C	5 minutes
4°C	∞

The PCR products were analysed by agarose gel electrophoresis (Section 2.1.4.)

2.5.1.7. Analysis of VP7 gene expression by recombinant MVA using Reverse Transcription (RT-PCR)

a) Elimination of DNA from extracted RNA preparations (Section 2.5.1.5.)

Prior to use in a One Step RT-PCR, RNA extracted using the Qiagen RNeasy mini kit was treated with DNase I Amplification

grade (Invitrogen) to remove any residual DNA from the preparation. The 40µl reaction mixture was prepared according to manufacturer's instructions;

RNA sample	4µg
10x DNase I reaction buffer	4µl
DNase I	4µl
Nuclease free water	Variable
Final volume	40µl

The mixture was incubated at room temperature for 15 minutes and inactivated by the addition of 2µl of 25mM EDTA solution in the reaction mixture heated at 65°C for 10 minutes.

b) One Step RT-PCR

RT-PCR was used to confirm the presence of VP7, VP7/VP2a200 or VP7/VP2a238 transcripts in MVA VP7 or MVA VP7VP2a infected cells. RT-PCR reactions were performed using SuperScript™One-Step RT-PCR with platinum® Taq and mRNA extracted as described in section 2.5.1.5 and DNase treated as described in section 2.5.1.7a.

The reaction mixture was prepared according to manufacturer's instructions;

2x Reaction Mix	25µl
Template RNA	6µl
Primer set 17 (Appendix I, Table A1)	1µl each
RT/platinum® <i>Taq</i> Mix	1µl
Nuclease free water	16µl
Final volume	50µl

The thermal cycling programme was as follows;

Step 1. 55°C	30 minute
Step 2. 94°C	2 minutes
Step 3. 94°C	15 seconds
Step 4. 57°C	30 seconds
Step 5. 72°C	1.12 minutes
Step 6. Go to step 3 (40 cycles)	
Step 7. 72°C	5 minutes
4°C	∞

The PCR products were analysed by agarose gel electrophoresis (Section 2.1.4.)

2.5.1.8. Inactivation of live MVA VP7

Inactivation of live MVA VP7 was performed by exposing the live MVA VP7 preparation (Table 2.2) to UV-C light (254nm) for 45 minutes. Live MVA VP7 (1ml) was added to a 35mm lidless dish and placed within a distance of 5cm to the light. The effectiveness of inactivation was confirmed by plaque assay (section 2.7.7.2).

2.6. Viruses

All of the viruses used in this thesis are listed below in Table 2.2.

Table 2.2: A list of viruses used in this study

Nomen- clature	Virus sample	Strain	Passage history	Other information
AHSV-3	AHSV-3	V1	BHK/BHK2	Obtained from The Pirbright Institute virus collection. Generated as described by (O'Hara, 1994)
AHSV-4	AHSV-4	Madrid, 1987 (Ricarda)	MB1/BHK4	Obtained from The Pirbright Institute virus collection
AHSV-6	AHSV-6	9AD	MB1/BHK4/ BHK2	Obtained from The Pirbright Institute virus collection
MVA VP7	Recombinant modified vaccinia Ankara (rMVA) expressing AHSV-4 VP7	VP7 from AHSV-4/ Madrid 1987	DF-1	Provided by Dr Javier Castillo-Olivares (Chiam <i>et al.</i> , 2009).
Wild-type MVA	Wild-type modified vaccinia Ankara	-	DF-1	Provided by Dr Javier Castillo-Olivares.
FB-VP7	Recombinant baculovirus expressing AHSV-4 VP7	VP7 from AHSV-4/ Madrid 1987	SF-9	Provided by Dr Javier Castillo-Olivares (Chiam <i>et al.</i> , 2009).

*Mouse brain (MB)

2.7. Animal Studies

All the animal work was performed under a relevant Home Office Project Licence and had been approved by The Pirbright Institute Ethical Review Committee / Animal Health Trust's Ethical committee (for pony studies). Specific details of each study are given below.

2.7.1. Animal experiments

2.7.1.1. Immunogenicity of baculovirus expressed AHSV-4 VP7 crystals in A129 mice, including challenge with AHSV-4 (at The Pirbright Institute, UK)

Ten groups of three mice (A129) were vaccinated (by intramuscular or intra peritoneal injection) on day 0 and 21 with VP7 crystal antigens (4µg, 8µg, 16µg), or cell debris (4µg) preparations (diluted in PBS, with or without equal volume of Montanide ISA 50 V2 adjuvant, Seppic – as specified in the text). The mice were subsequently challenged by subcutaneous injection on day 29 with AHSV-4 (see Table 2.2) (1×10^6 TCID₅₀). Serum samples were collected (see section 2.7.4.) on days 4, 21, 29, 31, 33 and 38 for analysis of the antibody response. Following virus challenge, clinical signs in the vaccinated and control animals were monitored closely (according to the clinical scoring system; section 2.7.2) and if the animal showed signs of distress, the animals were euthanized humanely. The experiment was terminated on day 38.

Antigen preparations for vaccination trials

Recombinant VP7 crystals were expressed using baculovirus (section 2.4.2) and purified from insect cells as described in section 2.4.3.

2.7.1.2. Immunogenicity of baculovirus expressed AHSV-4 VP7 crystals in ponies (The Animal Health Trust, Newmarket, UK)

Four groups of two Welsh mountain ponies (approximately 15 months of age) were vaccinated (by intramuscular injection) with 1ml of either: a) 25µg of VP7 crystals; b) 150µg of VP7 crystals; c) 25µg of sonicated VP7 crystals; or d) 150µg of sonicated VP7 crystals (diluted in PBS) on days 0, 28, 49 and 70. Whole blood samples were collected on days -3 (100mls), 28 (20mls), 35 (20mls), 42 (300mls), 49 (200mls), 63 (20mls), and 102 (300mls). The sera were obtained and provided by The Animal Health Trust (AHT) for VP7 antibody analysis.

Antigen preparations for vaccination trials

Recombinant VP7 crystals were expressed using baculovirus (section 2.4.2) and purified from insect cells as described in section 2.4.3. Crystals were fragmented by sonication as described in section 2.4.4.

2.7.1.3. Immunogenicity of MVA expressing AHSV-4 VP7 in IFNAR -/- A129 mice, including challenge with AHSV-3, AHSV-4 or AHSV-6 (at The Pirbright Institute, UK).

Interferon-alpha, receptor knock out (IFNAR -/-) strain A129 mice were vaccinated (by intra-peritoneal injection) with MVA AHSV-4 VP7 (1×10^7 PFU, diluted in DMEM), on day 0 and day 28. The mice were subsequently challenged (by subcutaneous injection) on day 38 with AHSV-3, AHSV-4 or AHSV-6 (see Table 2.2) (1×10^6 TCID₅₀, diluted in DMEM) together with non-vaccinated control mice. Serum samples were collected (see section 2.7.4.) on day 0 and 35 post-vaccination (pv) and on day 52 pv (14 days post-challenge); blood samples were collected for virus isolation on days 41, 43, 45 and 48 (days 3, 5, 7, and 10 post-challenge respectively). Clinical signs in the vaccinated and control

animals were monitored closely and if they reached clinical distress (according to the clinical scoring system described in section 2.7.2), they were euthanized humanely. The experiment was terminated on day 52.

Antigen preparations for vaccination trials

Live viral vector MVA expressing AHSV-4 VP7 (see Table 2.2) was obtained by Dr Javier Castillo-Olivares.

2.7.1.4. Immunogenicity of MVA expressing AHSV-4 VP7 in IFNAR -/- A129 and BALB/c mice including AHSV-4 challenge in IFNAR -/- A129 mice (at Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain)

Three groups of five BALB/c and interferon-alpha receptor knock out (IFNAR -/-) A129 mice were vaccinated (by intra-peritoneal injection on day 0 and 21) with live MVA AHSV-4 VP7, inactivated MVA AHSV-4 VP7 or inactivated MVA AHSV-4 VP7 plus wild-type MVA (1×10^7 PFU, diluted in DMEM). A fourth control group of mice (BALB/c and IFNAR -/- A129) were vaccinated twice with wild-type MVA (1×10^7 PFU, diluted in DMEM) on day 0 and on day 21 (by intra-peritoneal injection). Serum samples were collected (see section 2.7.4.) on day 35. The interferon-alpha receptor knock out (IFNAR -/-) A129 mice as well as the control mice (vaccinated with the wild-type MVA vector) were then subsequently challenged (by subcutaneous injection) on day 35 with AHSV-4 (see Table 2.2) (1×10^6 TCID₅₀, diluted in DMEM). Serum samples were collected (see section 2.7.4.) on day 50 from the surviving mice (terminal bleed) and blood samples were collected for virus isolation on days 38, 40, 42, 45, 47 and 49 (days 3, 5, 7, 10, 12 and 14 post-challenge respectively). Clinical signs in the vaccinated animals were monitored closely and if they reached clinical distress (according to the clinical scoring

system, section 2.7.2), they were euthanized humanely. The experiment was terminated on day 50.

Antigen preparations for vaccination trials

Live viral vector MVA expressing AHSV-4 VP7 (Table 2.2) and wild-type MVA (Table 2.2) was obtained by Dr Javier Castillo-Olivares. Inactivation of MVA expressing VP7 was performed as described in section 2.5.1.8.

2.7.1.5. Immunogenicity of live viral vector MVA VP7 in A129 mice

Three mice (A129) were vaccinated twice each (by intraperitoneal injection) on days 0 and 21 with live MVA AHSV-4 VP7 (2×10^7 PFU, diluted in DMEM). Whole blood was collected on day 35, sera was obtained for VP7 antibody analysis (see section 2.7.4).

Antigen preparations for vaccination trials

Live viral vector MVA expressing AHSV-4 VP7 (see Table 2.2) was obtained by Dr Javier Castillo-Olivares.

2.7.1.6. Immunogenicity of baculovirus expressed AHSV-4 VP7 in A129 mice

Three groups of three mice (A129) were vaccinated (by intraperitoneal injection) with either VP7 crystals (5 μ g) (with an equal volume of Imject Alum (Thermo scientific)) or 4M urea solubilised crystals (5 μ g) (without and without Imject Alum) on days 0, 21 and 42 (diluted in PBS). Whole blood was collected on day 35 and 56, sera were obtained for VP7 antibody analysis (see section 2.7.4).

Antigen preparations for vaccination trials

Recombinant VP7 crystals were expressed using baculovirus (section 2.4.2) and purified from insect cells as described in section 2.4.3. Urea (4M) solubilised VP7 crystals were

prepared for vaccination as follows; purified VP7 crystals (at the required vaccine dose) were spun down by centrifugation (10,000g for 10 minutes). The supernatant was carefully removed and the pellet re-suspended in half the original volume of 4M urea and incubated for 1 hour at room temperature.

2.7.1.7. Immunogenicity of VP7/VP2a200 crystals and the use of virus-like particles, capsomeres and keyhole limpet hemocyanin carrier proteins as vaccine platforms in the A129 mouse model

Twelve groups of three A129 mice were vaccinated (by intra-peritoneal injection) with 200µl of either a) VP7/VP2a200 crystals (5µg); b) 4M urea solubilised VP7/VP2a200 crystals (5µg) (with and without Imject Alum); c) Murine polyomavirus VP1 VLP expressing AHSV -4 VP2a (15µg, 5µg or 1µg) ; d) Murine polyomavirus VP1 capsomere expressing AHSV- 4 VP2a (15µg, 5µg or 1µg) or e) KLH bound to AHSV-4 VP2a (15µg, 5µg or 1µg) on days 0, 21 and 42. All the vaccines were administered with equal volumes of Imject Alum unless stated otherwise and diluted in PBS.

Whole blood was collected on days 35 and 56; sera were generated for VP7 (VP7/VP2a200 crystals only) and VP2a peptide specific antibody analysis (section 2.7.4).

Antigen preparations for vaccination trials

Recombinant VP7/VP2a200 crystals were expressed using baculovirus (section 2.4.2) and purified from insect cells (section 2.4.3). Urea (4M) solubilised VP7/VP2a200 crystals were prepared for vaccination as described above in section 2.7.1.6 for wild-type VP7 crystals.

Murine polyomavirus (MPV) VP1 based VLPs and capsomeres expressing AHSV-4 VP2a peptide (KEEGEDDTARQEIRKAWVK)

were generated and provided by The University of Queensland, Australia (Protein Expression Facility). A KLH carrier molecule conjugated to AHSV-4 VP2a peptide (KEEGEDDTARQEIRKAWVK) was provided by Genscript.

2.7.2. Post-challenge, clinical scoring system in mice

The challenged mice were monitored every day for clinical signs of infection. These signs were scored and recorded to determine the humane endpoint. Should the animal score a '2' or show severe clinical distress at any point the animal was immediately euthanized.

Clinical signs of AHS in mice	Score
Coat condition	0= normal glossy coat, 0.5=mildly ruffled coat, 1= ruffled coat, ungroomed
Separation from the group	0= absent, 1=present, 2= frequent
Reduced activity	0= absent, 1=present, 2=frequent
Ocular discharge	0=absent, 1=present
Nasal discharge	0=absent, 1=present
Respiratory rate	0=normal, 1= abnormal
Weight loss	0=absent, 1=present, 2=more than 20%/weight in grams
Dehydration	0=absent, 1=present
Periorbital swelling	0=absent, 1=present, 2=compromising vision
Tremors	0=absent, 1=present
Ataxia	0=absent, 1=present
Paralysis or Paresis	0=absent, 1=present
Circling gate	0=absent, 1=present
Weight (grams)	

An overall clinical score was given to each mouse at the end of the experiment. This score was calculated by adding together the clinical values per day, divided by the total number of days the mice survived.

2.7.3. Collecting blood from mice

Bleeding from the tail vein

A small laceration was made using a sterile scalpel blade in the tail vein of mice that had been restrained within a holding apparatus. The mouse tail was then gently massaged to encourage the bleed before the blood was withdrawn using a pipette. The amount of blood drawn from the tail vein complied with bleed restrictions on the project licence. The blood was placed in labelled sterile Eppendorf tubes, with or without ethylenediaminetetraacetic acid (EDTA) (Sigma).

2.7.4. Processing blood samples from mice

Collection of sera

Blood was collected in 1.5ml sterile Eppendorf tubes without EDTA. The blood samples were incubated over night at 4°C; this allowed sufficient time for the blood to clot. The samples were then centrifuged at high speed (13,000g) for 5 minutes to pellet the blood clot. The serum was collected and stored at -20°C.

Processing of EDTA blood for virus isolation of AHSV

One millilitre of sterile 1x PBS (Life Technologies, Invitrogen) was added to 100µl of blood sample containing EDTA (Sigma) (40µl of 0.05% EDTA (Sigma) per 50µl of blood) and mixed well. The sample was then centrifuged at 500g for 5 minutes. The supernatant was discarded carefully and the pellet re-suspended in 450µl of sterile distilled water. The sample was then incubated at room temperature for 5 minutes, before 50µl of 10x PBS (Life Technologies, Invitrogen) was added. The samples were then stored at 4°C.

2.7.5. Statistical analysis

Differences between the survival rates of vaccinated and control mice, following AHSV challenge, were examined using the Log-Rank test. Differences between the clinical scores and

vireamia among vaccinated and control mice following AHSV challenge were examined using the Kruskal-Wallis test ($P < 0.05$).

2.7.6. Antibody detection

2.7.6.1. AHS-VP7 ELISA (INGENASA)

The test is a 'blocking immune-enzymatic assay' based on the detection of antibodies to AHSV-VP7 in equine serum (INGENASA, Madrid, Spain). The manufacturer's instructions were followed using the reagents provided in the kit. Briefly, positive and negative control sera (100µl) were dispensed in duplicate into the first four wells of a 96 well plate (supplied with the kit and coated with pre-bound VP7). Wells E1 and F1 were left blank. Each of the remaining wells received 100µl of diluted serum (1:5) and the plate was incubated for 1 hour at 37°C. The plate was then washed five times using the washing solution provided. Horseradish peroxidase conjugate (pre-made and provided by the kit)(100µl) was added to each well except the blank wells and incubated for 30 minutes at 37°C. The plate was washed five times before the addition of the substrate solution (100µl). After 10 minutes at room temperature stop solution was added to the plates and optical densities read at 405nm. The test was considered valid if the positive controls had an OD lower than 0.2 and negative controls had an OD lower than 1.0. The blocking percentage (BP) for each sample was calculated as:

$$BP = \frac{\text{Abs (control-)} - \text{Abs (sample)}}{\text{Abs (control-)} - \text{Abs (control +)}} * 100\%$$

Samples with a blocking percentage lower than 45% were considered negative and sample showing a blocking

percentage higher than 50% were considered positive. Samples showing a blocking percentage between 45-50% were considered inconclusive.

2.7.6.2. AHSV VP7 crystal based indirect ELISA

This test is an indirect ELISA assay to detect antibodies against AHSV VP7 in mouse sera. Baculovirus expressed VP7 crystals (section 2.4.3) were diluted (9µg/ml) in coating buffer (0.05M Carbonate bicarbonate, pH 9.6) and added to the wells of a 96 well ELISA plate (CoStar) (50µl/well). The plate was left overnight at 4°C. The following day, the plate was washed three times with PBS-0.05% Tween 20 (Sigma) and blocked with blocking buffer (PBS-0.05% Tween20, 5% milk powder) for one hour at 37°C (200µl/well). The test sera was diluted in PBS-2% milk at the optimized dilution 1/50, alongside negative and positive sera, then added to the plate and incubated at 37°C for one hour. After which the plate was washed three times with PBS-0.05% Tween 20 and the secondary antibody conjugated to horseradish peroxidase (Appendix III, Table A3.3 , Antibody No 3.) was added to the plate (50µl/well) at a 1/2000 dilution. After one hour at 37°C, the plate was washed three times with PBS-0.05% Tween 20. Fifty microliters of TMB substrate (3,3',5,5'-tetramethylbencidine liquid supersensitive) (Sigma) was added to each well and the plate incubated for 7 minutes in the dark before the reaction was stopped with 50µl/well of H₂SO₄ 3N (VWR). The absorbance was read at 450 nm using a microplate reader (FLUOstar Omega (BMG LABTECH)). The cut off value for positive values was double the mean for the negative sera.

2.7.6.3. Western-blotting for detection of VP7-specific antibodies

The VP7 protein preparations were analysed by SDS PAGE then transferred onto a nitrocellulose membrane as described above (see Western blotting, section 2.1.15). The membrane was then cut into strips and incubated in blocking solution 1 (skimmed milk at a 5% w/v concentration, in 1x PBS) with gentle agitation for 30 minutes. Each strip was then incubated at room temperature overnight with gentle agitation in the primary antibody (test antibody diluted (1:100) in blocking solution 2 (skimmed milk at a 2% w/v concentration, in 1x PBS)). The next day, the membrane was washed three times with 20mls of prepared 1x Antibody Wash (Western breeze chromogenic kit, Invitrogen, Life Technologies) for 5 minutes. Each strip was then incubated with 10 ml of the appropriate species specific secondary antibody dilution (see Appendix III; Table A3.3) for 30 minutes before the membrane was rinsed and washed with 20 ml of Antibody Wash (Western breeze chromogenic kit, Invitrogen, Life technologies) three times for 5 minutes and with water twice for 2 minutes. Chromogenic substrate (5ml) (Western breeze chromogenic kit, Invitrogen, Life technologies) was then added to each strip. If the antigen was detected a purple band would appear. Upon development, the membrane was rinsed again twice with 20ml of water before being left to dry in open air at room temperature.

2.7.6.4 AHSV-4 peptide VP2a based indirect ELISA

This test is an indirect ELISA assay to detect antibodies against AHSV-4 peptide VP2a in mouse serum. Ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) bound AHSV-4 VP2a (OVA/VP2a, KLH/VP2a respectively) was diluted 0.2µg/ml in coating buffer (0.05M Carbonate bicarbonate, pH

9.6) and added to the wells of a 96 well ELISA plate (CoStar) (100µl/well). The plate was left overnight at 4°C. The following day, the plate was washed three times with PBS-0.05% Tween 20 (Sigma) and blocked with blocking buffer (PBS-0.05% Tween20, 5% milk powder) for one hour at 37°C (200µl/well). The test sera was diluted in PBS-2% milk at the optimized dilution 1/100, alongside negative and positive sera, added to the plate and incubated at 37°C for one hour. After which, the plate was washed three times with PBS-0.05% Tween 20 and the secondary antibody conjugated to horseradish peroxidase (Appendix III, Table A3.3, Antibody No. 3) added to the plate (50µl/well) at a 1/2000 dilution. After one hour at 37°C, the plate was washed three times with PBS-0.05% Tween 20. Fifty microliters of TMB substrate (3,3',5,5'-tetramethylbencidine liquid supersensitive) (Sigma) was added to each well and the plate incubated for 7 minutes in the dark before the reaction was stopped with 50µl/well of H₂SO₄ 3N (VWR) . The absorbance was read at 450 nm using a microplate reader (FLUOstar Omega (BMG LABTECH)). The cut-off value for positive values was double the mean of the negative sera.

2.7.6.5. Plaque reduction neutralisation test (PRNT)

Serial doubling dilutions of the test sera and the positive and negative serum control samples were made in duplicate wells in a 96 well plate using DMEM media as diluent. The diluted sera (50µl) were incubated with equal volumes of a virus suspension of AHSV-4 containing 100 TCID₅₀ for 1 hour at 37°C and 1 hour at 4°C. A serial titration of virus alone was carried out in duplicate to confirm the virus dose per well. The mix was added to wells of a 12 well plate seeded with Vero cells (80% confluent) and incubated for one hour at 37°C. After one hour the medium was removed and replaced with a 1ml agarose overlay (1%). The low melting agarose had been

previously melted and diluted 1/5 with DMEM. The overlay was left to set at room temperature. The plate was incubated at 37°C for four days. After which, the cell monolayer was fixed with 10% formaldehyde and stained with 2% crystal violet. Crystal violet stains live cell so the number of plaques could be calculated for each sample. The plaques generated from each test sera sample and control preparation were counted. The number of plaques counted for the test sera were compared with the number of plaques from the control preparations (with negative sera). The plaque reduction percentage value was calculated as follows:

Plaque reduction percentage value=

$$\frac{(\text{Control plaque number} - \text{test sera plaque number})}{\text{control plaque number}} * 100$$

The PRNT₅₀ titre of each serum sample was expressed as the reciprocal of the highest dilution of serum that results in a plaque reduction percentage value of 50% or more.

2.7.7. AHSV detection

2.7.7.1. Virus titration

One hundred microliters of Dulbecco modified minimum essential media (supplemented with antibiotics, L-Glutamine and HEPES (DMEM)) was added to each well of a 96-well plate. Twenty-five µl of virus inoculum was added in duplicate to appropriate wells (highest concentration). Fifty microliters of the virus inoculum was then serially diluted (1:3) per well across the plate. BHK (baby hamster kidneys) cells (CSU, The Pirbright Institute) supplemented with 10% foetal bovine serum) were added to the plates at 2×10^4 cells per well/100µl

and the plates incubated for 4-5 days at 37°C, 5% CO₂. The plates were examined for cytopathic effect at three days. Samples were considered positive if more than 50% CPE could be observed. The titres were calculated according to the Karber Formula (Kärber, 1931).

2.7.7.2. Plaque assay

Twelve well plates were seeded (0.4×10^6 per well) with vero cells and incubated overnight at 37°C. The following day different quantities of the virus inoculum were added to each well (100µl 50µl and 20µl). After one hour at 37°C, the inoculum was removed and the cell monolayer washed with unsupplemented DMEM media before a 2.5ml agarose overlay (1% low melting point agarose (Invitrogen)) was carefully added to the monolayer. The low melting agarose had been previously melted and diluted 1/5 with DMEM. The overlay was left to set at room temperature. The plate was then incubated for 5 days 37°C. After which, the cells were fixed by adding 10% formaldehyde on top of the agarose plug and incubated for 1 hour. After which, the agarose plug was removed and the monolayer stained with crystal violet (1% crystal violet in 80% methanol, 20% distilled water). After 15 minutes, the stain was removed and the plate wells washed with tap water and left to dry. Crystal violet stains live cells so the plaques could be calculated for each sample.

Chapter 3:

Characterisation of recombinant expressed AHSV-4 VP7

3.1. Introduction

The African horse sickness virus particle is comprised of three concentric protein layers, the outer capsid (VP2 and VP5), the outer core (VP7) and the inner core (VP3) – enclosing the RNA genome and proteins of the replicase complex (VP1, VP4 and VP6) (Roy *et al.*, 1994). During AHSV particle assembly monomers of VP7 spontaneously interact with each other to form spiralled trimeric units (260). These trimers are arranged with T=13 icosahedral symmetry, as a lattice of 5 and 6 membered rings (Grimes *et al.*, 1998), to complete the outer-core layer of the virion. This VP7 lattice layer increases particle stability and bridges the inner core (VP3) and outer virus (VP2 and VP5) layer (Roy *et al.*, 1994).

VP7 is considered to be a primary antigenic determinant of 'serogroup' for each virus species within the genus *Orbivirus*. VP7 is encoded by AHSV segment 8, is 349 amino acids residues in length and approximately 37.8kDa in size (Roy *et al.*, 1994; Roy, 1991). The structure of the VP7 molecule has been resolved to atomic resolution for both BTV and AHSV (top domain only) and is very similar. The amino acid sequence of VP7 is also highly conserved between different AHSV isolates (>98% identity) (Bachanek-Bankowska, 2012) but shows greater differences compared to other orbiviruses. For example, AHSV VP7 shows ~43% amino acid identity to VP7 of BTV, which is one of the most closely related *Orbivirus*

species (Basak *et al.*, 1996), and unsurprisingly the structure of their VP7 molecules is very similar.

During infection with either BTV or AHSV, VP7 monomers interact to form soluble trimers that then bind to the surface of the sub-core (composed of VP3) to form the outer surface of the core-particle (Roy *et al.*, 1994). However, unlike BTV, AHSV VP7 trimers can also aggregate to form crystalline-array structures, approximately 5µm in diameter, within the cell cytoplasm (Burroughs *et al.*, 1994). The function of these AHSV VP7 crystal structures is unknown. It is well documented that over expression of recombinant AHSV VP7 in insect cells similarly results in the generation of bi-dimensional VP7 crystals (Chuma *et al.*, 1992; Maree & Paweska, 2005). The advantages of using recombinant baculovirus for the expression of AHSV VP7 includes: high level expression of the recombinant protein, post-translation modifications similar to those in other insect or mammalian cells and the antigenic and functional similarity of the recombinant protein, to their authentic counterpart (Kost *et al.*, 2005).

Before investigating the immunogenicity of VP7 crystals *in vivo* (Chapter 4), it was considered necessary to characterise the expression of VP7 crystals *in vitro*. Chapter 3 therefore describes the expression of recombinant AHSV VP7 using baculovirus and MVA expression systems. Additionally, this chapter describes the generation of reagents to facilitate vaccination and challenge studies in Chapter 4. This includes purification of VP7 crystals from insect cells and treatment of VP7 crystals to reduced crystal size and increase solubility.

3.2. Results

3.2.1. Expression and characterisation of AHSV-VP7 in insect cells

Recombinant baculovirus expressing the AHSV-4 VP7 gene (FB-VP7, Table 2.2) was provided by Dr Javier Castillo-Olivares (Chiam *et al.*, 2009) and was used to infect Sf9 insect cells as described in Chapter 2, section 2.4. Light microscopy and examination of immunofluorescence verified the expression of VP7 which resulted in the generation of cytoplasmic bi-dimensional crystalline structures.

Light microscopy showed rod-shaped structures within the cytoplasm of Sf9 cells 48 hours after infection with FB-VP7 (Figure 3.1). These rod-like structures are rolled-up bi-dimensional sheets that unfold when the cells were lysed by the action of mild detergents. Multiple large crystals (1-4) were observed in different cells, as shown in Figure 3.1, Image B. Flat hexagonal crystals (i.e. those exceeding the diameter of the cells) could be observed in isolation having burst out of the cell.

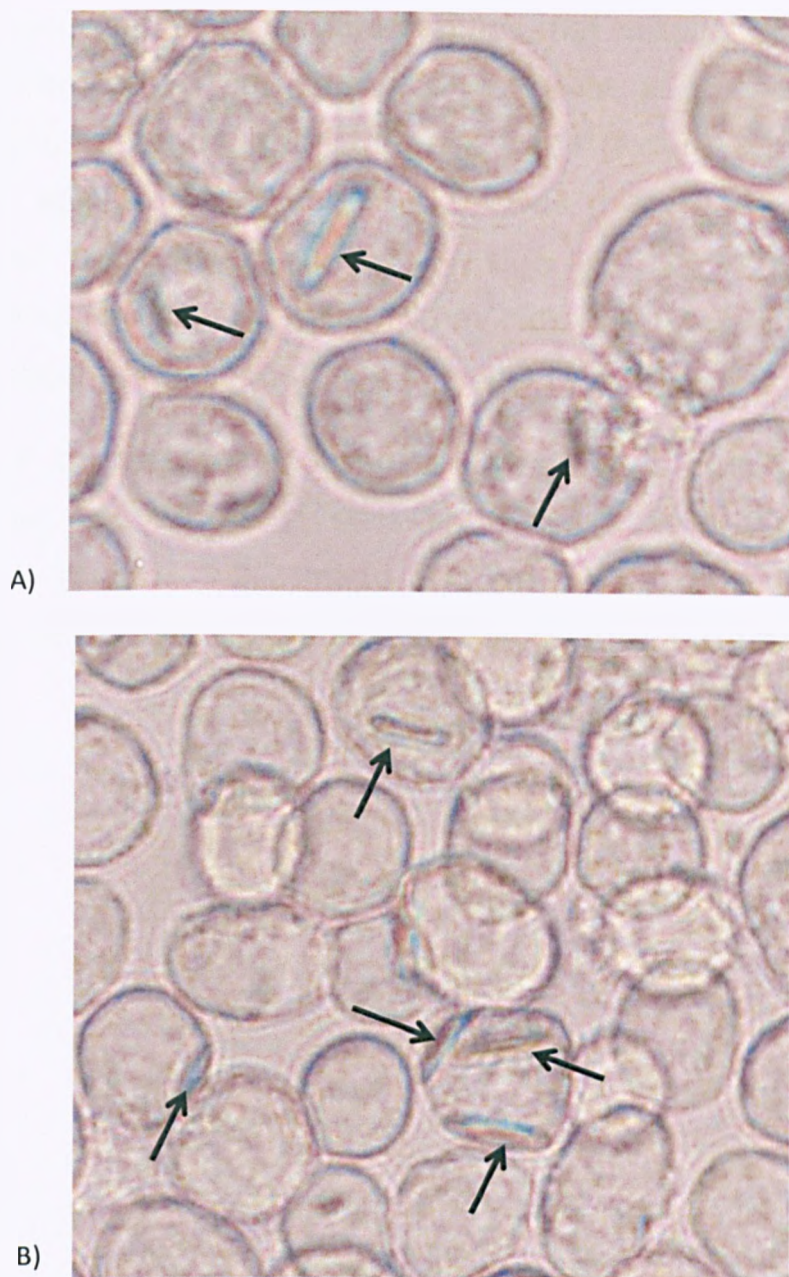


Figure 3.1: Detection of baculovirus expressed AHSV-4 VP7 inside Sf9 insect cells by light microscopy.

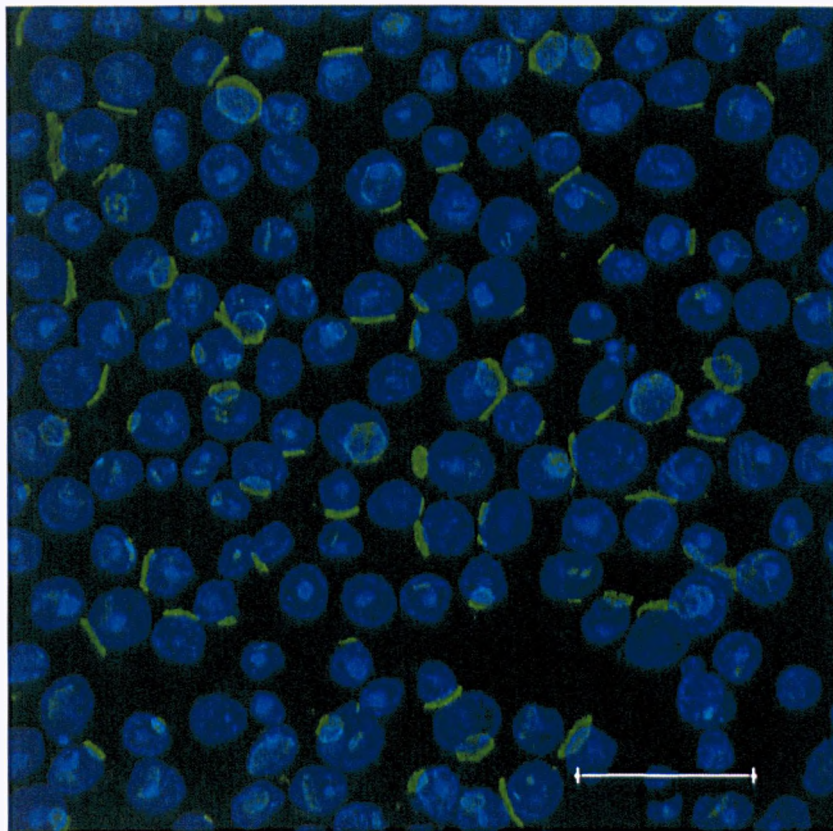
Sf9 cells were infected (MOI 1) with recombinant baculovirus (FB-VP7) and imaged at 48 hours post-infection by light microscopy. Inside the living infected insect cells the VP7 crystals appear as rod-like structure, as indicated by the black arrows.

The VP7 crystals were detected by immunofluorescence using a polyclonal anti-AHSV-9 antiserum (Appendix III, Table A3.1) within the cytoplasm of recombinant baculovirus infected cells.

Unlike visualisation by light microscopy, flat hexagonal crystals (in addition to the rod-like structures) were also observed by immunofluorescence inside the insect cell (Figure 3.2, Image A). The appearance of the crystals was not strictly uniform owing to their flexible nature. Due to their angle and positioning inside the cell, they did not always look symmetrical or hexagonal.

The VP7 crystals were observed by 48 days post-infection when the size of the crystals varied between $\sim 3\text{-}25\mu\text{m}$ in diameter (Figure 3.3, Image A). No VP7 expression was detected within 24 hours post-infection (Figure 3.3, Image B).

A)



B)

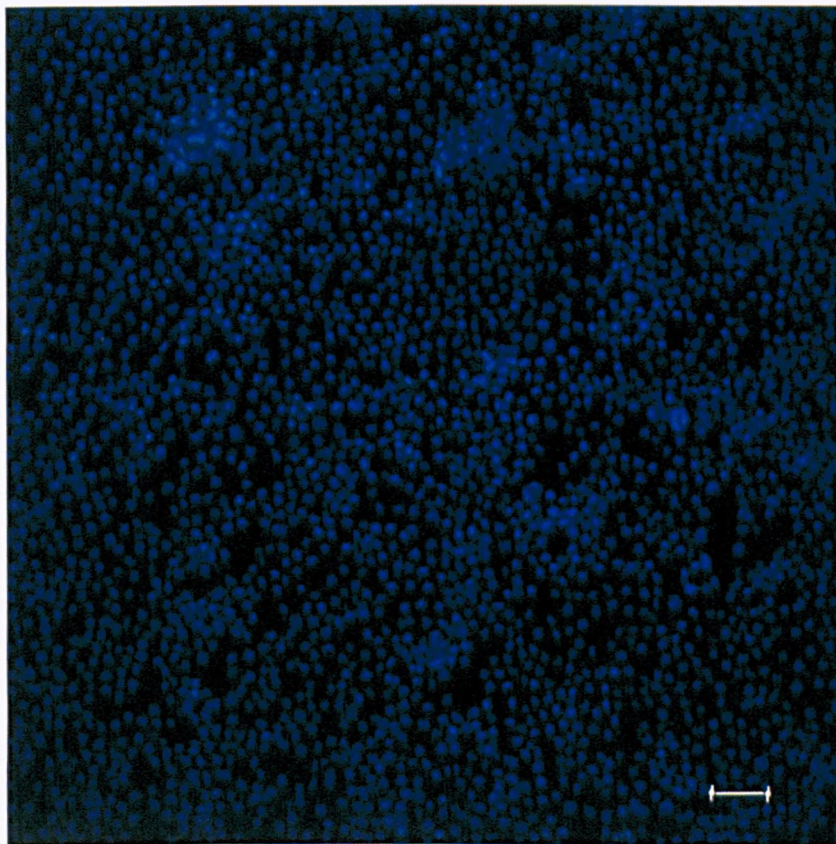


Figure 3.2: Detection of baculovirus expressed AHSV-4 VP7 in Sf9 insect cells by immunofluorescence.

Sf9 cells were infected with FB-VP7 (MOI 0.1), fixed at 3 days post-infection and subjected to indirect immunofluorescence, using polyclonal anti-AHSV-9 rabbit antiserum (Appendix III, Table A3.1) and Alexa-fluor 488 conjugated anti-rabbit antibodies. The cell nuclei are stained blue with DAPI. VP7 is stained green. Image A: SF9 insect cells infected with FB-VP7; Image B: Negative control: non-infected Sf9 cells. The white scale bar depicts 50µm

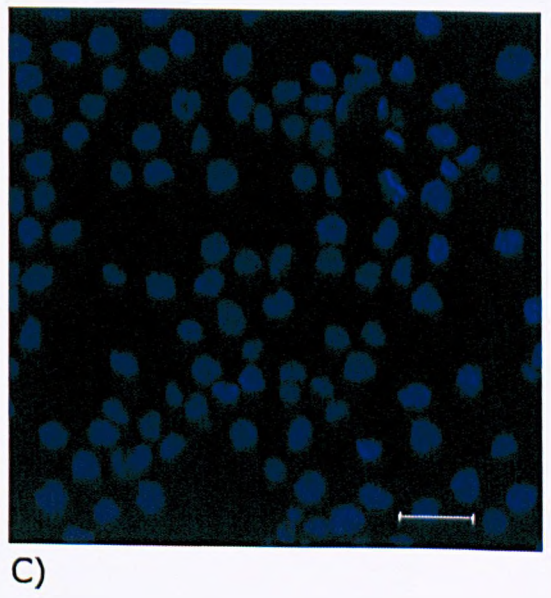
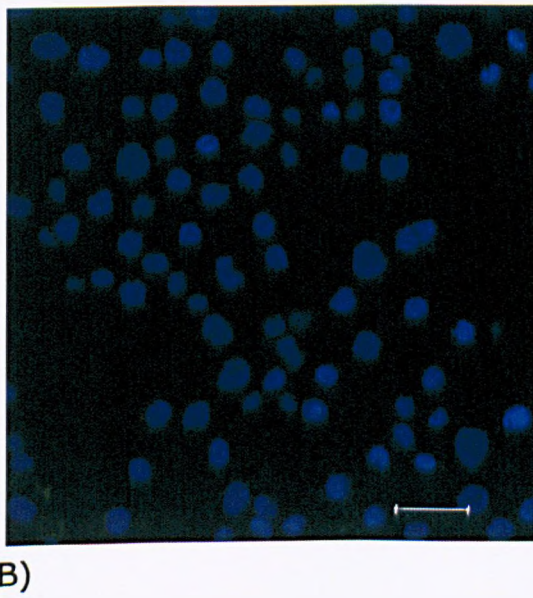
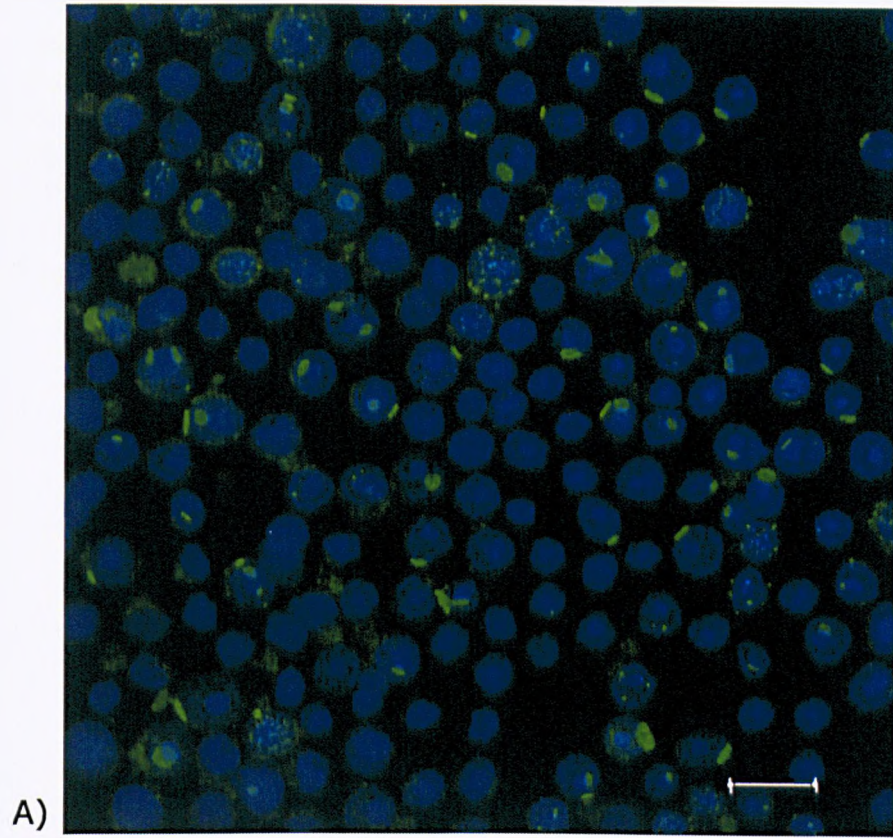


Figure 3.3: Detection of baculovirus expressed AHSV-4 VP7 in Sf9 insect cells by immunofluorescence at 24 and 48 hours post-infection.

Sf9 cells were infected with FB-VP7 (MOI 1), fixed at 24 and 48 hours post-infection and subjected to indirect immunofluorescence, using polyclonal anti-AHSV-9 rabbit antiserum and Alexa-fluor 488 conjugated anti-rabbit antibodies. Image A: Sf9 cells infected with FB-VP7 and fixed at 48 hours post-infection; Image B: Sf9 cells infected with FB-VP7 and fixed at 24 hours post-infection; Image C: Uninfected Sf9 cells. The cell nuclei are stained blue with DAPI. VP7 is stained green. The white scale bar depicts 25µm.

The antigenic reactivity of baculovirus expressed VP7, with anti-AHSV-9 antiserum, was confirmed by Western blot. A distinct band at ~37kDa was detected on the blot membrane and absent from a prepared mock infection as shown in Figure 3.4. This size corresponds to the predicted molecular weight of VP7. The extra bands observed on the blot are probably due to non-specific reactivity of the polyclonal antibody with other cellular proteins in the lysate preparation. At 72 hours post-infection, the infected cell lysates were subjected to centrifugation (10,000g for 10 minutes). The large majority of the expressed VP7 was in an aggregated form and could be recovered in the pellet. Minimal quantities of soluble VP7 were detected in the supernatant as shown in Figure 3.5.

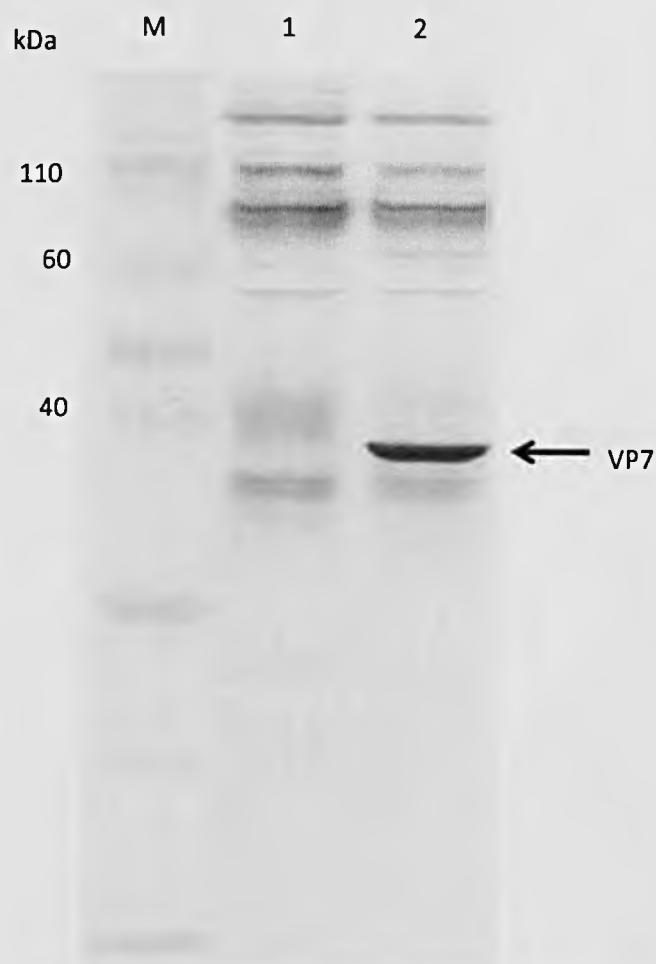


Figure 3.4: Detection of VP7 in FB-VP7 baculovirus infected cells by Western blot.

Sf9 cells were infected with FB-VP7 (MOI 1), harvested at 3 days post infection, lysed with 0.25% NP40 then analysed by SDS PAGE and Western blot using polyclonal anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Mock infected cell lysate; Lane 2: FB-VP7 infected cell lysate. The arrow indicates the expected molecular weight of VP7 (~37kDa).

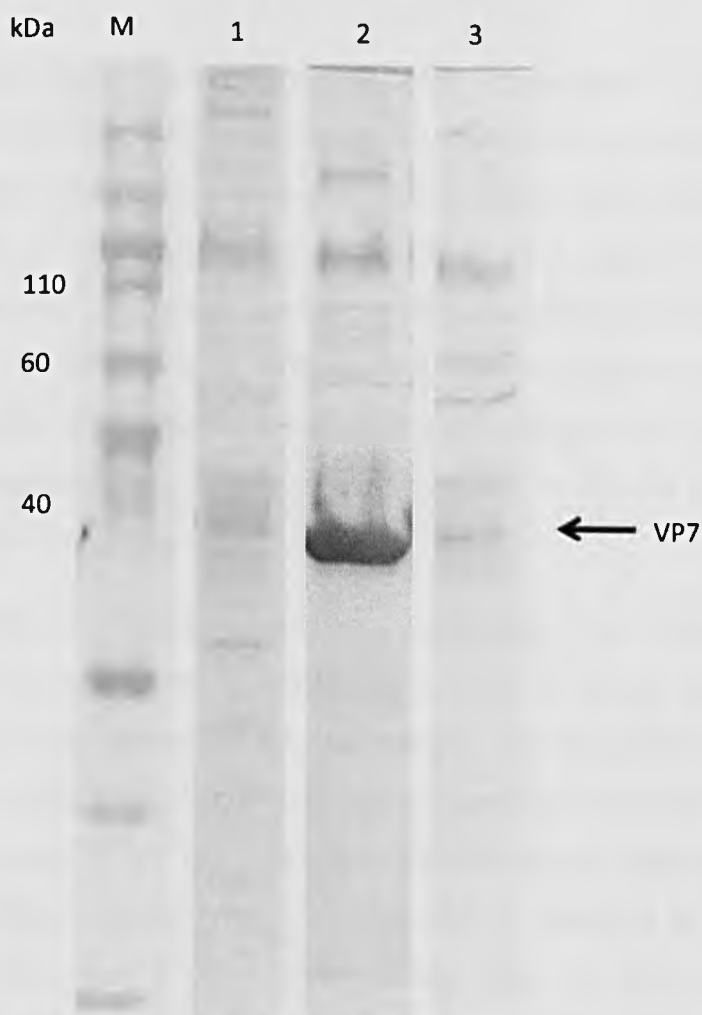


Figure 3.5: Detection of VP7 in the pellet and supernatant of FB-VP7 baculovirus infected cell lysate by Western blot.

Sf9 cells were infected with FB-VP7 (MOI 1), harvested at 3 days post infection, lysed with 0.25% NP40 and centrifuged at 10,000g for 10 minutes. The supernatant was collected and the pellet and supernatant analysed by SDS PAGE and Western blot using polyclonal anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Mock infected cell lysate; Lane 2: FB-VP7 infected cell lysate pellet; Lane 3: FB-VP7 infected cell lysate supernatant. The arrow depicts the expected molecular weight of VP7 (~37kDa).

3.2.2. Purification of VP7 crystals from insect cells

In order to conduct vaccination studies and to assess its immunogenic properties (Chapter 4), AHSV-VP7 crystals had to be purified from the recombinant baculovirus infected insect cell culture. The basic protocols used for this procedure were

based on those described by Maree and co-workers (2005) with various modifications that were investigated during this project. It was difficult to separate the crystals from the cell debris without compromising crystal yield. The use of lysis buffers containing different detergents (including sarkosyl, Triton X-100 or NP40) and differential centrifugation steps (including ultracentrifugation using sucrose gradients (40%-68%)) were explored with little improvement in either purity or yield (results not shown).

A slightly different procedure was assessed by pelleting together the nuclear and cytoplasmic fraction in order to maximise yield and simplify the procedure without affecting purity. The method combined the use of detergent NP40 and freeze-thaw cycles (-80°C) followed by filtration through a sterile gauze filter (as described in Chapter 2, section 2.4.3). The homogenised cell lysate (containing the crystals) was frozen at -80°C and thawed slowly resulting in the aggregation of the cell debris upon thawing, leaving a fraction of the crystals remaining in the supernatant. The supernatant was then passed directly through a gauze filter ($\sim 40\mu\text{m}$ gauze). The crystals in suspension passed freely through the filter while the cell debris 'clump' was retained. The purity of the crystal preparation was sub-optimal but cell debris contamination was no less than that what was observed using the other methods. The purity of the crystal preparation was assessed by SDS-PAGE and protein staining. A band of the correct size for VP7 ($\sim 37\text{kDa}$) was observed, with minimal contaminating protein bands, as shown in Figure 3.6. The basic structural preservation of the purified crystal after treatment was confirmed by differential interference contrast (DIC) microscopy (Figure 3.7).

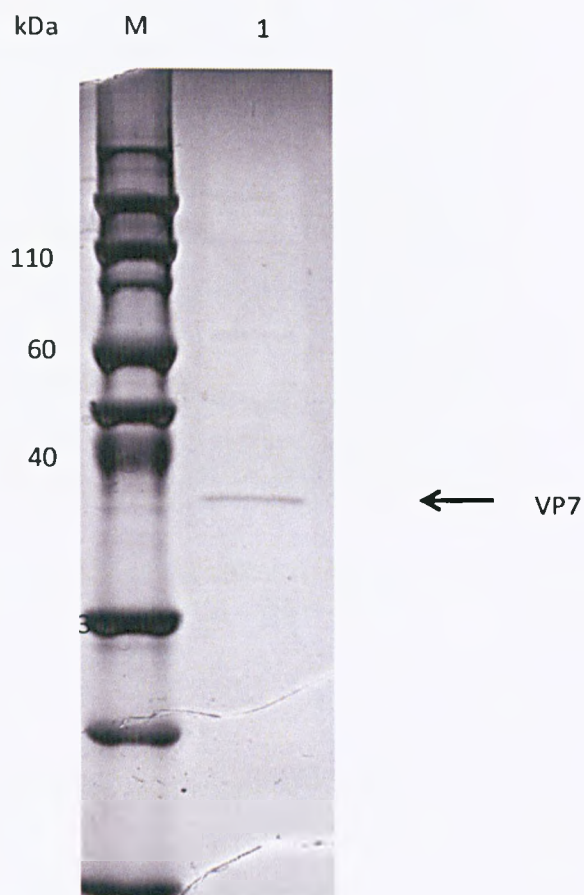


Figure 3.6: Detection of purified VP7 crystals by protein staining.

The purified VP7 crystals, obtained through combined use of detergents and filters, were separated by SDS page and stained with SimplyBlue Safestain [™]. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Purified VP7 crystals. The arrow depicts the expected molecular weight of VP7 (~37kDa).

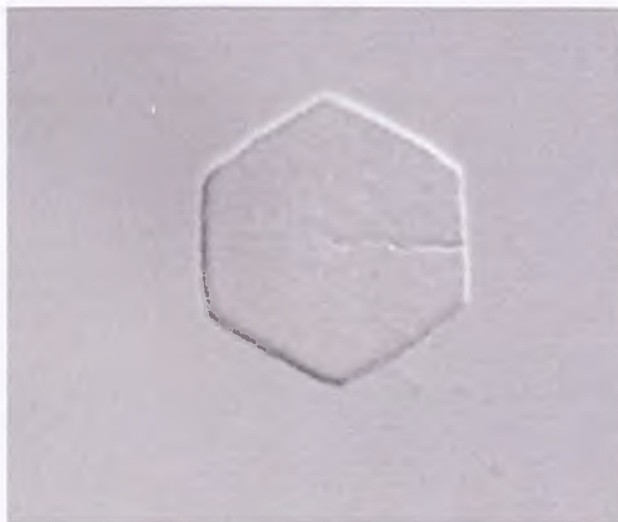


Figure 3.7: Baculovirus expressed AHSV-4 VP7 crystal.
Image obtained by differential interference contrast (DIC) microscopy.

3.2.3. Quantification of purified VP7 crystals

The quantity of total protein in each preparation of VP7 was initially confirmed by the BCA protein quantification assay (Pierce) whereby an approximate crystal to cell debris ratio in each preparation was confirmed by protein staining (Figure 3.6). The VP7 concentration was also determined by SDS-PAGE alongside known BSA concentration standards as described in Chapter 2, section 2.1.18 (Figure 3.8). The purified yield of a 175 cm² tissue culture flask of Sf9 cells infected with FB-VP7 (5 MOI) and harvested at 94 hours post-infection was approximately 500µg of purified protein per flask, as determined by the BCA assay (Chapter 2, section 2.1.17), and approximately 250µg of purified VP7 crystals determined by comparisons to the BSA standards.

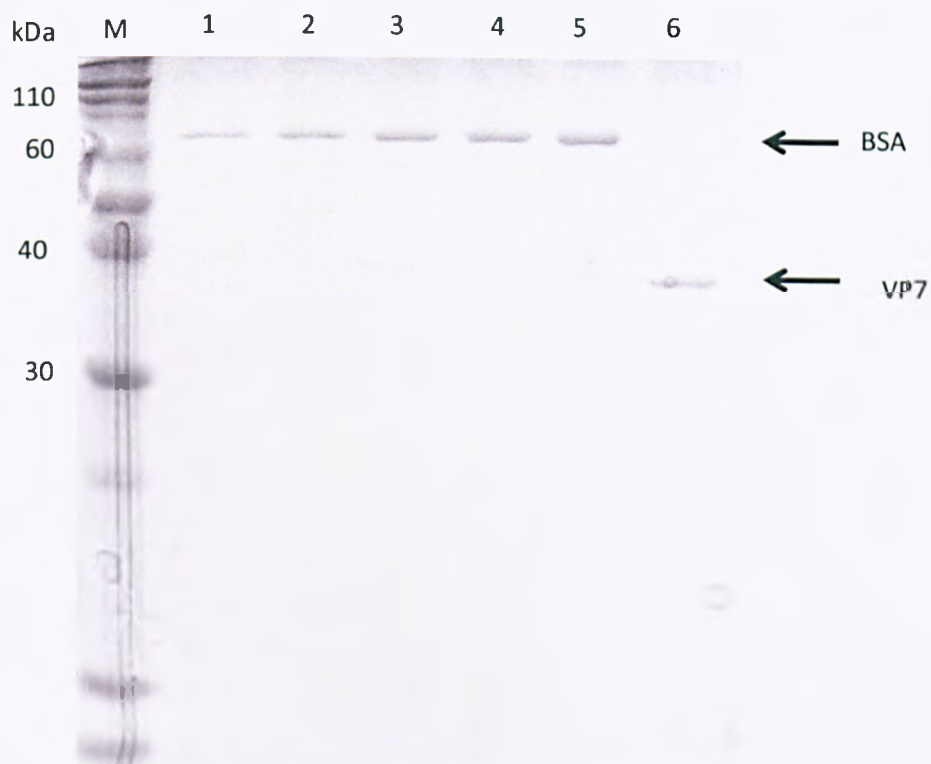


Figure 3.8: Detection and quantification of purified VP7 crystals by protein staining.

The purified VP7 crystals were separated by SDS page and stained with SimplyBlue Safestain [™]. M: Protein marker Novex® Sharp Protein Standard; Lane 1-5: BSA concentrations 25ng, 50ng, 100ng, 200ng, 250ng respectively; Lane 6: Purified VP7 crystals. The arrow depicts the expected molecular weight of VP7 and BSA (~37kDa and ~69kDa respectively).

The retention of antigenic epitopes recognised by polyclonal anti-AHSV-9 antiserum within the purified crystal preparations of VP7 was confirmed by Western blot. A band corresponding to size of VP7 (~37kDa) was present on the membrane after probing with the antiserum and absent after probing with normal rabbit antiserum (Appendix III, Table A3.1), as shown in Figure 3.9, verifying the presence of VP7 within the crystal preparation.

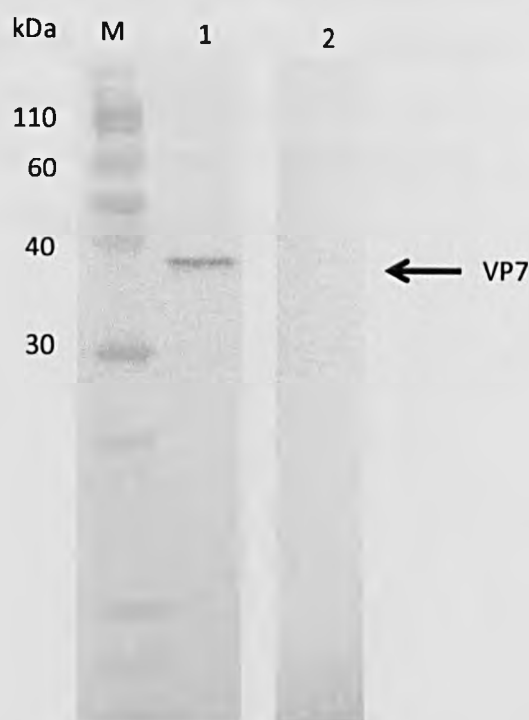


Figure 3.9: Detection of purified VP7 crystals by Western blot.

Purified crystals were separated by SDS PAGE and analysed by Western blot using anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard, Lane 1: Purified VP7 crystals probed with anti-AHSV-9 antiserum; Lane 2: Purified VP7 crystals probed with normal rabbit serum (Appendix III, Table A3.1).

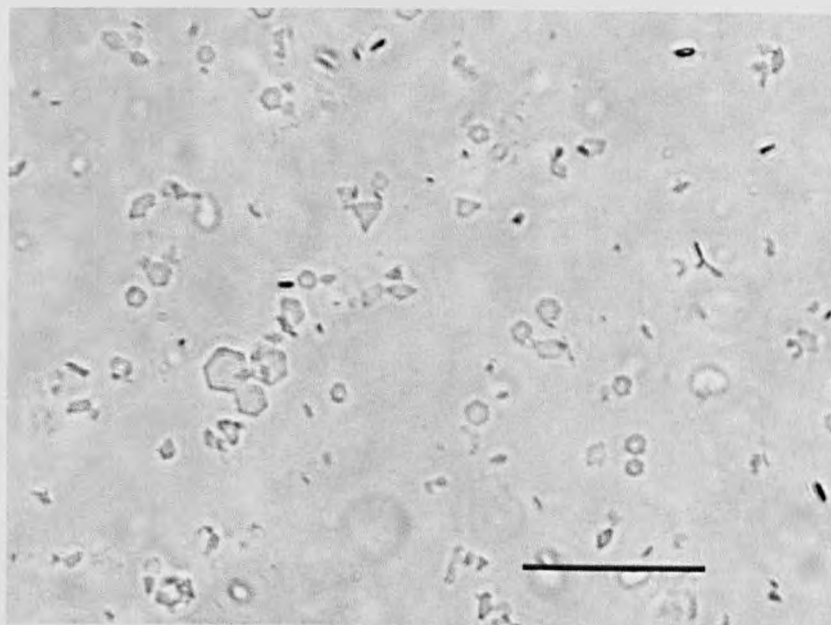
3.2.4. Sonication of VP7 crystals

To study the significance of the size of the VP7 crystals in relation to their immunogenicity *in vivo* (Chapter 4), baculovirus expressed VP7 crystals were subjected to sonication to generate smaller fragments and reduce the size of the antigen assemblies (Chapter 2, section 2.4.4). Successful sonication of the crystal material was confirmed by light microscopy and by Western blot.

Sonication broke the intact VP7 crystal into smaller protein crystal fragments. Pulsing (30 seconds) for 4 minutes and 30 seconds fragmented the VP7 crystals, as assessed by light

microscopy (Figure 3.10). The sonicated VP7 protein still reacted with polyclonal anti-AHSV-9 antiserum by Western blot. A clean single band representing VP7 (~37kDa) was evident on the blot membrane, indicating that sonication had not degraded VP7 (Figure 3.11).

A)



B)

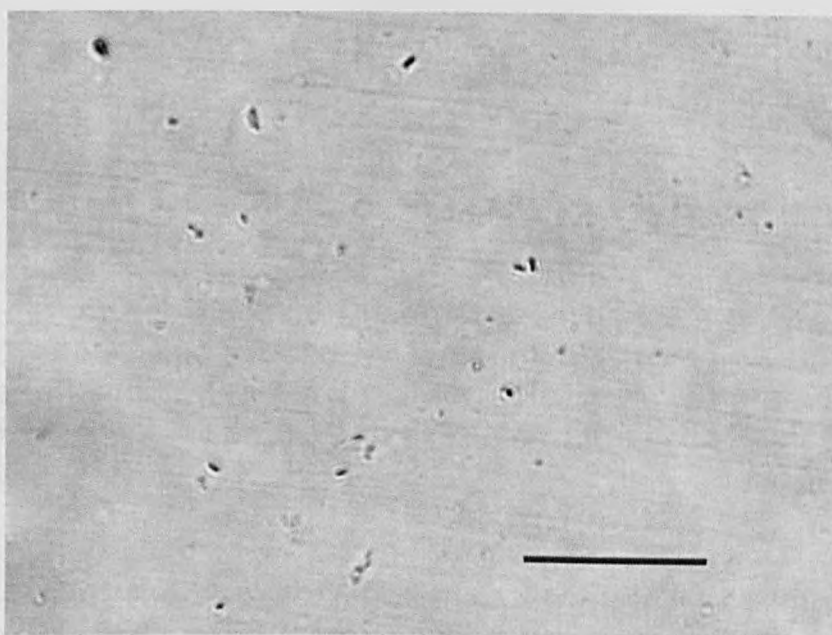


Figure 3.10: Visualisation of fragmented VP7 crystals after sonication by light microscopy.

Purified VP7 crystals were sonicated for 4 minutes and 30 seconds, as described in the Chapter 2, section 2.4.4, and visualised by light microscopy after the crystals/ crystal fragments had been allowed to settle for 30 minutes. Image A: VP7 crystals pre-sonication treatment; Image B: VP7 crystals post-sonication treatment. The black scale bar depicts 50 μ m.

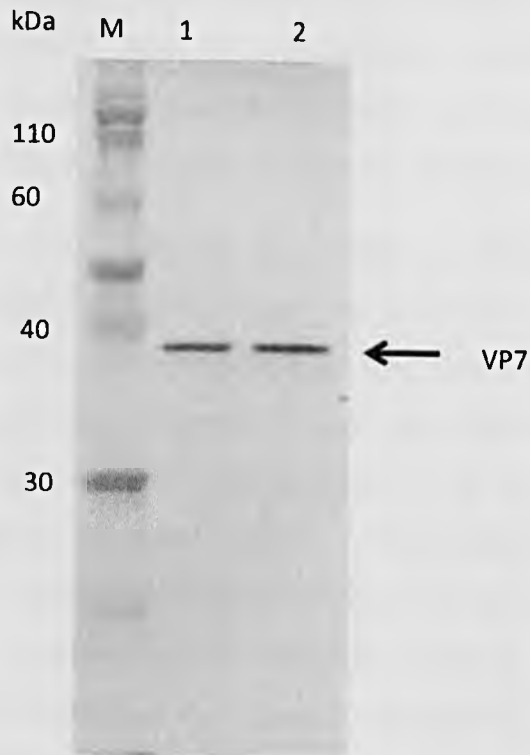


Figure 3.11: Detection of VP7 antigen after sonication, by Western blot.

Purified VP7 crystals were sonicated for 4 minutes and 30 seconds, as described in Chapter 2, section 2.4.4, separated by SDS PAGE and probed by Western blot using anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Intact VP7 crystals; Lane 2: sonicated VP7 crystals.

The sizes of the crystal fragments were initially estimated by light microscopy as shown in Figure 3.10. The crystal preparations were allowed to settle for 30 minutes before imaging. It was observed that the intact crystals sedimented much more rapidly than the fragmented crystals. The estimated average size of intact crystals by light microscopy (Figure 3.10, Image A) was approximately $5\mu\text{m}$ which corresponded to the size of crystals described in the literature (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree, 2000; Maree, 2005). The estimated average size of the sonicated fragmented crystals was approximately $1\text{-}2\mu\text{m}$ as determined

by light microscopy (Figure 3.10, Image B). Noting that this was not the most accurate way of determining particle size, intact and sonicated crystal preparations were also analysed by Digital light scattering (DLS) analysis for re-confirmation.

DLS analysis (Figure 3.12, Image A) shows a sharp peak (~20%) between ~3500-6500nm (peak at 5000nm) for the intact crystal preparation, corresponding to the size of crystals determined by light microscopy (see above) and described in the literature (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree, 2005). Two small peaks (~10%, ~4%) were present between ~ 70-300nm and ~250-900nm respectively but are likely to represent contaminating cellular protein in the preparation after purification. For the fragmented crystal preparation (Figure 3.12, Image B) the peak of intensity has shifted left to ~600-2500nm in size (peak between 1000-2000nm) at ~12.5%. This value corresponded to the size estimated by light microscopy. A small peak of intensity (~4%) remained between ~ 4000- 6500nm (peak between 5000-6000nm) even after sonication. A peak of ~10% intensity was also observed, at <1000nm in size (between 50-150nm), in the untreated crystal preparation but both peaks are thought likely to represent contaminating cellular proteins.

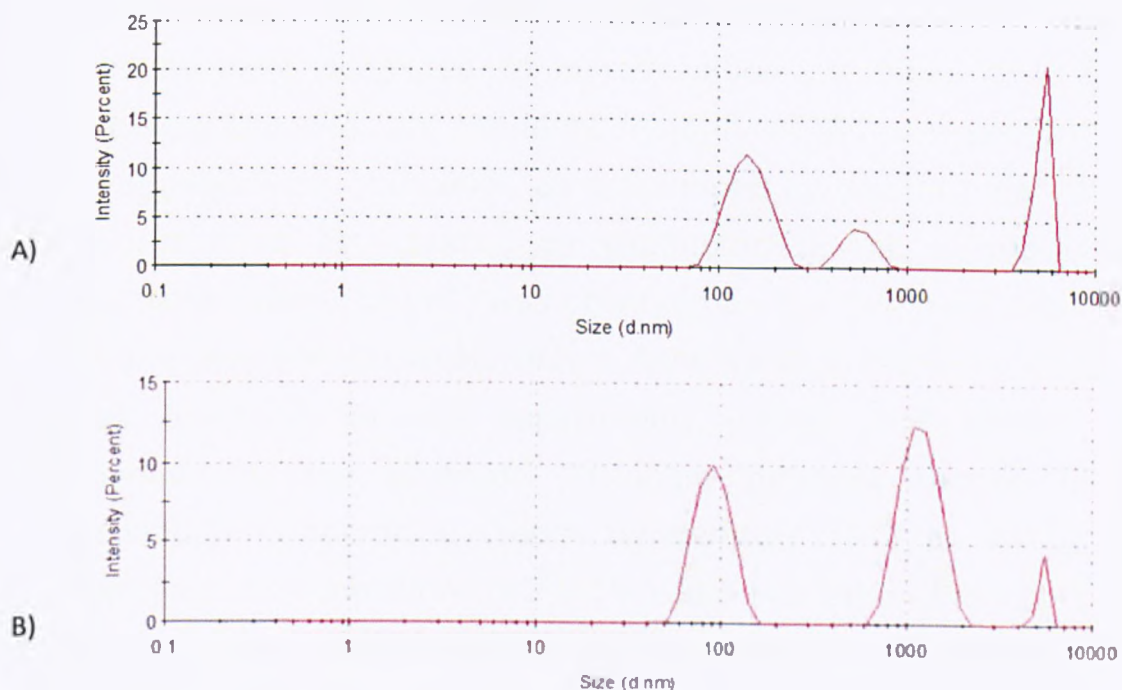


Figure 3.12: Estimating the size of intact and sonicated crystal fragments by Digital Light Scattering (DLS) analysis.

Purified intact and fragmented VP7 crystal preparations were diluted 1/10 in PBS and 100 μ l added to a UV micro cuvette and analysed at 5 reads (40 seconds each read) using The Malvern Zetasizer. Image A: Intact VP7 crystal preparation; Image B: Sonicated VP7 fragmented crystal preparation

3.2.5. Solubilisation of VP7 crystals

Sonication of VP7 crystal material resulted in smaller particulate crystal fragments. In order to reduce the size of the VP7 crystals from a particulate structure to a soluble protein the purified VP7 crystals were subjected to treatment with sarkosyl (0.5% sarkosyl in 0.05M Tris/HCl pH 8, 150mM NaCl) for 30 minutes at 25°C, based on the solubilisation method described by Rutkowska and co-workers (2011). By light microscopy and Western blot it became apparent that sarkosyl treatment led to minimal solubilisation of the crystals. By light microscopy, there was no detectable difference in the quantity of crystals observed after treatment in comparison to

mock-treated crystals without sarkosyl. After treatment the crystals were subjected to centrifugation (10,000g for 10 minutes) showing that the large majority of VP7 was present as aggregates in the pellet, as determined by Western blot. A distinct band at ~37kDa, corresponding to the estimated molecular weight of VP7, was observed on the blot membrane for the preparation pellet. Only a faint band of the same size was observed for the supernatant (results not shown). Changes to the protocol (including different ranges of incubation temperature (room temperature (RT) to 45°C), detergent concentration (0.2%-1%) and incubation time (1-3 hours)) made very little change to the degree of solubilisation using sarkosyl (results not shown).

Solubilisation using urea treatment was also attempted. It was observed that after one hour at room temperature 8M urea had completely solubilised the crystals, as confirmed by light microscopy and Western blot. After treatment the crystals were no longer observed by visual inspection. Figure 3.14 shows how treatment of the VP7 crystals with 8M urea completely solubilises the crystals. Post-treatment the crystals could no longer be visualised by light microscopy. Additionally, after the treated crystals had been subjected to centrifugation (10,000g for 10 minutes) it was found that there was an approximately equal fraction of VP7 present in the pellet and supernatant as shown in Figure 3.13. This figure also demonstrates that after one hour the degree of solubilisation is directly related to the concentration of urea.



Figure 3.13: Detection of VP7 in the pellet and supernatant after solubilisation of VP7 crystals with urea by Western blot.

Purified VP7 crystals were solubilised with 2M, 4M, 8M urea for one hour at room temperature alongside a mock-treated preparation. The preparations were centrifuged (10,000g for 10 minutes). The supernatant was collected and the pellet and supernatant analysed by SDS PAGE and Western blot using anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: 8M treated crystals (supernatant); Lane 2: 4M treated crystals (supernatant); Lane 3: 2M treated crystals (supernatant); Lane 4: Mock-treated crystals (supernatant); Lane 5: 8M treated crystals (pellet); Lane 6: 4M treated crystals (pellet); Lane 7: 2M treated crystals (pellet); Lane 8: Mock-treated crystals (pellet)

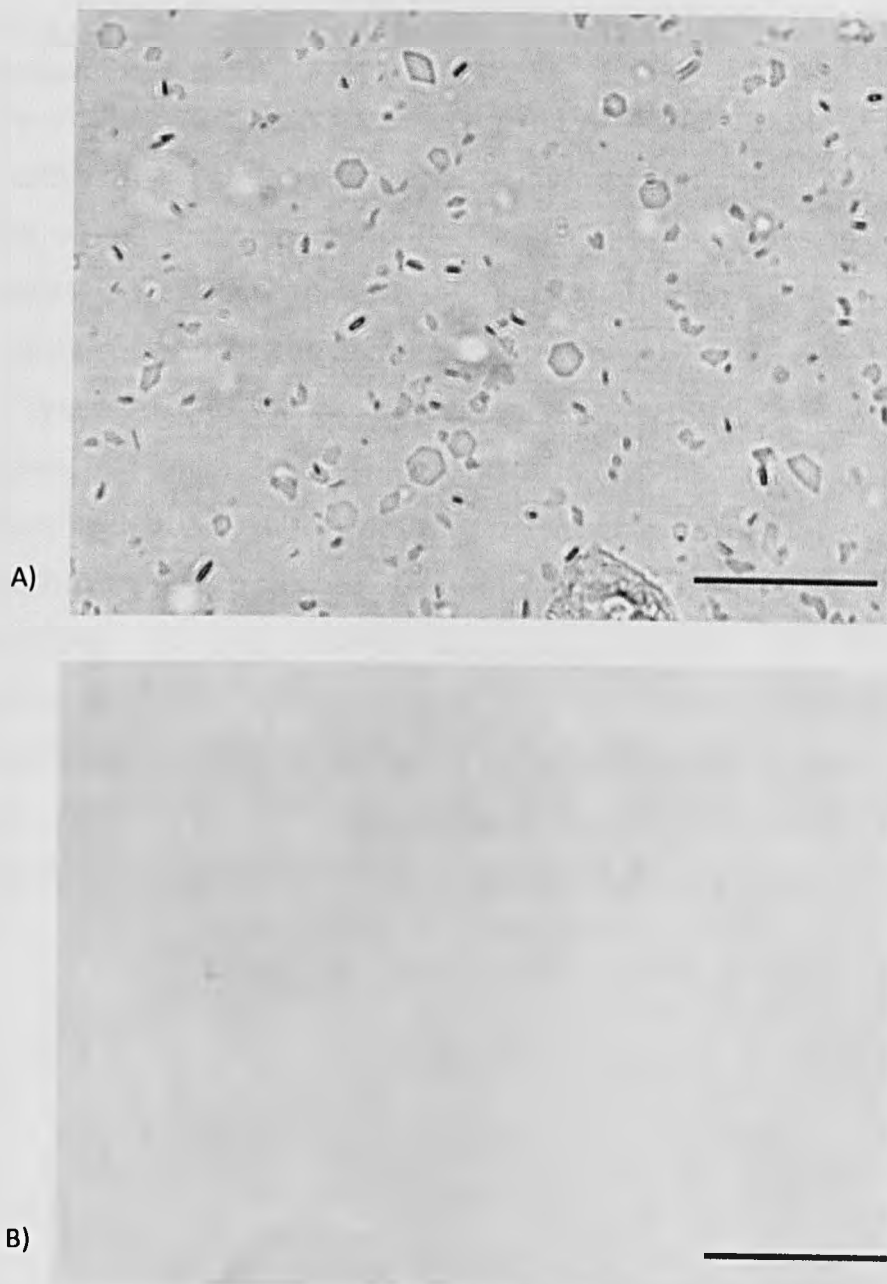


Figure 3.14: Visualisation of VP7 crystals before and after crystal solubilisation with 8M urea by light microscopy.

Purified VP7 crystals were solubilised with 8M urea (as described in Chapter 2, section 2.4.6). The crystals were visualised by light microscopy before and after treatment. Image A: VP7 crystals pre- urea treatment, Image B: VP7 crystals post- urea treatment. The black scale bar depicts 50µm.

3.2.6. Expression and characterisation of the live MVA vector expressing AHSV-4 VP7

Live viral vector MVA expressing AHSV-4 VP7 (Table 2.2) was obtained from Dr Javier Castillo-Olivares (Chiam *et al.*, 2009). This virus stock was amplified and used to infect DF-1 cells. Expression of recombinant VP7 was confirmed by detection of a protein band of the expected size for AHSV VP7 (~37 kDa) by Western blot, using polyclonal anti-AHSV-9 antiserum as shown in Figure 3.15. This band was absent from a prepared mock-infection. At 24 hours post-infection the infected cell lysates were subjected to centrifugation (10,000*g* for 10 minutes). It was found that the majority of VP7 has aggregated and was retained in the pellet, as was also observed for VP7 expressed from recombinant baculovirus (see section 3.2.1.). Minimal quantities of soluble VP7 were detected in the supernatant as shown in Figure 3.16.

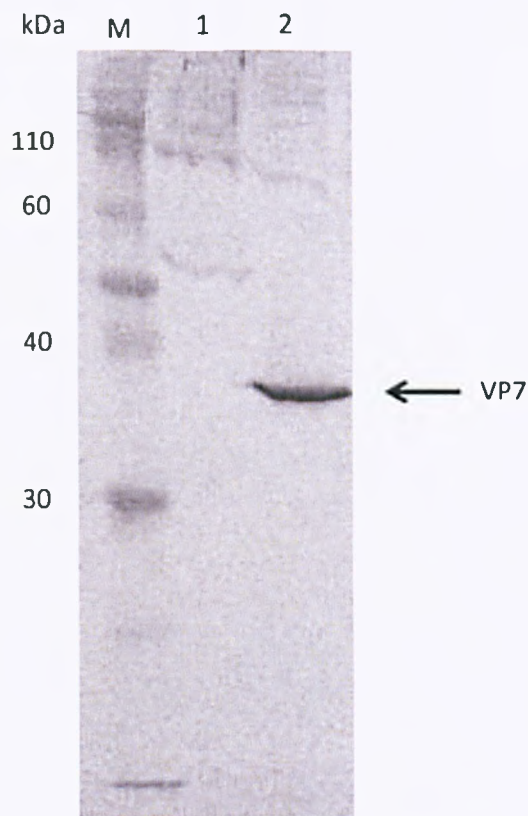


Figure 3.15: Detection of VP7 in MVA-VP7 infected DF-1 cells by Western blot.

MVA-VP7 infected, and mock- infected DF-1 cells, were harvested at 24 hours post infection, lysed with 0.25% NP40 then analysed by SDS PAGE and Western blot using anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Mock infection; Lane 2: MVA VP7 infected cell lysate.

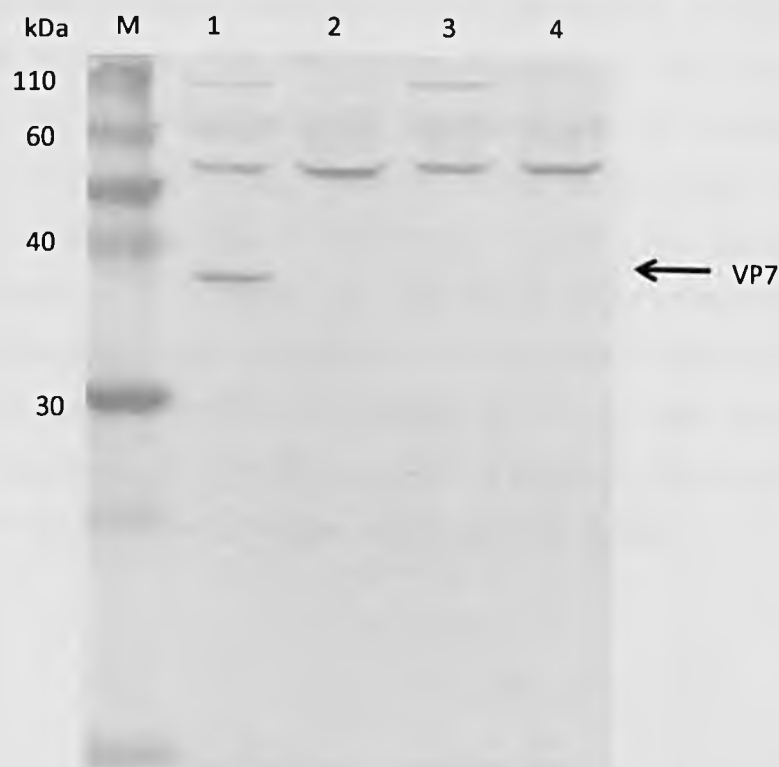
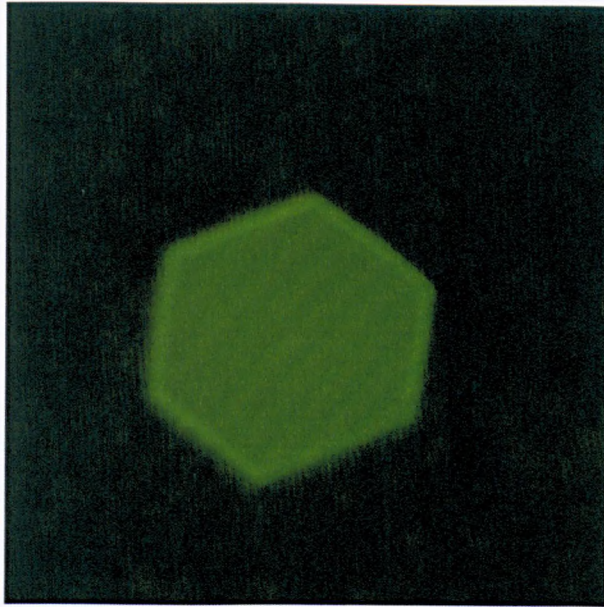


Figure 3.16: Detection of VP7 in the pellet and supernatant after centrifugation of MVA VP7 infected cell lysate by Western blot.

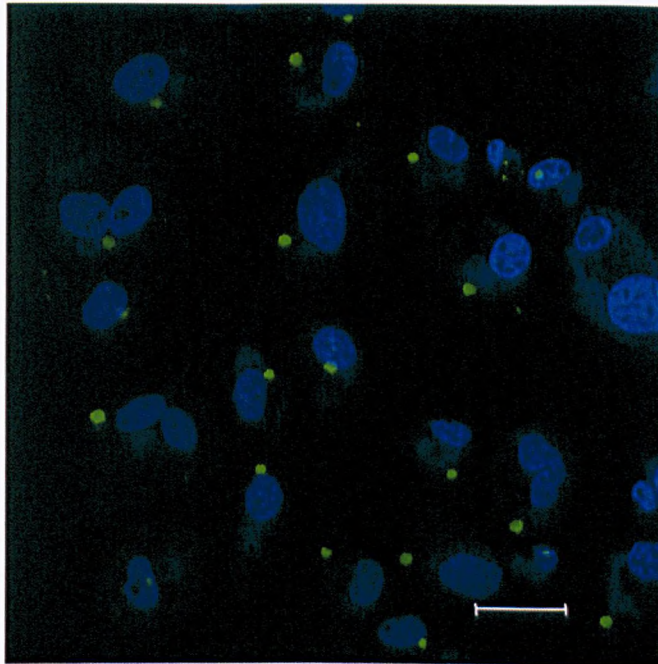
DF-1 cells were infected with MVA VP7 (MOI 1), harvested at 24 hours days post infection, lysed with 0.25% NP40 and centrifuged (10,000g for 10 minutes).The supernatant was collected and the pellet and supernatant analysed by SDS PAGE and Western blot, using anti-AHSV-9 antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: MVA VP7 infected cell lysate pellet, Lane 2: Mock infected cell lysate pellet, Lane 3: MVA VP7 infected cell lysate supernatant, Lane 4: Mock infected cell lysate supernatant.

The capacity of the AHSV VP7 protein to form hexagonal crystalline structures when expressed by recombinant MVA in DF-1 avian cells (*in vitro*) was confirmed by immunofluorescence using polyclonal anti-AHSV-9 antiserum. Hexagonal arrays were clearly visible in the MVA VP7 infected cell cytoplasm (Figure 3.17, Image A, B). Wild-type MVA was tested with polyclonal anti-AHSV-9 antiserum but showed very minimal cross-reactivity, as seen in Figure 3.17, Image C.

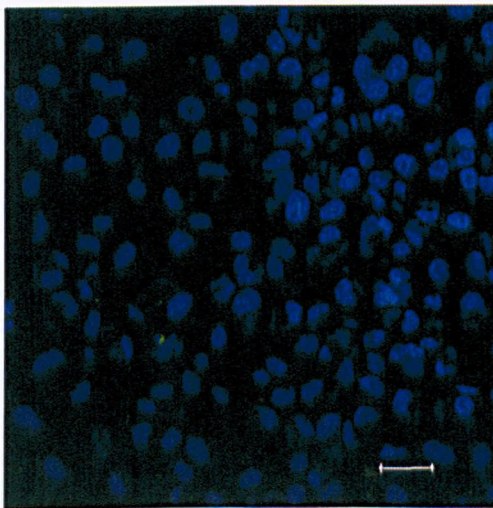
Unlike the VP7 crystals generated by recombinant baculovirus expression in insect cells, the large majority of VP7 crystals generated by recombinant MVA appeared as flat hexagonal structures and not rod-like, most likely due to the relative size of the mammalian cell. Typically, each cell contained approximately 1-2 crystals by 24 hours post-infection. In addition, the size of the crystals did not appear to exceed 6µm by 24 hours post-infection. Expression of VP7 by MVA could be detected as early as 4 post-infection (Figure 3.18, Image A) by which time VP7 had already begun to aggregate.



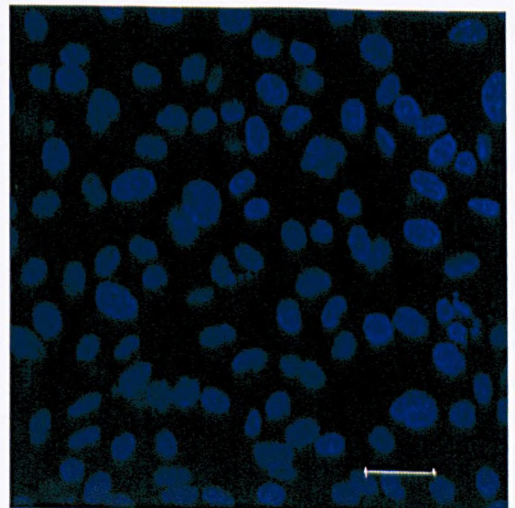
A)



B)



C)

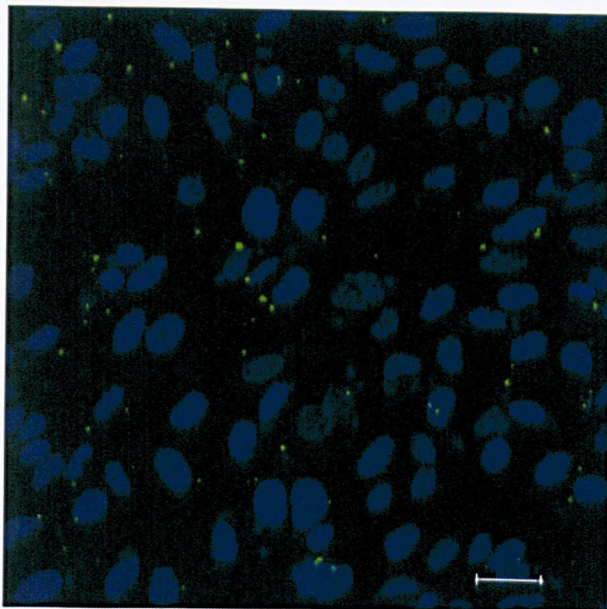


D)

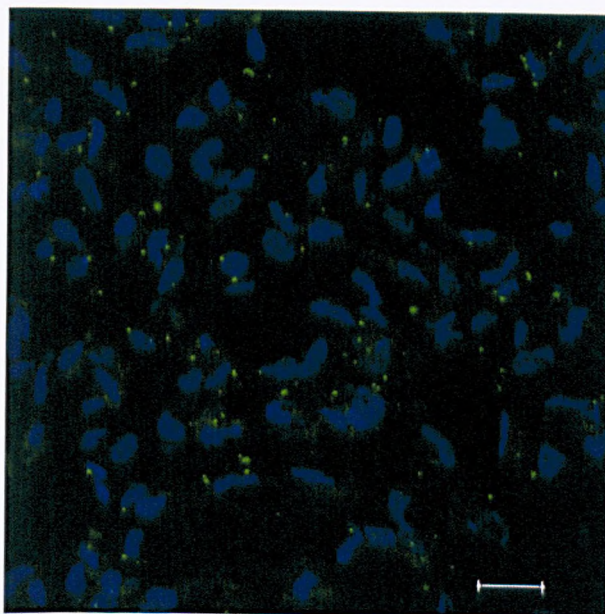
Figure 3.17: Detection of MVA expressed AHSV-4 VP7 in DF-1 avian cells by immunofluorescence.

DF-1 cells were infected with MVA expressing AHSV-4 VP7 (MOI 0.1) and fixed at 24 hours post-infection. The crystal antigens were detected using polyclonal anti-AHSV-9 rabbit antiserum followed by anti-rabbit antibodies conjugated with Alexa 488. Whilst in the fixed cell the crystals appear predominately as flat hexagonal structures. The cell nuclei are stained blue with DAPI. VP7 is stained green. Image A: DF-1 cells infected with MVA VP7 and labelled with polyclonal anti-AHSV-9 antiserum; Image B: DF-1 cells infected with MVA VP7 and labelled with polyclonal anti-AHSV-9 antiserum; Image C: DF-1 cells infected with wild-type MVA (MOI 0.1) and labelled with polyclonal anti-AHSV-9 antiserum; Image D: DF-1 mock infection labelled with polyclonal anti-AHSV-9 antiserum. The white scale bar depicts 25µm.

A)



B)



C)

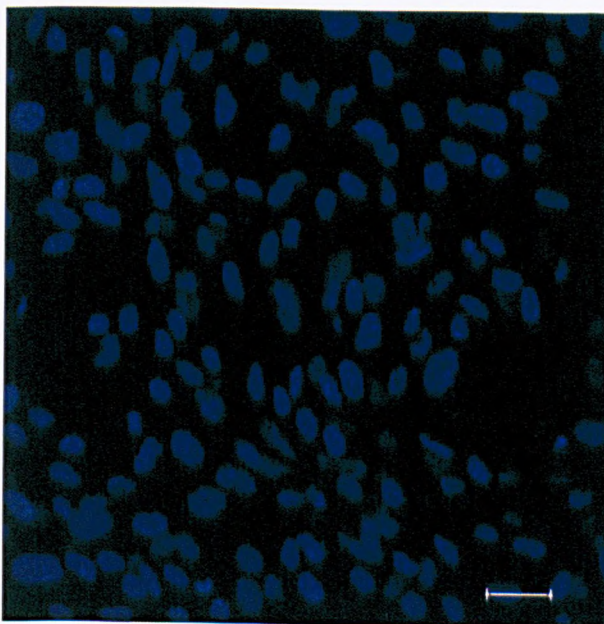


Figure 3.18: Detection of MVA expressed AHSV-4 VP7 in DF-1 avian cells by immunofluorescence at 4 and 8 hours post-infection.

DF-1 cells were infected with MVA expressing AHSV-4 VP7 (MOI 1) and fixed at 4 and 8 hours post-infection. The crystal antigens were detected using polyclonal anti-AHSV-9 antiserum followed by anti-rabbit antibodies conjugated with Alexa 488. The cell nuclei are stained blue with DAPI. VP7 is stained green. Image A: DF-1 cells infected with MVA VP7, fixed at 4 hours post-infection; Image B: DF-1 cells infected with MVA VP7, fixed at 8 hours post-infection; Image C: DF-1 cell mock infection. The white scale bar depicts 25µm.

3.2.7. Expression of AHSV-VP7 during AHSV infection

To verify that the formation of VP7 crystals occurs during AHSV infection (*in vitro*) BHK cells were infected with AHSV-4 and examined by immunofluorescence staining with polyclonal anti-AHSV-9 antiserum. The results depicted in Figure 3.19, Image A shows that VP7 crystals were indeed generated following AHSV infection and that these could be detected in the cytoplasm of infected cells within 24 hours post-infection. Both flat hexagonal and rod-like structures of approximately 3-5µm in diameter could be observed. Multiple crystals were observed per cell. As the primary antibody used in the assay was serum obtained from a rabbit experimentally infected with AHSV-9 diffuse staining was also observed which includes any soluble VP7.

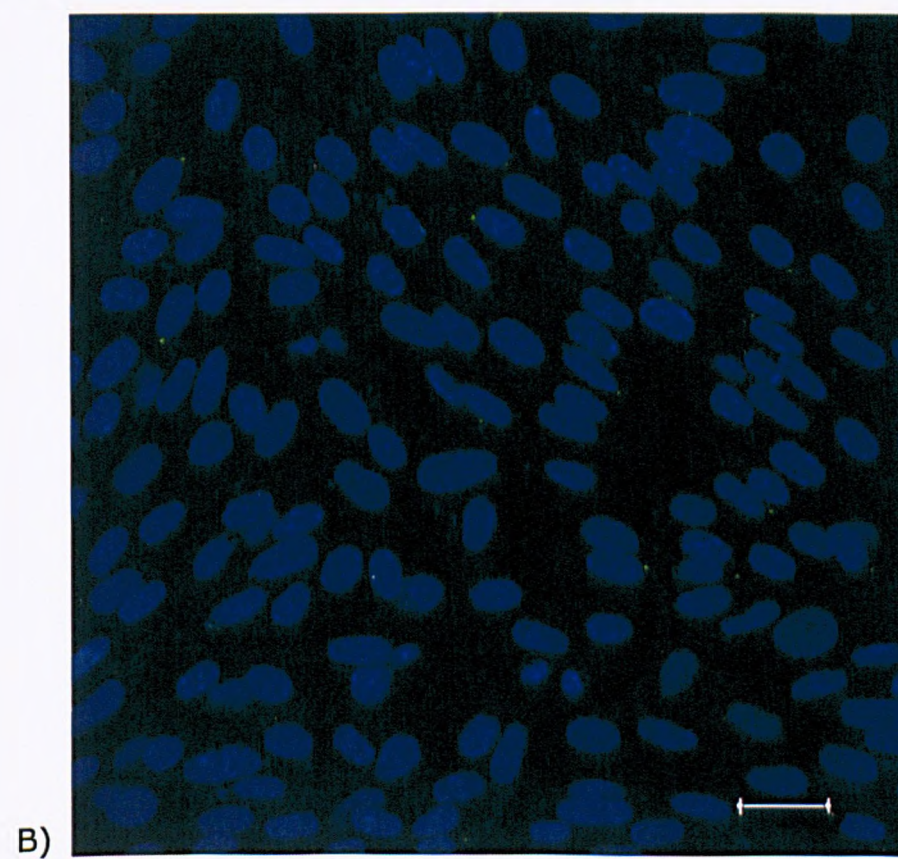
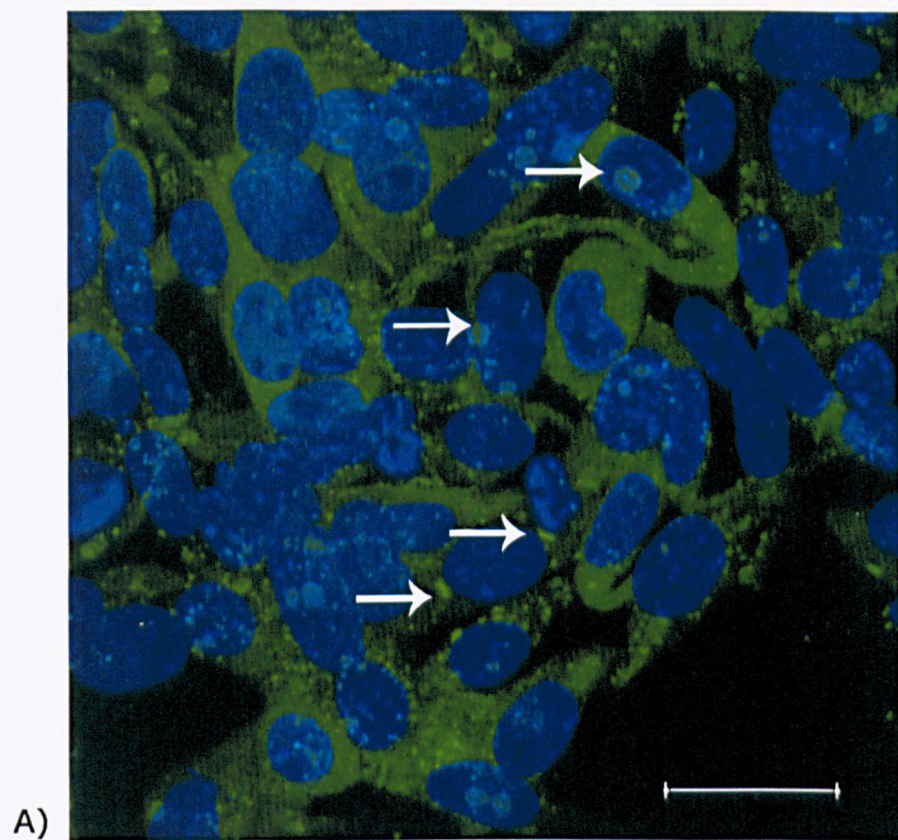


Figure 3.19: Detection AHSV-4 expressed VP7 hexagonal arrays in BHK cells by immunofluorescence.

BHK cells were infected with AHSV-4 (MOI 0.1) and fixed at 24 hours post-infection. The crystal antigens were detected using anti-AHSV-9 antiserum, followed by anti-rabbit antibodies conjugated with Alexa 488. Whilst in the fixed cell, the crystals appear as rolled up rod shaped and flat hexagonal structures. The cell nuclei are stained blue with DAPI. The white arrows depict VP7 crystals. Image A: AHSV-4 infected BHK cells labelled with polyclonal anti-AHSV-9 antiserum. Image B: A negative control of non-infected BHK cells labelled with polyclonal anti-AHSV-9 antiserum. The white scale bar depicts 25µm.

3.3 Discussion

Expression and characterisation of recombinant AHSV-VP7

Before investigating the immunogenicity of recombinant VP7 *in vivo* (in the horse and mouse model), it was first necessary to characterise the expression of recombinant VP7 *in vitro* and prepare reagents accordingly.

Recombinant baculovirus expression

A recombinant baculovirus expression system was used to express VP7 in insect cells (Sf9 cells). The results re-confirmed that expression of VP7 in insect cells results in the generation of bi-dimensional hexagonal crystals. The average size of the VP7 crystals were approximately 5µm in diameter, although the size of the crystals can range up to ~25µm as previously described by Chuma *et al.* 1992, Maree *et al.* 2005 and Burroughs *et al.* 1994. By light microscopy inside the cells the crystals can be observed as rod-shaped structures highlighting the possible flexibility of the crystal structures. Within the core particle the joints between VP7 trimers must be flexible in order to achieve a closed icosahedron. Flexibility within the trimer-trimer interaction may facilitate the crystals to roll allowing them to appear as rod shaped when space is limited, for example inside the insect cell. Additionally, as the crystals are composed of flat sheets of VP7 trimers (Maree, 2000), the capacity for the crystals to roll also suggests that the crystal depth must only a few trimer sheets thick or less. Maree (2000) describes the depth of chimeric VP7 crystals that form flat hexagonal crystals, similar to that observed for wild-type VP7, as being approximately 200nm (Maree, 2000). When visualised by immunofluorescence the crystals appear as both flat hexagonal and rod-like structures. The inability for flat crystals to be observed inside the insect cell by light microscopy is due to a lack of contrast within the cell.

The strong stained band observed after SDS-PAGE, representing VP7 from the infected cell lysate and the multiple crystals per cell, reflects the high level of expression obtained with the baculovirus expression systems. The polyhedron promoter is strong but expression is confined to the later stages of the infection cycle starting around 20 hours post-infection (O'Reilly, 1994). This would explain why expression of VP7 occurred between 24 and 48 hours post-infection. The ability of recombinant VP7 to form large crystals within this time reflects the tendency of VP7 trimers to interact with each other and the stability of this interaction.

Purification of VP7 crystals from insect cells

In order to utilise VP7 crystals for immunological studies the VP7 crystals expressed by recombinant baculovirus needed to be purified and isolated from the insect cells. The crystals were released from the cells by cell lysis and homogenisation followed by a freeze/ thawing method and filtration through a cell strainer to separate the crystal from the cell debris. This method was simple and time-effective and achieved a semi-pure crystal preparation but neither the yield nor purity was optimal. It was very difficult to separate the crystals from the cell debris which could have been due to simple protein interactions, e.g. hydrophobic, hydrophilic and charged interaction between the crystal surface and the insect cell proteins. Additionally, due to the small yield of crystals generated from the cytoplasmic fraction alone, the cytoplasmic and nuclear fraction also containing crystals were pelleted together. Lysis and disruption of the nucleus during homogenisation and freeze cycles resulted in the presence of DNA in the preparation which would have contributed to an inability to separate the crystal from the debris. If time

permitted treatment with DNAase may have helped resolve this problem.

Sonication of VP7 crystals

Reducing crystal size would make it possible to investigate the effect of VP7 crystal size on the immunogenicity of the VP7 protein. From the results it was revealed that the crystals could be fragmented into small crystal fragments by sonication. The energy generated from the process was sufficient to fragment the structures by mechanical force but the energy was too low to disrupt or denature the integral monomer-monomer or trimer-trimer interactions. From DLS analysis it also observed that a small fraction of the VP7 crystal preparation was not fully fragmented indicating that sonication did not ensure a uniform treatment of the preparation. The results generated may therefore reflect the different sizes of the VP7 crystals within the preparation. Additionally, it highlights the strength of the crystal structures.

Difficulties were experienced when attempting to accurately estimate the average fragment crystal size. As expected, neither the crystals nor the crystal fragments could be run without further pre-treatment in a native PAGE gel due to their size and particulate nature. The purified crystal preparations were known to be contaminated with cellular proteins from the insect culture cells. This made estimating the size of the fragmented crystals by DLS analysis very difficult and the interpretation of the results presented here is speculative. After sonication there was a shift of the peak, indicating a reduction in crystal size, which coincided with observations by light microscopy.

However, the presence of contaminating proteins in the preparation may have influenced the result. It was also observed by light microscopy that the larger crystals sedimented more rapidly than the fragmented crystals which also made accurate comparisons of particle size very difficult by DLS and light microscopy.

In an attempt to minimise difficulties caused by size and rapid sedimentation rates (since a primary determinant of sedimentation rate is size), DLS analysis was run at shorter reads. Additionally, VP7 crystal size can exceed 5-6µm in size (Chuma *et al.*, 1992; Maree & Paweska, 2005) yet no particles greater than this size were observed by DLS analysis. Firstly, the larger crystals (>5µm) may be in the minority and therefore would not necessarily be represented by a major peak. Secondly, much larger crystals/ larger fragmented crystals may not have been efficiently detected by DLS analysis because they may have sedimented too quickly to be registered. Thirdly, any particle size (greater than 10µm (10,000nm)) would have been dismissed because of the size limit of DLS potentially underestimating the overall size range of the intact/ fragmented crystals.

Solubilisation of VP7 crystals

Solubilisation of VP7 would offer the opportunity to investigate the effect of size and structures on the capacity of VP7 to generate an antibody response. Rutkowska and co-workers (2011) describe the efficient solubilisation of chimeric AHSV VP7 protein constructs using sarkosyl treatment (0.5% sarkosyl). However, the result shows that sarkosyl treatment induced minimal solubilisation for wild-type VP7 crystals. The VP7 crystal structures appeared relatively stable in these ionic conditions. Sarkosyl is a mildly denaturing ionic detergent

that had been shown not to disrupt VP7 trimer formation (Rutkowska *et al.*, 2011). The ability for sarkosyl treatment to efficiently solubilise chimeric or modified VP7 particles (Rutkowska *et al.*, 2011) but not wild-type VP7 crystals, may reflect a difference in their structure and the strength of the trimer-trimer interaction between the two forms of the protein.

Difficulties have been experienced attempting to solubilise VP7 using detergents (Basak *et al.*, 1996) because VP7 is considered a highly insoluble antigen. This was reflected in the ability of these authors to resolve only the top domain of AHSV VP7 during x-ray crystallography (Basak *et al.*, 1996). The results presented here show that urea treatment (8M) completely solubilises the VP7 crystals. This was not unexpected as urea is a powerful protein denaturant. Direct interaction between urea and the target protein appears to be the driving force behind protein denaturation, disrupting hydrophobic interactions and hydrogen bonds (Stumpe & Grubmuller, 2007). The results show that as the concentration of urea decreased the degree of solubilisation also decreased. It is important to establish what form of VP7 is maintained after treatment with different concentrations of urea. Procedure that could facilitate further investigation includes native page or sedimentary velocity centrifugation alongside appropriate markers.

Live viral vector MVA expressing AHSV-4 VP7

Recombinant MVA was used to express VP7 in an avian cell line (DF-1 cells). The results confirm that expression of AHSV VP7 by a live viral vector MVA results in the generation of VP7 crystals mimicking the structures observed during natural AHSV infections and recombinant VP7 expression in insect

cells. The crystals appeared more uniform in size (approximately 3-5µm) in comparison to the large range of crystals sizes (up to ~25µm) observed after VP7 synthesis by a recombinant baculovirus. This is perhaps owing to the vv early/late promotor P7.5 providing a moderate level of expression throughout infection in comparison to the high expression level by recombinant baculovirus that is restricted to the later stages of the infection cycle. The detection of VP7 antigen as early as 4 hours post-infection in recombinant MVA infected DF-1 cells could be due to the use of this early/late promoter. By 4 hours, VP7 had already started to aggregate and by 24 hours post-infection the large majority of VP7 had aggregate into crystalline form highlighting the strength of the VP7 trimer-trimer interactions.

AHSV-4 infection expression of VP7

Mammalian cells (BHK 21) were infected with AHSV-4 *in vitro*. The results re-confirm that during AHSV infection, VP7 expression results in the generation of VP7 crystals. The ability to detect the crystals using sera raised against a different AHSV serotype (AHSV-9) re-confirms that VP7 is a sero-group specific antigen and that the VP7 protein is highly conserved among AHSV serotypes. If resources and time permitted, and to fully characterise the expression of VP7 during AHSV infection *in vitro*, an immunofluorescence assay would be conducted, using a VP7 specific polyclonal/ monoclonal antibody.

In conclusion, the work in this chapter shows that the methods developed to generate and purify recombinant expressed AHSV-VP7 were suitable for preparation of reagents and their

further characterisation by vaccination and challenge studies in the horse and mouse models.

Chapter 4:

Immunogenicity of AHSV-VP7 crystals *in vivo*

4.1. Introduction

AHSV VP7 is an immuno-dominant antigen which induces high levels of antibody during AHSV infection of its vertebrate hosts (Martinez-Torrecuadrada *et al.*, 1997) and has therefore been used as the basis for antibody based AHSV diagnostic assays (Maree & Paweska, 2005; OIE, 2012). It has been documented that VP7 is one of the last antigens to be detected by the equine host during AHSV infection (Martinez-Torrecuadrada *et al.*, 1997). There is no evidence so far that VP7 specific antibodies are virus neutralising (Mertens *et al.*, 1996; Wade-Evans *et al.*, 1997).

Wade-Evans and co-workers (1997) demonstrated that vaccination of mice with AHSV VP7 crystals purified from AHSV infected cell cultures resulted in the generation of VP7 specific antibodies (Wade-Evans *et al.*, 1997). Vaccination of ponies with MVA expressing AHSV-4 VP7 similarly induced VP7-specific antibodies (Chiam *et al.*, 2009). The protective role of VP7 during AHSV infection has not been well documented. However, Wade-Evans and co-workers (1997) demonstrated the protective capacity of purified AHSV VP7 crystals against heterologous AHSV challenge in mice. Partial protection was observed and was considered to be associated with cell-mediated immunity and not humoral immunity.

In order to understand the immunogenicity of VP7 based antigen delivery mechanisms, it was necessary to study the factors that can influence the immunogenicity of VP7 crystals.

In Chapter 3, it was confirmed that recombinant baculovirus expression of VP7 in insect cells resulted in the generation of VP7 crystals, which could be subsequently purified from the infected insect cell culture. These VP7 crystals were mechanically fragmented or chemically solubilised. In Chapter 3, the capacity of expressed VP7 to form crystals in MVA VP7 infected cells was also demonstrated.

Purified crystals (expressed by recombinant baculovirus) and recombinant MVA VP7 viruses provide tools to investigate the immunogenic properties of VP7. This chapter explores the effects that the size and structure of VP7 assemblies have on the immune response to VP7 in equine and mouse animal models (using exogenous administration of pre-formed or treated antigen / crystals; or *de novo* synthesis of VP7 within the host cells after MVA VP7 inoculation).

4.2. Results

4.2.1 Immunogenicity of baculovirus expressed AHSV-4 VP7 crystals in A129 mice, including AHSV-4 challenge.

A pilot experiment was designed to optimise the conditions (including dose, the effect of adjuvant and route of inoculation) required to induce an antibody response to AHSV-VP7 crystals in the A129 mouse model.

Table 4.1: Groups of mice vaccinated with VP7 crystals and challenged with AHSV-4

Group number (Mouse ID number)	Route of administration	Vaccination		AHSV challenge (1x10 ⁶ TCID ₅₀)
		Prime (Day0)	Boost (Day21)	Challenge (Day29)
Group 1 (M1.1-1.3)	IP (200µl)	4µg Cell debris with adjuvant	4µg Cell debris with adjuvant	AHSV-4 (Table 2.2)
Group 2 (M2.1-2.3)		4µg Cell debris	4µg Cell debris	
Group 3 (M3.1-3.3)		4µg VP7 crystals *	4µg VP7 crystals*	
Group 4 (M4.1-4.3)		4µg VP7 crystals	4µg VP7 crystals	
Group 5 (M5.1-5.3)		8µg VP7 crystals*	8µg VP7 crystals*	
Group 6 (M6.1-6.3)		8µg VP7 crystals	8µg VP7 crystals	
Group 7 (M7.1-7.3)		16µg VP7 crystals*	16µg VP7 crystals*	
Group 8 (M8.1-8.3)		16µg VP7 crystals	16µg VP7 crystals	
Group 9 (M9.1-9.3)	IM(50µl)	4µg VP7 crystals*	4µg VP7 crystals*	
Group 10 (M10.1-10.3)		4µg VP7 crystals	4µg VP7 crystals	

(*administered with Montanide ISA50 V2, Seppic)

Ten groups of three mice (A129) were vaccinated on day 0 and 21 by intra-peritoneal or intra-muscular injection with purified AHSV-4 VP7 crystals (4µg, 8µg, 16µg), or preparations of Sf9 cell debris, with or without Montanide ISA50 V2, Seppic, (as shown in Table 4.1 and described in Chapter 2, section 2.7.1.1). The A129 mice strain was used,

as it is the 'background strain' for the interferon alpha receptor gene knock- out (IFNAR -/-) strain used in challenge studies with AHSV and BTV (Calvo-Pinilla *et al.*, 2009b; Castillo-Olivares *et al.*, 2011; Jabbar *et al.*, 2013). Sera obtained on day 28 were analysed for VP7 specific antibodies using the commercially available AHSV VP7 ELISA kit (INGENASA) which has been validated for equine sera. The test is an indirect competition ELISA which facilitates its use for multiple different species, including mice (as previously tested in house).

Analysis of sera from the mouse vaccinates by ELISA showed that after two vaccinations, VP7 specific antibodies were undetectable (blocking percentage <50%) in all of the mouse vaccinates. In the absence of VP7 specific antibody preparations and in order to maximise the amount of information that could be gathered from the experiment, the mice were subsequently challenged on day 29 with AHSV-4 (1×10^6 TCID₅₀; as described in Chapter 2, section 2.7.1.1). After challenge, the mice were observed for development of clinical signs and the experiment was terminated on day 38. All of the mice, including the control mice (mice vaccinated with cell debris alone), survived the AHSV-4 challenge with no observable clinical signs. Only one mouse (M5.2) vaccinated with 8µg VP7 crystals plus Montanide ISA50 V2 (IP) mildly seroconverted by day 38 post-vaccination (nine days post AHSV-4 challenge), as shown in Figure 4.1.

In conclusion, VP7 specific antibodies were not detected by ELISA (INGENASA) after two vaccinations of A129 mice with VP7 crystals, but were detected post AHSV-4 challenge in at least one mouse. Additionally, the results show that A129 mice are unsuitable as an AHSV challenge model.

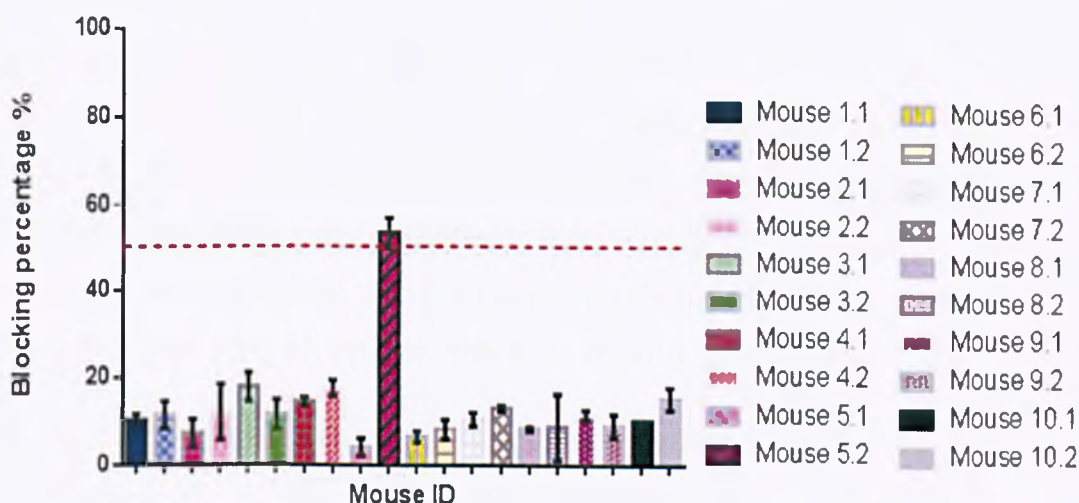


Figure 4.1: Generation of VP7 specific antibodies against VP7 crystals in A129 mice after vaccination and AHSV-4 challenge.

Serum samples taken on day 38 (9 days post-challenge) from mice vaccinated on day 0 and 21 with VP7 crystals and Sf9 cell debris (control mice) (as shown in Table 4.1), and subsequently challenged with AHSV-4 (1×10^6 TCID₅₀) on day 29 (as described in Chapter 2, section 2.7.1.1.) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage).

4.2.2. Analyses of sera from ponies vaccinated with MVA VP7

In view of the failure to detect VP7 antibodies in A129 mice vaccinated with recombinant purified VP7 crystals (section 4.2.1), the VP7 ELISA was used to test sera from MVA VP7 vaccinated ponies, which had previously been shown to contain VP7 specific antibodies, by Western blotting techniques (Chiam et al, 2009). These antisera (Appendix III, Table A3.1) were re-tested by Western blot and by the INGENASA ELISA.

VP7 specific antibody analysis

Analysis of the equine test sera by Western blot showed that VP7 specific antibodies had been raised against recombinant

MVA expressed AHSV-4 VP7 (Figure 4.2). By Western blot, both ponies tested sero-negative for VP7 antibodies on day 0 (confirmed by the lack of band corresponding to the size of VP7 ~37kDa). By day 98 post-vaccination (pv) both ponies had seroconverted by Western blot (pony 5859 and pony 5717, by day 42 and 98 pv respectively) generating a band matching the size of VP7 on the blot membrane (Figure 4.2).

Analysis of test sera from the pony vaccinates using the INGENASA VP7 ELISA did not demonstrate VP7 antibodies because each pony showed a blocking percentage <50% (positive cut-off as described by INGENASA) and was therefore considered negative. By ELISA, test serum obtained from pony 5859 on day 98 was only considered inconclusive (with a blocking percentage between 45-50%, as determined by the INGENASA assay) (Figure 4.3).

In conclusion, a VP7 specific antibody response was detected by Western blot in ponies following at least two vaccinations with MVA expressing AHSV-4 VP7. However, VP7 specific antibodies were not detected using the INGENASA ELISA, which is the OIE prescribed serological diagnosis test kit, suggesting that the kit has low sensitivity. This finding implies that failure to detect VP7 specific antibodies post- VP7 crystal vaccination (as described in section 4.2.1) may have been associated with ELISA sensitivity and not the complete absence of VP7 specific antibodies in the mouse vaccinates.

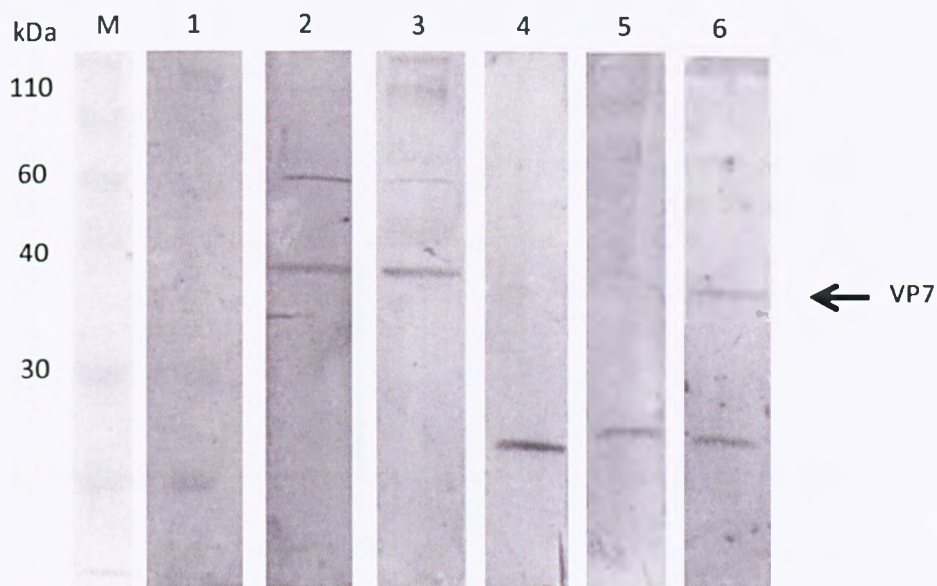


Figure 4.2: Detection of VP7 with MVA-VP7 vaccinated pony sera by Western blot.

Purified crystals of baculovirus expressed VP7 were analysed by SDS PAGE and Western blot. The blots were probed with antisera (Appendix III, Table A3.1) from ponies vaccinated on days 0, 35 and 77 with MVA VP7 as described by Chiam *et al.* 2009. M: Protein marker Novex® Sharp Protein Standard; Lane 1: probed with serum from pony 5859 on day 0; Lane 2: probed with serum from pony 5859 on day 42 pv; Lane 3: probed with serum from pony 5859 on day 98 pv; Lane 4: probed with serum from pony 5717 on day 0; Lane 5 : probed with serum from pony 5717 on day 42 pv; Lane 6: probed with serum from pony 5717 on day 98 pv. The black arrow depicts the expected molecular weight of VP7 (~37kDa).

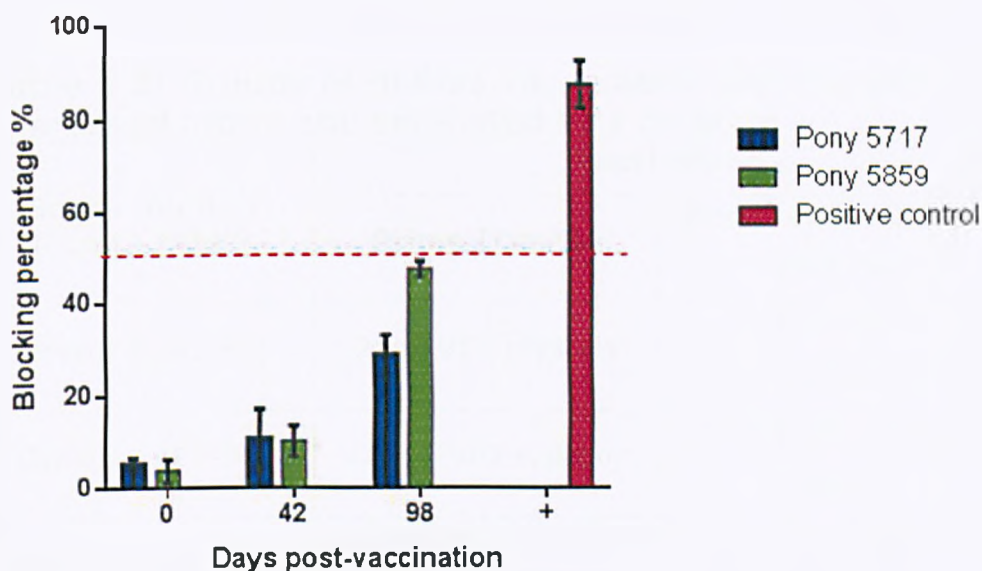


Figure 4.3: Generation of VP7 specific antibodies against AHSV VP7 in ponies after vaccination with MVA VP7.

Serum samples obtained on days 0, 42 and 98 (Appendix III, Table A3.1) from ponies vaccinated on days 0, 35 and 77 with MVA VP7 (as described by Chiam *et al.* 2009) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The positive control was serum obtained from a horse experimentally infected with AHSV-4 (Appendix III, Table A3.1). The red line depicts the positive cut-off point (50% blocking percentage).

4.2.3 Immunogenicity of baculovirus expressed AHSV-4 VP7 crystals in ponies

In light of detecting VP7 specific antibodies against AHSV-4 VP7 (by MVA VP7 vaccination in ponies) by Western blot, this experiment was designed to optimise the conditions necessary for inducing a VP7 antibody response in ponies against pre-formed VP7 crystals (in terms of dose and the number of vaccinations). The immunogenicity of VP7 crystals, in relation to crystal size, was also investigated. The VP7 crystal antigens used in this experiment were those described in Chapter 3.

Table 4.2: Groups of ponies vaccinated with baculovirus expressed intact and sonicated VP7 crystals

Group number (Pony number)	Vaccination	
	Prime (Day 0)	Boost (Days 28, 49 and 70)
Group 1 (P1-P2)	25µg VP7 crystals	25µg VP7 crystals/dose
Group 2 (P3-P4)	150µg VP7 crystals	150µg VP7 crystals/dose
Group 3 (P5-P6)	25µg sonicated VP7 crystals	25µg sonicated VP7 crystals/ dose
Group 4 (P7-P8)	150µg sonicated VP7 crystals	150µg sonicated VP7 crystals/ dose

Four groups of two Welsh mountain ponies were vaccinated with one of the following antigens: a) VP7 crystals (25 µg / dose); b) VP7 crystals (150 µg / dose); c) sonicated VP7 crystals (25 µg / dose); or d) sonicated VP7 crystals (150 µg / dose) in the absence of adjuvant as shown in Table 4.2. Ponies were vaccinated on days 0 and 28, 49 and 70 by intramuscular injection (as described in Chapter 2, section 2.7.1.2). Whole blood samples were collected on days -3, 28, 35, 42, 49, 56 and 102. Sera were obtained for VP7 specific antibody analysis by Western blot and INGENASA ELISA.

VP7 specific antibody analyses

Sera from the ponies vaccinated with purified VP7 crystals (25 µg / 150 µg per dose) were tested on days -3, 42, 56 and 102 post-vaccination (pv), by western blotting, for reactivity against the components of an MVA VP7 infected cell lysate. Even after four vaccinations, no obvious band corresponding to the size of VP7 (37kDa) was observed. However, there was

high degree of cross reactivity between other components of the lysate and the antiserum, with many 'extra' bands detected on the blot membrane. A band corresponding to ~37kDa was observed for the positive control serum (serum from a horse experimentally infected with AHSV-4 (Appendix III, Table A3.1) (results not shown).

The pony sera were also tested by the INGENASA ELISA for VP7 specific antibodies to re-confirm that a VP7 specific antibody response had not been generated. All of the pony sera tested negative for VP7 specific antibodies (blocking percentage <50%), while the positive control sera tested positive (Figure 4.4).

In conclusion, after four vaccinations with either sonicated or intact VP7 crystals, a VP7 specific antibody response could not be detected in the ponies by Western blot or by INGENASA VP7 specific ELISA.

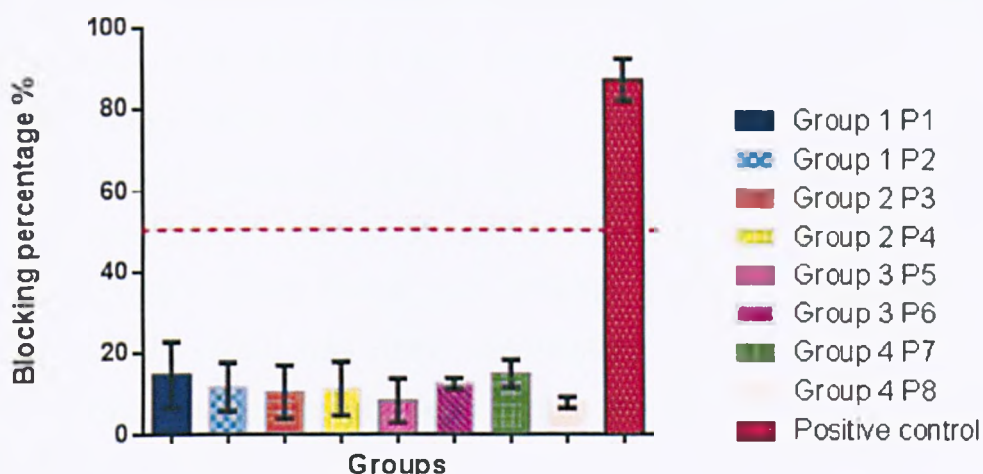


Figure 4.4: Generation of VP7 specific antibodies against sonicated and intact AHSV VP7 in ponies.

Serum samples obtained on day 102 from ponies vaccinated four times with: a) 25µg of VP7 crystals (Group 1); b) 150µg of VP7 crystals (Group 2); c) 25µg of sonicated VP7 crystals (Group 3); or d) 150µg of sonicated VP7 crystals (Group 4) (as described in Chapter 2, section 2.7.1.2) were analysed for VP7 specific antibodies by the INGENASA AHSV VP7 ELISA. The positive control was serum obtained from a horse experimentally infected with AHSV-4 (Appendix III, Table A3.1). The red line depicts the positive cut-off point (50% blocking percentage).

4.2.4. Immunogenicity of AHSV-4 VP7 expressed by MVA in IFNAR -/- A129 mice, including challenge with AHSV-3, AHSV-4 or AHSV-6

Vaccination of ponies with purified recombinant expressed AHSV-4 VP7 crystals failed to generate detectable levels of VP7 antibodies. However, previous studies (Chiam *et al.*, 2009) and evidence from this study (section 4.2.2), indicate that MVA VP7 vaccination could induce VP7 specific antibody responses in ponies.

The next objective of this study was to determine whether VP7 specific antibody responses could be generated in mice, following vaccination with MVA VP7. AHSV VP7 is a sero-group specific antigen (cross-reacts between AHSV serotypes), and

previous studies have indicated partial cross-protection after vaccination with AHSV-9 VP7 crystals (Wade-Evans *et al.*, 1997; Wade-Evans *et al.*, 1998). The protective efficacy of MVA VP7 was therefore tested against homologous (AHSV-4) and heterologous (AHSV-3, AHSV-6) challenge. Interferon alpha receptor gene knock out (IFNAR $-/-$) A129 mice were used, as this strain has been established as suitable for BTV and AHSV challenge studies (Calvo-Pinilla *et al.*, 2009b; Castillo-Olivares *et al.*, 2011).

Five IFNAR $-/-$ A129 strain mice were vaccinated (by intra-peritoneal injection) with MVA AHSV-4 VP7 on days 0 and day 28 (as described in Chapter 2, section 2.7.1.3 and shown in Table 4.3). On day 35, whole blood was taken and the sera used for VP7 antibody detection by Western blot. The mice were subsequently challenged, together with non-vaccinated control mice (by sub-cutaneous injection) on day 38, with AHSV-3, AHSV-4 or AHSV -6 (as described in Chapter 2, section 2.7.1.3). The protective efficacy of MVA VP7 against AHSV challenge was determined by analysis of clinical scores, viraemia and survival rate. The level of viraemia in the blood was measured by virus titration. The clinical score was calculated according to the morbidity scoring system (Chapter 2, section 2.7.2) and the survival rate was expressed as a percentage per group. Whole blood was obtained from the surviving mice upon termination of the experiment and the sera were used for VP7 antibody analysis.

Table 4.3: Groups of IFNAR -/- A129 mice vaccinated with MVA VP7 and challenged with AHSV-3, -4 or -6.

Group number (Mouse number)	Vaccination (1×10^7 PFU/dose)		AHSV Challenge (Table 2.2) (1×10^6 TCID ₅₀)
	Prime (Day0)	Boost (Day28)	Challenge (Day38)
Group 1			
(M1.1- M1.5)	MVA VP7	MVA VP7	AHSV-3
(M1.6- M1.10)	-	-	AHSV-3
Group 2			
(M2.1-M2.5)	MVA VP7	MVA VP7	AHSV-4
(M2.6-M2.10)	-	-	AHSV-4
Group 3			
(M3.1-3.5)	MVA VP7	MVA VP7	AHSV-6
(M3.6-3.10)	-	-	AHSV-6

Prior to AHSV challenge, three mice (Mouse 1.7, 2.2, 3.3) died post-vaccination. The cause of death was unknown although, since they were IFNAR -/- mice, they were more susceptible to infection.

4.2.4.1. Analysis of VP7 antibody responses to MVA VP7 vaccination

VP7 specific antibody responses in the IFNAR -/- mice vaccinated with MVA VP7 AHSV were analysed by Western blot. The test serum from each vaccinated mouse (collected on day 35 post-vaccination) and test sera obtained from the two surviving vaccinates on day 52 post-vaccination (14 days post AHSV-4 challenge) were tested for reactivity with purified VP7 crystals by Western blot. A band corresponding to the size of VP7 (~37kDa) was not observed in the sera of any of the mouse vaccinates pre-challenge. VP7 antibodies were detected (evidenced by the display of a clear band after western blotting, corresponding to ~37KDa) in antisera from the surviving mice challenged with AHSV-4, as shown in Figure 4.5

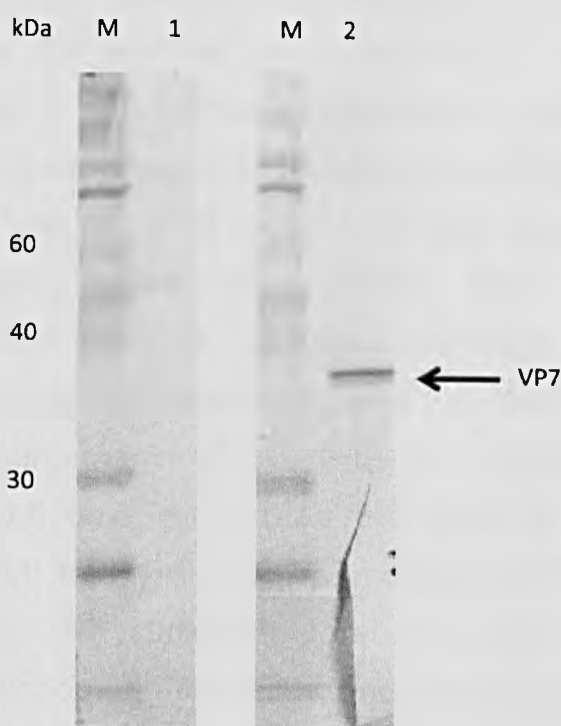


Figure 4.5: Detection of antibodies to VP7 in sera from MVA VP7 vaccinated mice by Western blot.

Purified crystals of baculovirus expressed VP7 were analysed by SDS PAGE and western blotting. The blots were probed with antisera from mice vaccinated with MVA VP7 only, or from mice vaccinated and subsequently challenged with AHSV-4 (as described in Chapter 2, section 2.7.1.3). M: Protein marker Novex® Sharp Protein Standard; Lane 1: probed with pooled sera from MVA VP7 vaccinates on day 35; Lane 2: probed with antisera from mice vaccinated with MVA VP7 and challenged with AHSV-4 on day 52 pv (14 days post-challenge). The black arrow depicts the expected molecular weight of VP7 (~37kDa).

4.2.4.2. Evaluation of the protective efficacy of MVA VP7 vaccinates

Survival rate

The survival rates of the mice vaccinated with MVA VP7 then challenged with homologous and heterologous AHSV serotypes are shown in Figure 4.6. Group 1 vaccinates (heterologous challenge with AHSV-3) had an overall survival percentage of 0%, with an average survival rate of 4.2 days. This is 0.3

days less than that of the AHSV-3 challenged control mice, which also had a 0% survival percentage by 10 days post-challenge. Group 2 vaccinates (homologous challenge with AHSV-4) had an overall survival percentage of 50% with an average survival rate of 10.75 days, 3.95 days more than the AHSV-4 challenged control mice, which had a survival percentage of 0% by day 10 post-challenge. Group 3 vaccinates (heterologous challenge with AHSV-6) had an overall survival percentage of 0% with an average survival rate of 8 days, 0.6 days more than the AHSV-6 challenged control mice, which had a survival percentage of 0% by day 10 post-challenge. No statistical differences were observed between any corresponding vaccinate and control groups when the data was examined using Log Rank tests.

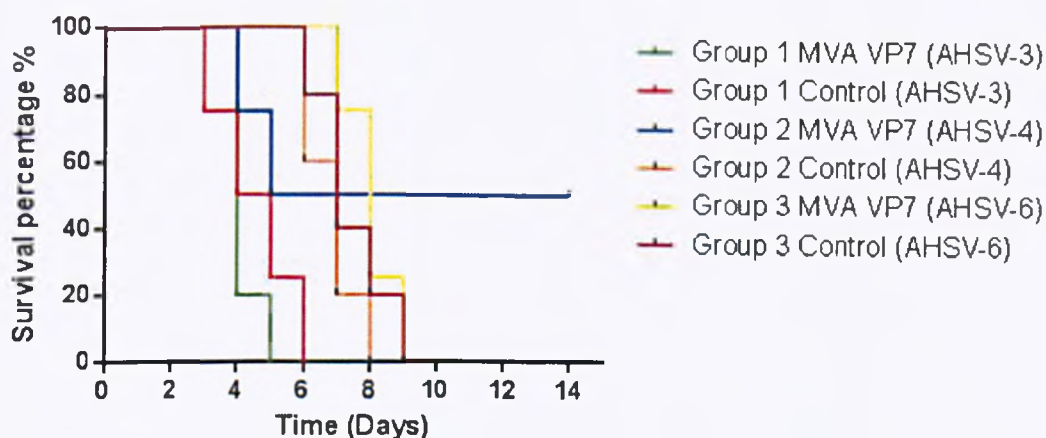


Figure 4.6: Survival rates of IFNAR $-/-$ A129 mice vaccinated with MVA expressing AHSV-4 VP7 and challenged with AHSV 3, 4 and 6.

Three groups of MVA AHSV-4 VP7 vaccinated mice were challenged with AHSV-3, AHSV-4 or AHSV-6 (as described in Chapter 2, section 2.7.1.3) on day 38 post-vaccination, alongside an appropriate control group for each serotype (Table 4.3). The survival rates are represented by lines expressing the percentages of surviving mice per day.

Clinical scores

The clinical scores for the AHSV challenged mice were calculated by collating their morbidity scores collected during the course of the experiment and dividing it by the number of days the animal survived (as described in Chapter 2, section 2.7.2). The mean clinical scores per group are presented in Figure 4.7. The mean clinical score of Group 1 vaccinates (AHSV-3 challenge) was 1.594, 0.369 less than that of the AHSV-3 challenged control mice (1.963). The mean clinical score of Group 2 vaccinates (challenged with AHSV-4) was 1.090, 0.375 less than that of the AHSV-4 challenged control mice (1.465). The mean clinical score of Group 3 vaccinates (AHSV-6 Challenge) was 0.810, 0.534 less than that of the AHSV-6 challenged controls (1.344). No statistical differences were observed between the groups when the data was examined using Kruskal Wallis tests.

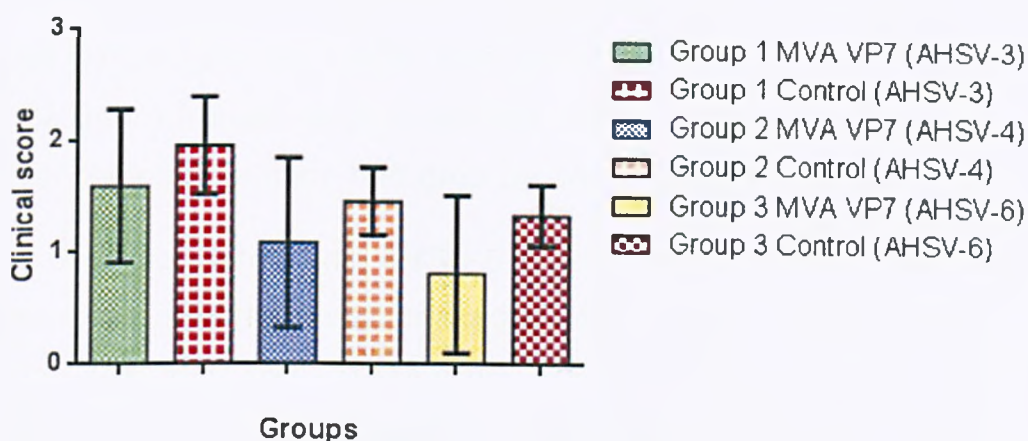


Figure 4.7: Mean clinical scores of IFNAR -/- A129 mice vaccinated with MVA expressing AHSV-4 VP7 and subsequently challenged with AHSV-3, -4 and -6.

Three groups of MVA VP7 vaccinated mice were challenged with AHSV-3, -4 or -6 (as described in Chapter 2, section 2.7.1.3) on day 38 post-vaccination, alongside an appropriate control group for each serotype (Table 4.3). The clinical score for each animal was calculated according to the number of clinical points obtained (from the clinical scoring system) throughout the duration of the study divided by the number of days each animal survived. The mean clinical scores per group are represented by the histogram. Clinical scores were monitored for up to 14 days post-challenge.

Virus in the blood

The level of viraemia in the challenged mice was determined by virus titration. Circulating virus was detected in all of the challenged mice by day 3 post-challenge. For vaccinated and AHSV-3 challenged mice, the average titre peaked by day 3 post-challenge, with a mean titre of 2.950 TCID₅₀/ml, this value matched that of the AHSV-3 challenged control mice (2.950 TCID₅₀/ml). None of the Group 1 mice survived for a bleed at day 5 (Figure 4.8).

For vaccinated and AHSV-4 challenged mice, the average viraemia peaked at day 7 post-challenge, with a mean titre of 2.762 TCID₅₀/ml, 0.105 less than that of the controls (2.867

TCID₅₀/ml). By day 10, the average virus titre had dropped by 1.687 TCID₅₀/ml to 1.075 TCID₅₀/ml in the blood of the two mice that survived virus challenge until day 10 post-challenge. All of the control mice had died by day 8 (Figure 4.8).

For the vaccinated and AHSV-6 challenged mice, the average titre of virus in the blood peaked by day 3 post-infection with a mean titre of 0.762 (TCID₅₀/ml), 0.638 TCID₅₀/ml lower than that of the controls at day 3. For the control mice, the titre of AHSV-6 in the blood peaked later, at day 5 post-challenge, with a value of 1.550 TCID₅₀/ml, 1.038 more than that of the vaccinated and AHSV-6 challenged mice by day 5. By day 7, the virus titres had reduced from the day 5 values by 0.063 TCID₅₀/ml and 0.475 TCID₅₀/ml for both the vaccinated (0.450 TCID₅₀/ml) and control mice (1.075 TCID₅₀/ml) respectively. All of the mice had died by day 9 post-challenge. The mean virus titres in the blood of the mice are represented by line plots in Figure 4.8. No statistical differences were observed between the groups when the data was examined using Kruskal Wallis tests.

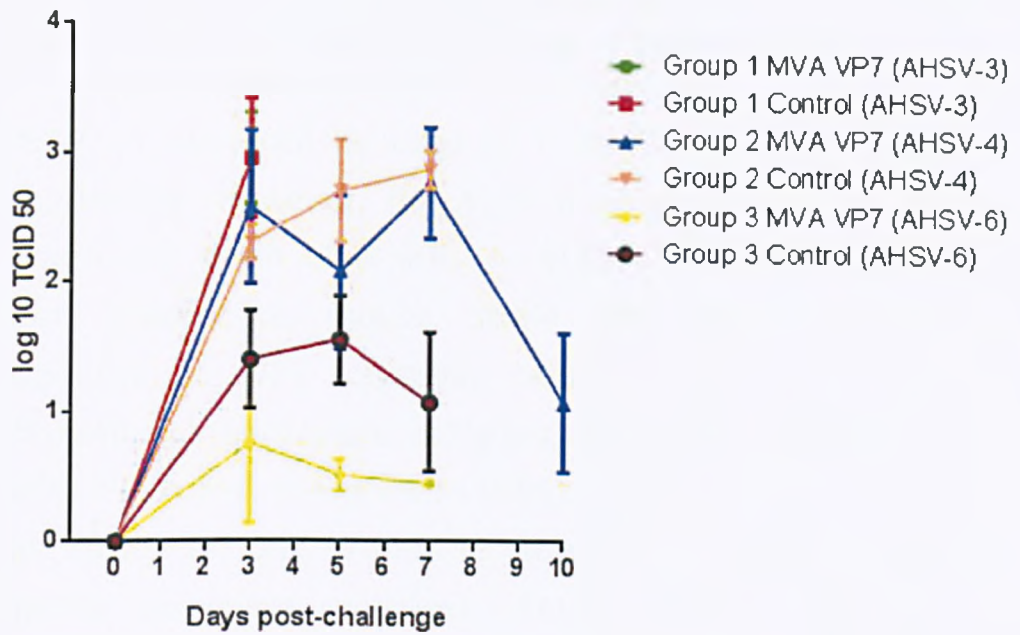


Figure 4.8: Mean viraemia levels in IFNAR $-/-$ A129 mice vaccinated with MVA expressing AHSV-4 VP7 and challenged with AHSV-3,-4 or -6.

Three groups of MVA VP7 vaccinated mice were challenged with AHSV-3, -4 or -6 (as described in Chapter 2, section 2.7.1.3) on day 38 post-vaccination, alongside an appropriate control group for each serotype (Table 4.3). The mean viraemia levels in the blood of the mice (per group) are represented by line blots expressing the $\log_{10}$ TCID₅₀ /ml values of the samples collected for each day.

In conclusion, VP7 specific antibodies were not detected in the IFNAR $-/-$ A129 mice after MVA VP7 vaccination. However, VP7 specific antibodies were detected in the surviving mice post AHSV-4 challenge. The protective efficacy of MVA VP7 is uncertain. Post- AHSV challenge no significant differences were observed in the clinical scores, survival rates or viraemia between the MVA VP7 vaccinates and the corresponding control mice. However, the observations suggest that recombinant VP7 may afford partial protection against homologous AHSV-4 challenge, with a survival rate of 50%. No protection was observed against heterologous challenge.

4.2.5. Immunogenicity and protective efficacy of MVA AHSV-4 VP7 in IFNAR -/- A129 and BALB/c mice

The experiment described in section 4.2.4, confirmed that IFNAR -/- mice can be used as a model for AHSV challenge experiments. However, the A129 mouse strain (which is the background strain of IFNAR -/- A129 mice) may not be the most appropriate mouse strain for analysing antibody responses to VP7 crystals, because mice can respond differently to particulate antigens, according to Milich *et al.* 1986. Milich and co-workers (1986) reported huge variation in the ability of certain mouse strains to induce antibodies against particulate antigens. BALB/c mice represented a mouse strain capable of inducing high titres of antibodies to both particulate and non-particulate antigens (Milich & McLachlan, 1986). Additionally, BALB/c mice represent a mouse strain capable of raising antibodies to VP7 crystals (Wade-Evans *et al.*, 1997).

A primary objective of this experiment was to determine whether there is any variation in the immunogenicity of VP7 crystals associated with the mouse strain used. In addition, the immune response to VP7 synthesised 'de novo' within cells of the vaccinated animal was studied using different MVA VP7 based preparations (live MVA VP7, inactivated MVA VP7 and inactivated MVA VP7 plus wild-type MVA) and compared with that generated in response to pre-formed VP7.

Four groups of five IFNAR -/- A129 or BALB/c mice were vaccinated on day 0 and 21 (by intra-peritoneal injection) with: MVA VP7 live; MVA VP7 inactivated; MVA VP7 inactivated with live wild-type MVA; or wild-type MVA as shown in Tables 4.4 and 4.5 respectively. Sera were obtained from whole blood taken on day 35, for serological analysis by Western blot,

INGENASA AHSV VP7 ELISA and VP7 crystal based indirect ELISA. The IFNAR -/- A129 mice were subsequently challenged with AHSV-4 (as described in Chapter 2, section 2.7.1.4 and shown in Table 4.4) on day 35.

Table 4.4: Groups of IFNAR -/- A129 mice vaccinated with MVA VP7 and challenged with AHSV-4

Group number (Mouse number)	Vaccination (1x10 ⁷ PFU/dose)		AHSV Challenge (1x10 ⁶ TCID ₅₀)
	Prime (Day0)	Boost (Day21)	Challenge (Day35)
Group 1 (M1.1-1.5)	Live MVA VP7	Live MVA VP7	AHSV-4 (Table 2.2)
Group 2 (M2.1-2.5)	Inactivated MVA VP7	Inactivated MVA VP7	
Group 3 (M3.1-3.5)	Inactivated MVA VP7 plus wild-type MVA	Inactivated MVA VP7 plus wild-type MVA	
Group 4 (M6.1-6.6)	Live wild-type MVA	Live wild-type MVA	

Table 4.5: Groups of BALB/c mice vaccinated with MVA VP7

Group number (mouse number)	Vaccination (1x10 ⁷ PFU/dose)	
	Prime (Day 0)	Boost (Day 21)
Group 1 (M1.1-1.5)	Live MVA VP7	Live MVA VP7
Group 2 (M2.1-2.5)	Inactivated MVA VP7	Inactivated MVA VP7
Group 3 (M3.1-3.5)	Inactivated MVA VP7 plus wild-type MVA	Inactivated MVA VP7 plus wild-type MVA
Group 4 (M6.1-6.4)	Live wild-type MVA	Live wild-type MVA

4.2.5.1. Analysis of VP7 antibody responses to live and/or inactivated MVA VP7 vaccination in IFNAR -/- A129 and BALB/c mice

Initial analysis of VP7 antibody responses in the mice vaccinated with live and/or inactivated MVAs, was conducted using a VP7 crystal based indirect ELISA (as described in Chapter 2, section 2.7.6.2). Sera from the vaccinated mice together with known positive sera (from mice experimentally infected with AHSV-4) (Appendix III, Table A3.1) and negative (pre-immunisation) control sera, were tested accordingly. The negative and positive sera reacted as expected with low (<0.2 O.D) and positive O.D values (>0.4 O.D) respectively (results not shown). However, there was significant cross-reactivity between the plate coated with the VP7 antigen and sera from mice vaccinated with wild-type MVA (results not shown). Efforts were made to minimise this background reactivity (e.g. pre-absorption of the coating antigen with antibodies raised against wild-type MVA only, alternative blocking buffer and variation in coating antigen concentration and dilutions of test sera). None of these attempts enabled an accurate judgment to be made, because the background absorbance was so high. In light of this, the AHSV VP7 ELISA (INGENASA) and a Western blot assay was performed with the test sera, alongside a positive control (antisera from mice experimentally infected with AHSV-4).

By Western blot, no bands corresponding to the size of VP7 (~ 37 kDa) were observed for any of the test sera, from the MVA vaccinated mice, indicating that VP7 specific antibodies had not been generated. However, a band corresponding to the size of VP7 (~ 37 kDa) was observed after testing the positive control sera (post-infected mice) (results not shown). The INGENASA ELISA confirmed a lack of VP7 specific

antibodies in the MVA vaccinated mice (<50% blocking percentage), while the positive control sera tested positive as shown in Figure 4.9.

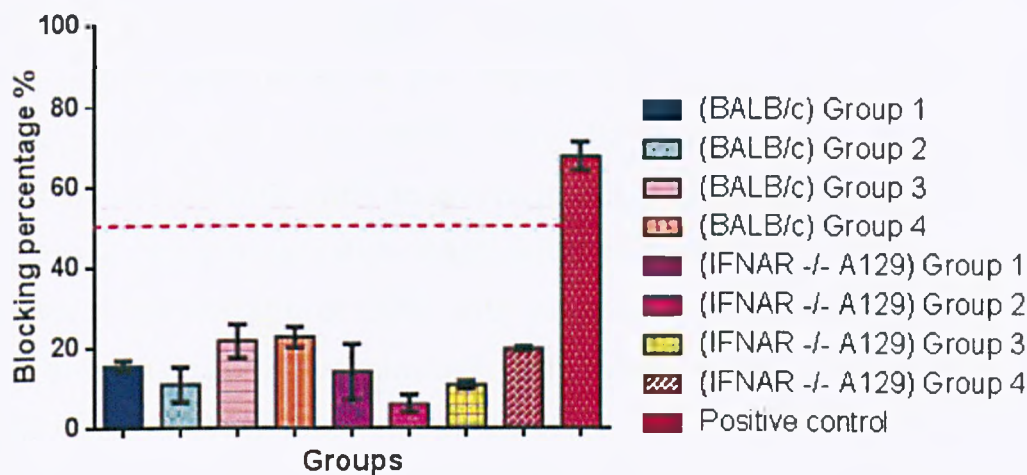


Figure 4.9: Generation of VP7 specific antibodies against VP7 in BALB/c and IFNAR -/- A129 mice after vaccination with different MVA VP7 preparations.

Serum samples taken on day 35 from eight groups of five MVA VP7 vaccinated or wild-type MVA vaccinated IFNAR -/- A129 and BALB/c mice: vaccinated on day 0 and 21 with live MVA VP7 (Group 1); inactivated MVA VP7 (Group 2); inactivated MVA VP7 plus wild-type MVA (Group 3) or wild-type MVA only (control group -Group 4) (as described in Chapter 2, section 2.7.1.4) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The positive control was antisera obtained from IFNAR -/- A129 mice experimentally infected with AHSV-4 (Appendix III, Table A3.1) The red dotted line depicts the positive cut-off point (50% blocking percentage).

4.2.5.2 Analyses of the protective efficacy of live and inactivated MVA AHSV-4 VP7 preparations in IFNAR -/- A129 mice against homologous AHSV-4 challenge

Evaluation of protective efficacy

Survival rate

The survival rates of the groups of IFNAR -/- A129 mice vaccinated with live and/or inactivated MVA AHSV-4-VP7, then challenged with AHSV-4 are shown in Figure 4.10. Group 1 (vaccinated with live MVA VP7) had an overall survival percentage of 60% with an average survival period of 12 days. Group 2 (vaccinated with inactivated MVA VP7) had an overall survival percentage of 80% with an average survival period of 13 days. Group 3 (vaccinated with inactivated MVA VP7 plus wild-type MVA) had an overall survival percentage of 60% with an average survival period of 11.6 days. Group 4 (vaccinated with wild-type MVA) had an overall survival percentage of 20% with an average survival period of 9 days.

Group 1's average survival period was 0.4 and 3 days longer than that of Groups 3 and 4 respectively. Group 2's average survival period was 1, 1.4 and 4 days longer than that of Groups 1, 3 and 4 respectively. Group 3's average survival period was 2.6 days longer than that of Group 4. Group 4's average survival period was 9 days. No statistical differences were observed between the groups when these data were examined using Log Rank tests.

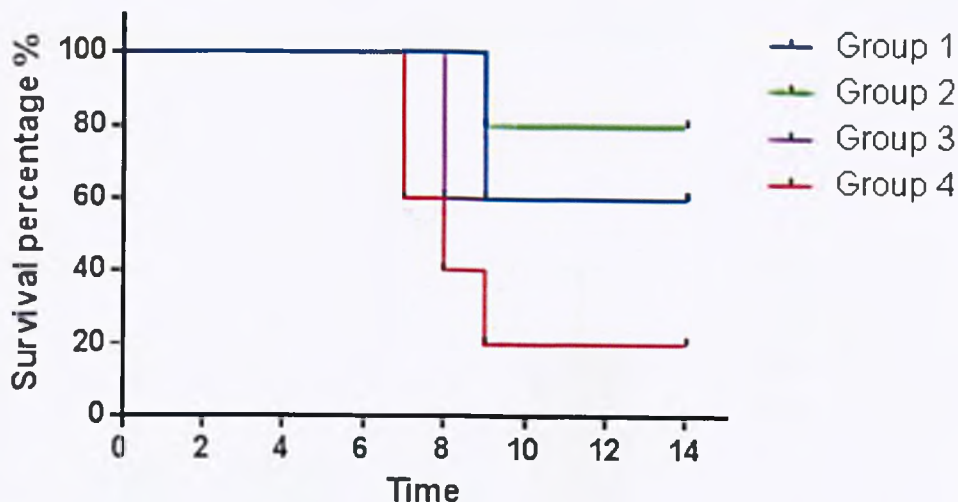


Figure 4.10: Survival rates in IFNAR $-/-$ A129 mice vaccinated with MVA VP7 and subsequently challenged with AHSV-4.

Four groups of five IFNAR $-/-$ A129 mice were vaccinated with MVA VP7 and/or wild-type MVA: vaccinated with live MVA VP7 (Group 1); with inactivated MVA VP7 (Group 2); with inactivated MVA VP7 plus wild-type MVA (Group 3) or with wild-type MVA (control group-Group 4) and challenged with AHSV-4 on day 35 post-vaccination (as described in Chapter 2, section 2.7.1.4). The survival rates are represented by lines expressing the percentages of surviving mice per day.

Clinical scores

The clinical scores were calculated by collating the morbidity score collected during the course of the experiment and dividing it by the number of days the animal survived. The mean clinical scores per group are represented in Figure 4.11. The mean clinical scores of Group 1, 2, 3 and 4 were 1.358, 1.332, 1.080 and 2.160 respectively. The clinical score of Group 1 was larger than the value of Group 2 (by 0.026) and 3 (by 0.278) but smaller than Group 4 (by 0.802). The clinical score of Group 2 was larger than that of Group 3 (by 0.252) but smaller than that of Group 4 (by 0.828). The clinical score of Group 3 was lower than that of Group 4 (by 1.08). No

statistical differences were observed between the groups when the data was examined using Kruskal Wallis tests.

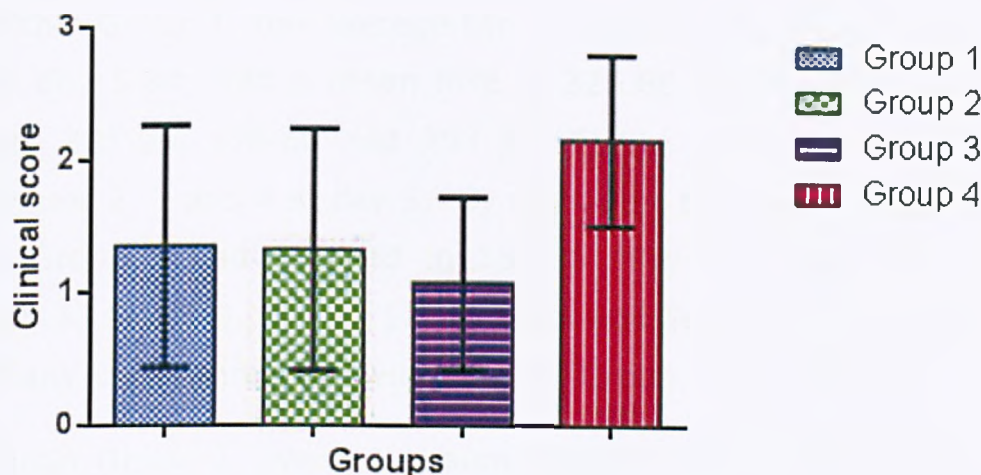


Figure 4.11: Mean clinical scores in IFNAR $-/-$ A129 mice vaccinated with MVA VP7, and subsequently challenged with homologous AHSV-4.

Four groups of five IFNAR $-/-$ A129 mice were vaccinated with MVA VP7 and/or wild-type MVA: vaccinated with live MVA VP7 (Group 1); with inactivated MVA VP7 (Group 2); with inactivated MVA VP7 plus wild-type MVA (Group 3) or with wild-type MVA (control group-Group 4) and challenged with AHSV-4 on day 35 post-vaccination (as described in Chapter 2, section 2.7.1.4). The clinical score for each animal was calculated according to the number of clinical points obtained (from the clinical scoring system) throughout the duration of study divided by the number of days each animal survived. The mean clinical scores per group are represented by the histogram. Clinical scores were monitored for up to 14 days post-challenge.

Detection of virus in the blood.

The virus titre in the blood of the MVA VP7 vaccinated and AHSV-4 challenged IFNAR $-/-$ A129 mice were determined by plaque assay. As shown in Figure 4.12, no virus was detected in the blood of any mice taken on day 3 in Groups 1,2 and 3 (mice vaccinated with live MVA VP7; inactivated MVA VP7; or

inactivated MVA VP7 plus wild-type VP7 respectively). Virus was detected in the blood of all mice from Group 4 (MVA only vaccinated mice) on day 3. All of the mice in Groups 1, 2 and 3 developed viraemia by day 5 post-challenge (pc).

Within Group 1, the average titre of virus in the blood peaked by day 5 pc, with a mean titre of 326.68 PFU/ml. This value was 283.26, 176.66 and 303.32 PFU/ml lower than that of Groups 2, 3 and 4 at day 5. By day 10 pc the mean virus titre in Group 1 had dropped to 158.33 PFU/ml (a reduction of 168.35 PFU/ml). By day 12, no virus was detected in the blood of any of the three surviving mice of Group 1 (Figure 4.12).

Within Group 2, average viraemia peaked by day 5 pc with a mean of 609.94 PFU/ml: 238.26 and 106.6 PFU/ml higher than Groups 1 and 3, and 20.06 PFU/ml lower than Group 4. The mean virus titre in Group 2 dropped to 62.50 PFU/ml by day 12 pc (a reduction of 547.44 PFU/ml). By day 14 no virus was detected in the blood of any of the four surviving animals in Group 2, by plaque assay (Figure 4.12).

Within Group 3, viraemia peaked by day 5 pc, with a mean virus titre of 503.34 PFU/ml: 126.66 and 106.60 PFU/ml lower than the virus titre of Group 4 and Group 2 respectively, and 176.66 PFU/ml higher than Group 1 on day 5 pc. The mean virus titre had dropped by 470.01 PFU/ml by day 12 (33.33 PFU/ml) and by day 14, no virus was detected in the blood of any of the three surviving animals (Figure 4.12).

Within Group 4, viraemia peaked by day 5 pc (630.00 PFU/ml), 303.32, 20.06 and 126.66 PFU/ml higher than Groups 1, 2 and 3 respectively. By day 10, only one mouse in group 4 survived, with a virus titre of 175.00 PFU/ml (455.00 PFU/ml less than the mean virus titre of day 5). By day 12, no

virus was detected in the blood of the surviving mouse (Figure 4.12).

No statistical differences were observed between the different groups when the data was examined using Kruskal Wallis tests.

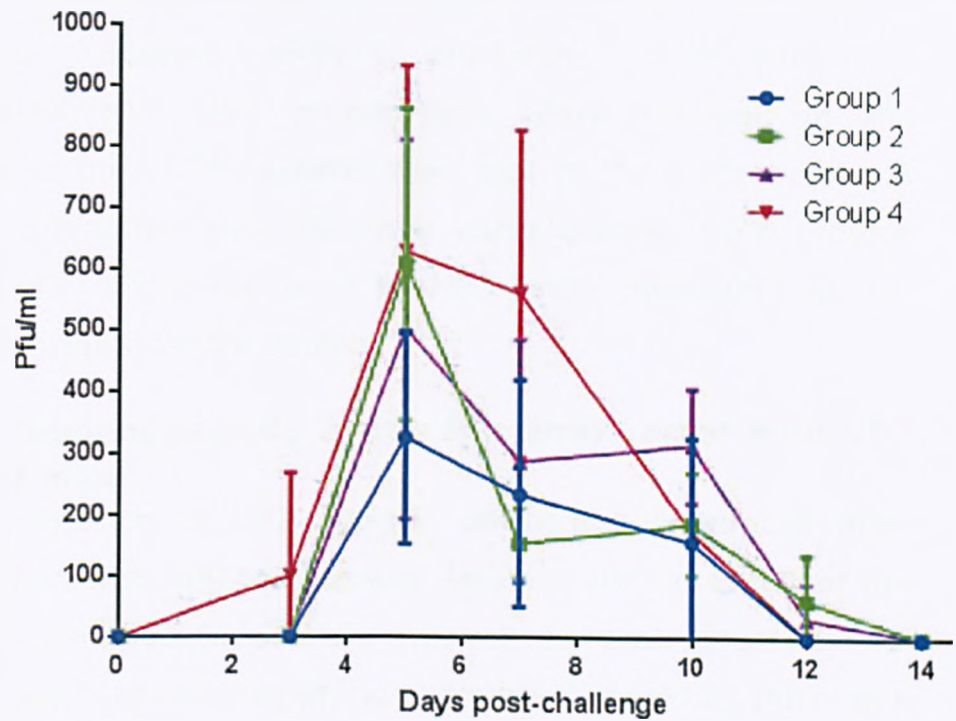


Figure 4.12: Mean viraemia levels in IFNAR $-/-$ A129 mice vaccinated with MVA VP7 and subsequently challenged with homologous AHSV-4.

Four groups of five IFNAR $-/-$ A129 mice were vaccinated with MVA VP7 and/or wild-type MVA: vaccinated with live MVA VP7 (Group 1); with inactivated MVA VP7 (Group 2); with inactivated MVA VP7 plus wild-type MVA (Group 3) or with wild-type MVA (control group-Group 4) and challenged with AHSV-4 on day 35 post-vaccination (as described in Chapter 2, section 2.7.1.4). The mean viraemia levels in the blood of the mice (per group) are represented by line blots expressing the PFU /ml values of the samples collected for each day.

In conclusion, VP7 antibodies were not generated against MVA VP7 vaccination in IFNAR $-/-$ A129 or BALB/c mice. However, VP7 specific antibodies were detected in IFNAR $-/-$ A129 mice

post-challenge. The protective efficacy of VP7 vaccination is uncertain. Although the survival rates were higher, survival periods were longer and clinical signs were lower in the vaccinated mice that received VP7, statistical analyses indicate that there were no significant differences between the VP7 vaccination groups and control mice. However, these observations suggest that recombinant VP7 may afford partial protection against AHSV-4 challenge, particularly the inactivated MVA VP7 preparation, which resulted in the survival of mice 60% greater than that of the control mice. A larger vaccination and challenge study/groups might provide statistically valid evidence of at least partial protection against the homologous AHSV strain.

4.2.6. Immunogenicity of the live viral vector MVA VP7 in A129 mice

In the absence of VP7 specific antibodies generated after vaccination with MVA expressing AHSV-4 VP7, in either of the studies described in section 4.2.4 and 4.2.5, a small pilot experiment was conducted, vaccinating three A129 mice with a higher dose of MVA VP7 (2×10^7 PFU) than previously used (1×10^7 PFU). The A129 mice strain was used as the mouse model, because A129 mice are the background strain for the interferon alpha receptor gene knock- out (IFNAR $-/-$) mice that generated VP7 specific antibodies post-challenge with AHSV-4, as described in section 4.2.4 and 4.2.5. One group of three A129 mice was vaccinated on day 0 and 21 (by intra-peritoneal injection) with MVA expressing AHSV-4 VP7 (2×10^7 PFU) (as shown in Table 4.6 and described in Chapter 2, section 2.7.1.5). Sera were obtained from whole blood taken on day 35 for serological analysis by INGENASA AHSV VP7 ELISA.

Table 4.6: Groups of A129 mice vaccinated with MVA VP7

Group number (Mouse number)	Vaccination (2x10 ⁷ PFU/dose)	
	Prime (Day 0)	Boost (Day 21)
Group 1 (M1-M1.3)	Live viral vector MVA expressing AHSV-4 VP7	Live viral vector MVA expressing AHSV-4 VP7

Analyses of VP7 specific antibody responses

VP7 specific antibody responses were analysed by INGENASA AHSV-4 VP7 ELISA. Figure 4.13 shows that no VP7 specific antibodies were detected after two vaccinations with 2x10⁷ PFU of MVA expressing AHSV-4 VP7.

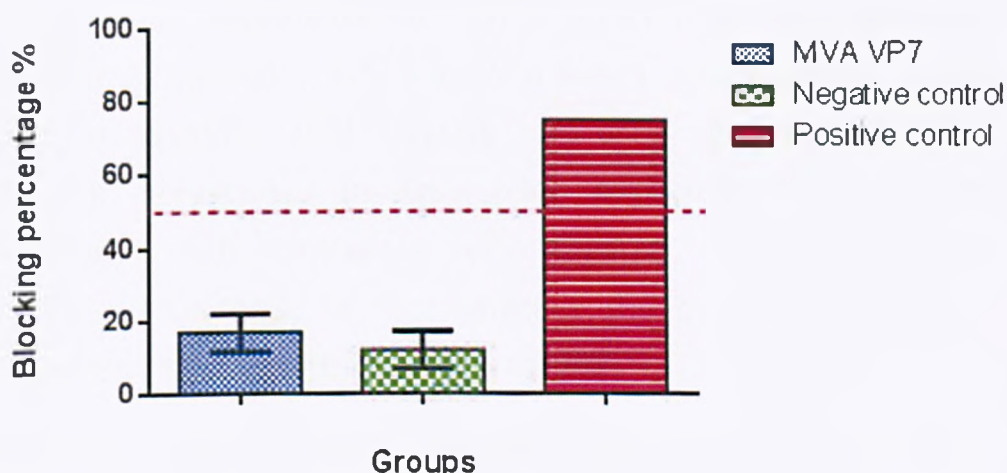


Figure 4.13: Generation of VP7 specific antibodies, in A129 mice after vaccination with MVA VP7.

Serum samples taken on day 35 from mice vaccinated with MVA VP7 (as described in Chapter 2, section 2.7.1.5) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage). The positive control was sera obtained from mice experimentally infected with AHSV-4 (Appendix III, Table A3.1). The negative control was sera obtained from non-vaccinated A129 mice

4.2.7. Immunogenicity of baculovirus expressed AHSV-4 VP7 in A129 mice

In the absence of VP7 antibodies generated in response to vaccination with recombinant baculovirus expressed and purified VP7 crystals, a pilot experiment was designed to optimise conditions (including the effect of three vaccinations, the use of adjuvant and increasing VP7 crystal solubility) necessary for inducing an antibody response to AHSV VP7 crystals in the A129 mouse model. During natural infection, soluble VP7 trimers are synthesised within the host cells, which assemble to form the VP7 crystals and the outer core layer of the virus capsid. Improving the solubility of VP7 crystals, expressed in the baculovirus expression system, could have an effect on immunogenicity and may offer insights into what fraction of VP7, generated during natural AHSV infection, is responsible for VP7's dominant immunogenicity. Chapter 3, section 3.2.5 described how urea treatment can be used to solubilise VP7 crystals (4M urea partially solubilised the VP7 crystals). In this pilot experiment, mice were vaccinated with untreated VP7 crystals or with 4M urea solubilised crystals, to test whether the partial solubilisation improved the immune response to VP7.

Three groups of three A129 mice were vaccinated by intra-peritoneal injection with different VP7 based preparations on days 0, 21 and 42 (as described in Chapter 2, section 2.7.1.6 and shown in Table 4.7). Urea (4M) solubilised crystals (5µg) were administered either with or without adjuvant (Imject Alum, Thermo Scientific). VP7 crystals (5µg) were administered with adjuvant only. Sera were obtained from whole blood taken on day 35 and 56 for serological analysis by the INGENASA AHSV VP7 ELISA.

Table 4.7: Groups of A129 mice vaccinated with VP7

Group number (Mouse number)	Vaccination		
	Prime (Day 0)	Boost (Day 21)	Boost (Day 42)
Group 1 (M1.1-1.3)	VP7 crystals (5µg) *	VP7 crystals (5µg) *	VP7 crystals (5µg) *
Group 2 (M2.1-2.3)	Urea solubilised VP7 crystals (5µg)	Urea solubilised VP7 crystals (5µg)	Urea solubilised VP7 crystals (5µg)
Group 3 (M3.1-3.3)	Urea solubilised VP7 crystals (5µg) *	Urea solubilised VP7 crystals (5µg) *	Urea solubilised VP7 crystals (5µg) *

(*administered with Imject Alum, Thermo Scientific)

Analyses of VP7 specific antibody responses

Figure 4.14 shows that after 35 days post-vaccination, the mean blocking percentage of Group 1 (whole crystals with adjuvant) was inconclusive (49%). The mean blocking percentage of Group 2 (partially solubilised VP7 crystals without adjuvant) was marginally positive (53.12%), while the mean blocking percentage of Group 3 (partially solubilised VP7 crystals with adjuvant) was clearly positive, with a blocking percentage of 76.92%.

Figure 4.15 shows that after day 56 post-vaccination, Group 1, 2 and 3 tested positive for VP7 specific antibodies with a mean blocking percentage of 68.38, 85.12 and 96.84% respectively. Negative sera (non-vaccinated A129 mouse serum) tested negative for VP7 antibodies in each instance. In order to establish if there were any significant differences between the different groups, the serum of the mice from Groups 1, 2 and 3 was tested at different dilutions. Figure 4.16 shows that when the sera was diluted to 1 in 125, all the mice tested negative for VP7 specific antibodies, except for the sera from the three mice in Group 3, which had a mean blocking percentage of 77.98%. After a 1 in 25 dilution, the mean

blocking percentage of all the groups was positive. The differences between the mean blocking percentage of Groups 1 and 2 were marginal with a mean blocking percentage of 54.51% and 57.27% respectively. In summary, VP7 specific antibodies were detected in all of the mice vaccinated with VP7 crystals, when they were administered with adjuvant. After two vaccinations VP7 specific antibodies were detected in the mice vaccinated with 4M urea treated crystals. After three vaccinations untreated VP7 crystals (with adjuvant) induced similar antibody titres to the 4M urea treated crystals (without adjuvant). The highest immune response was to the 4M urea treated VP7 crystals, with adjuvant.

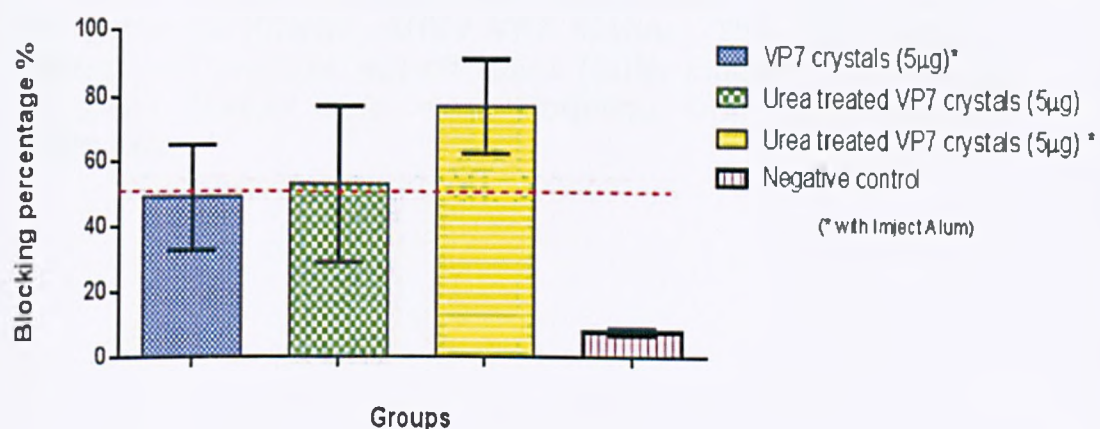


Figure 4.14: Generation of VP7 specific antibodies against VP7 in A129 mice on day 35 post-vaccination.

Serum samples were taken on day 35 post-vaccination from mice vaccinated twice with 5µg VP7 crystals (with Imject Alum); 5µg 4M urea solubilised VP7 crystals (with or without Imject Alum) (as described in Chapter 2, section 2.7.1.6). These sera were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage). Negative control sera were obtained from non-vaccinated A129 mice.

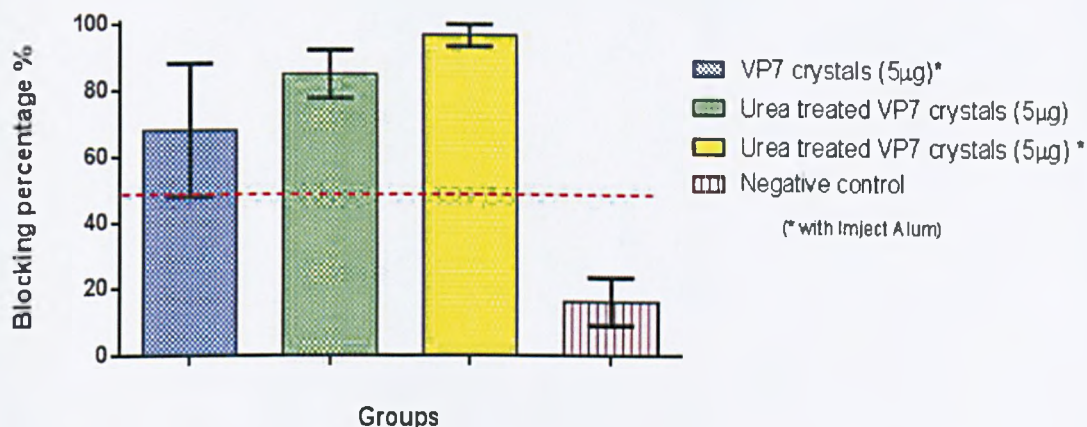


Figure 4.15: Generation of VP7 specific antibodies against VP7 in A129 mice on day 56 post-vaccination.

Serum samples were taken on day 56 post-vaccination, from mice vaccinated three times with 5µg VP7 crystals (with Imject Alum); 5µg 4M urea solubilised VP7 crystals (with or without Imject Alum) (as described in Chapter 2, section 2.7.1.6). These sera were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage). Negative control sera were obtained from non-vaccinated A129 mice.

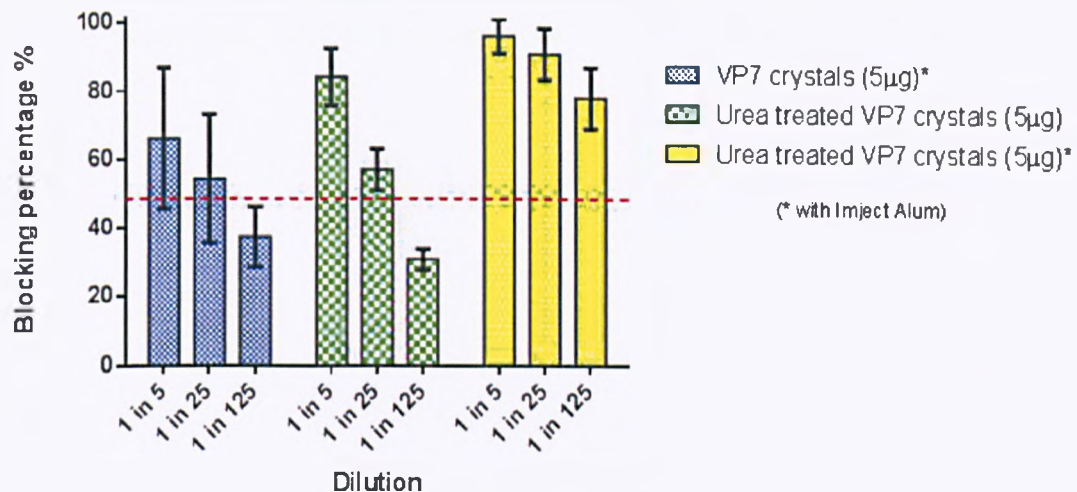


Figure 4.16: Dilution of VP7 specific antiserum raised against AHSV-4 VP7 in A129 mice on day 56 post-vaccination.

Serum samples were taken on day 56 post-vaccination, from mice vaccinated three times with 5µg VP7 crystals (with Imject Alum); 5µg 4M urea solubilised VP7 crystals (with or without Imject Alum) (as described in Chapter 2, section 2.7.1.6). These serum samples were diluted in a fivefold series (in diluent provided in the INGENASA kit). These sera were then analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage).

To conclude, a summary of the results from the VP7 crystal vaccination experiments in both mice and ponies is presented below in Table 4.8.

Table 4.8: A summary of the results from the VP7 crystal vaccination experiments

Species/ Strain	Inoculum	Dose/Number of vaccinations	Route of administration	Adjuvant?	VP7 antibody response?	Comments
Mouse/A129	Purified VP7 crystals	4,8 or 16 µg/2	IP/IM	Montanide ISA50 V2, Seppic	ELISA=(-)	See section 4.2.1
Ponies	Purified VP7 crystals	25 or 150µg/4	IM	No	ELISA=(-) WB=(-)	See section 4.2.3
Mouse/ IFNAR -/- A129	MVA VP7	1x10 ⁷ /2	IP	No	WB=(-)	See section 4.2.4
Mouse/ IFNAR -/- A129 or BALB/c	MVA VP7	1x10 ⁷ /2	IP	No	ELISA=(-) WB= (-)	See section 4.2.5
Mouse/ A129	MVA VP7	2x10 ⁷ /2	IP	No	ELISA=(-)	See section 4.2.6
Mouse/ A129	Purified VP7 crystals	5µg/3	IP	Imject Alum, Thermo Scientific	ELISA=(+)	See section 4.2.7
	Urea solubilised VP7 crystals	5µg/3	IP	Imject Alum	ELISA=(+)	
	Urea solubilised VP7 crystals	5µg/3	IP	No	ELISA=(+)	

* IP= intra-peritoneal, IM=intra-muscular

4.3 Discussion

AHSV VP7 is considered a highly immuno-dominant antigen, evidenced by the high titre of VP7 antibodies generated during natural AHSV infection in the horse. Unique to AHSV, virus replication or expression of VP7 in mammalian or insect cells (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree & Paweska, 2005) results in the aggregation of VP7 trimers to form VP7 crystals that are approximately 5µm in diameter. The crystals are highly ordered, with a repetitive crystalline structure. This suggests that it may share certain characteristics with antigens capable of cross-linking BCRs and inducing a T-cell independent, as well as a T-cell dependent, antibody response (Bachmann *et al.*, 1995; Fehr *et al.*, 1997; Fehr *et al.*, 1998; Milich & McLachlan, 1986). Vaccination with VP7 crystals was therefore considered likely to induce a strong VP7 specific antibody response. However, the results show that recombinant VP7 crystals are poorly immunogenic in the absence of adjuvant and multiple vaccinations.

Vaccination with pre-formed baculovirus expressed VP7 crystals in A129 mice failed to induce VP7 specific antibody responses that were detectable by the INGENASA ELISA, even after two vaccinations and in the presence of adjuvant. An explanation for the lack of VP7 antibodies generated is most likely to be associated with the size of the crystals. VP7 crystals may be too large to be efficiently processed by the immune system. Due to their size, the crystals might be unable to travel from the peripheral tissue at the site of inoculation to the secondary lymphoid organs, without first being processed by antigen presenting cells (APCs). Antigens greater than approximately 200nm in size depend on cellular transport to enter the lymphatic drainage system (Manolova *et al.*, 2008). APCs can squeeze through the gaps between the

endothelial cells of the lymph vessel (Bachmann & Jennings, 2010; Manolova *et al.*, 2008). The optimal size of antigen uptake by antigen presenting cells is typically between 20nm-3µm (pathogen-like dimensions) (Bachmann & Jennings, 2010). Macrophages can phagocytose antigens up to 5µm in diameter (Tabata & Ikada, 1988).

Although the average size of the crystals (~5µm) is in the upper limits of the macrophages uptake range, the size of the crystals in the baculovirus expressed preparation is not uniform. Baculovirus expressed VP7 crystal size can range up to 25µm in diameter (Chuma *et al.*, 1992; Maree & Paweska, 2005). If the crystals are not efficiently processed, the overall effective dose of VP7 being delivered to the immune system could be (much) lower than expected. In this instance the dose may not be sufficient to generate a response, particularly if processing of the crystals is slow. It might therefore be expected that with multiple vaccinations (more than two) a VP7 specific antibody response may be generated. However, after four vaccinations of ponies with crystals and sonicated crystals fragments (smaller crystals approximately 1-2µm in size) no VP7 specific antibody responses were detected. It is possible that the size of the fragmented crystals was still too big to be efficiently processed by the immune system, especially if sonication had not uniformly fragmented the crystals to the same size.

As described earlier, inefficient processing of the antigen by APCs might result in a lower effective dose being delivered to the immune system. Even after four vaccinations no antibodies were detected, indicating a lack of responsiveness post-vaccination. Inadequate activation of T-cells and co-stimulatory molecules can lead to tolerance (Bachmann *et al.*,

1998; Storni *et al.*, 2005). In this instance and in order to establish the background immunogenicity of VP7 crystals, adjuvants had not been used. However, the results indicate that adjuvant can enhance the immunogenicity of the VP7 crystals.

The results from the vaccination experiment described in section 4.2.7 shows that in the presence of adjuvant (Imject Alum) and after three vaccinations, a VP7 specific antibody response was detected in A129 mice. It is well documented that aluminium based adjuvants promote the induction of strong Th2 type responses (Brewer *et al.*, 1999). It has also been reported that aluminium adjuvants act as an immunopotentiator, boosting the immune response by the induction of uric acid, an immunological danger signal (Kool *et al.*, 2008). Improved antibody responses may be associated with improved antigen processing via the enhanced activation and recruitment of immune cells, for example macrophages and dendritic cells, for antigen processing and uptake (Kool *et al.*, 2008).

Imject Alum is a formulation of aluminium hydroxide and magnesium hydroxide. Aluminium hydroxide is an insoluble salt; however the addition of magnesium hydroxide makes the adjuvant more soluble in comparison to other aluminium-based adjuvants. This may have influenced the rate of VP7 crystal proteolysis, enhancing crystal antigen processing by antigen presenting cells. However, with the requirement of three vaccinations for the induction of a VP7 specific antibody response the rate of antigen processing may still not be optimal to induce a rapid antibody response. The dose may also be sub-optimal.

Interestingly the results show that the immunogenicity of VP7 crystals can be enhanced when they are partially solubilised using 4M urea. The results from a pilot experiment showed that after two vaccinations VP7 antibodies were detected in the mice vaccinated with 4M urea treated crystals. Additionally, after three vaccinations, 4M urea treated crystals administered in the absence of adjuvant, induced a high antibody responses similar to that generated against intact VP7 crystals (with adjuvant). This suggests that the soluble portion of the preparation is responsible for the observed increase in VP7 immunogenicity.

To fully understand effects of solubility on the immunogenicity of VP7, it is important to establish what form VP7 takes after the urea treatment (e.g. monomer, dimer, trimer, fully denatured). It is also important to recognise that other factors may be influencing the results, for example variation in the effective dose. Although the initial starting dose was the same between VP7 crystals and urea treated crystals after solubilisation, the many VP7 molecules contained within the crystals may potentially now be more readily available to the immune system in comparison to the VP7 molecules still contained within an aggregated form. If time permitted and in order to see if the heightened immunogenicity that was observed is associated with a larger effective dose, an animal experiment would be conducted to assess if the response is dose dependent. Additionally, although urea treatment only partially solubilises the crystal preparation, it is possible that urea treatment affects the stability of the crystals which may improve the rate of proteolysis and processing of the crystals to the immune system which could also influence the effective dose. It is also possible that as the urea was not dialysed

from the vaccine preparation in order to prevent re-aggregation, the urea might have provided its own immunological 'danger signal', enhancing the immune response still further.

As described in Chapter 3, expression of VP7 by the live MVA vector *in vitro* results in the generation of crystals *in situ* within the host cell. Analyses of sera raised against MVA VP7 by vaccination of ponies (Chiam *et al.*, 2009) detected VP7 specific antibodies. However, VP7 specific antibodies were not detected after MVA VP7 vaccination in either of the mouse models tested (IFNAR -/- A129, BALB/c and A129 mice).

Even though VP7 specific antibodies were detected after vaccination of ponies with MVA VP7, the antibody response was relatively weak and was only detected by Western blot and not by INGENASA ELISA. The result may reflect a heightened sensitivity for VP7 antibody detection by Western blot in comparison to the ELISA. The results also suggest that the VP7 specific antibody response generated by MVA VP7 is not particularly strong. This matches the data described above for baculovirus expressed pre-formed VP7 crystals.

Despite the difficulties encountered raising antibodies against VP7 crystals, VP7 specific antibodies were generated in the mouse model post-challenge with AHSV-4. This result suggests that an alternative form of VP7 that is generated during natural infection (other than the VP7 crystals), for example soluble VP7, may be responsible for inducing a VP7 specific antibody response post-challenge. Soluble VP7 protein would be synthesised within the host cell during live viral vector MVA VP7 infection. However, during MVA VP7 infection VP7 quickly aggregates as early as 4 hours post-

infection, as described in Chapter 3. If the immune system does respond more efficiently to soluble VP7 in comparison to the crystals, exposure to soluble VP7 during infection may be limited due to a high rate of aggregation.

A pilot challenge experiment was conducted to explore the protective efficacy against heterologous or homologous virus challenge, in mice vaccinated with recombinant VP7 (recombinant MVA and baculovirus). The results suggest that A129 mice are not highly susceptible to AHSV infection and therefore are unlikely to be a suitable model strain for use in future vaccination and AHSV challenge experiments. However, IFNAR $-/-$ A129 mice do succumb to AHSV infection and are therefore suitable as a challenge model, as previously described (Calvo-Pinilla *et al.*, 2009a; Castillo-Olivares *et al.*, 2011; Jabbar *et al.*, 2013).

There appeared to be no statistically significant differences in the viraemia, survival rates or clinical scores between vaccinates and control mice, after homologous and heterologous AHSV challenge. However MVA VP7 did appear to induce partial protection against homologous AHSV-4 challenge. A larger experiment, with more animals would be required to generate statistically significant results.

VP7 is a serogroup specific antigen, so it might also be expected that partial protection would also be observed against heterologous challenge, as described by Wade-Evans *et al.*, 1997. This was not the case, although the results may indicate variations in the virulence and pathogenicity associated with differences in viraemia, clinical score and survival rate, between the different challenge viruses (different serotypes). At this stage it is difficult to estimate

whether there is a protective role of VP7, or whether the observed differences are simply normal variations in the experimental system.

The results show that the protective efficacy of VP7 deserves further investigation. However, the absence of VP7 specific antibodies after vaccination, suggests that any protection afforded by the intact VP7 crystals would be associated with a cell mediated immune response and not due to humoral immunity which was also suggested by Wade-Evans *et al.* 1997.

In conclusion, the work generated in this chapter indicates that AHSV VP7 crystals are not immunogenic in the absence of multiple vaccinations and adjuvant but immunogenicity can be improved when the crystals are treated with 4M urea, 'partially solubilising' the crystal preparation. The results suggest that if soluble VP7 is highly immunogenic as suggested, the virus (AHSV) may generate the crystals (during replication) as a mechanism to hide VP7 from the animal's immune system. This will be discussed further in Chapter 7.

Chapter 5: Use of modified VP7 as a vaccine platform; Generation and characterisation of modified VP7 constructs

5.1. Introduction

Recent advances in DNA technologies have facilitated the expression of individual recombinant antigens, individual peptides, and even full virus-like particles (VLP). The use of immunologically important peptides has been somewhat limited for use in vaccination, owing to poor immunogenicity. However, several studies have demonstrated that the immunogenicity of target peptides can be enhanced when incorporated and displayed within a delivery platform for example a VLP (Clarke *et al.*, 1987; Fehr *et al.*, 1998; Whitacre *et al.*, 2009).

The most common method used to incorporate peptides/epitopes into the scaffold of the recipient antigen is by genetic insertion. An important advantage of this method is that successful incorporation can potentially display the target peptide in the same conformation and at the same density as the recipient molecule (Chackerian, 2012). The success rate for chimeric particle generation relies on the compatibility between the recipient molecule and the target peptide (both structurally and chemically). For example, over half of the peptides tested were not compatible with chimeric Hepatitis B core antigen (HBcAg) assembly (Billaud *et al.*, 2005; Jegerlehner *et al.*, 2002). Woodchuck hepatitis virus core

antigens are able to tolerate insertions at a greater number of positions in comparison with the Hepatitis B virus core antigen, demonstrating the importance of selecting an appropriate carrier antigen (Billaud *et al.*, 2005). Factors that can influence the success of chimeric antigen construction include peptide size, hydrophobicity and charge (Barcena, 2013; Karpenko *et al.*, 2000). In addition peptide insertion must be at a site that will not impair the correct folding and protein assembly; equally the insertion site must be externally presented for effective B- cell activation (Donaldson, 2015). Immuno-dominant viral hydrophilic epitopes, which are normally located on 'looped' structures, are typically targeted as insertion sites on the recipient antigen molecules (Barcena, 2013).

An alternative approach to foreign peptide display, is by attaching the relevant peptides to carrier antigens, using chemical conjugation (Jegerlehner *et al.*, 2002), for example by using biotin-streptavidin complexes (Chackerian *et al.*, 2008). The advantages of this approach over genetic insertion include the potential for conjugation of larger and more complex molecules. For example, a large number of immunologically important epitopes can be displayed to the immune system, without disrupting the original structure of the protein carrier. This may be important for targeting complex B-cell epitopes. However, manufacturing of chemically conjugated chimeric particles is also more complex.

The structure of the upper domain of the AHSV VP7 monomer and trimer unit has been resolved at an atomic level (Basak *et al.*, 1996). Rutkowska and co-workers (2011) have investigated the use of soluble chimeric AHSV VP7 trimers as a vaccine delivery platform, inserting viral peptides into the

natural hydrophilic loops on the top domain of the VP7 molecule. Rutkowska and co-workers (2011) inserted a 110 amino acid peptide sequence, without disrupting trimer formation. They also showed that soluble chimeric VP7 induced an insert specific antibody response in guinea-pigs (Rutkowska *et al.*, 2011).

A primary aim of this thesis is to investigate the use of the AHSV VP7 particulate crystals as a vaccine delivery platform. VP7 is considered to be an immuno-dominant antigen during natural AHSV infection and the structure of the VP7 crystals, with its repetitive epitope patterns, corresponds with other antigens capable of cross-linking BCRs, thereby inducing a T-cell independent and T-cell dependent antibody response (Bachmann *et al.*, 1995; Milich & McLachlan, 1986). The crystals could therefore serve to enhance the immunogenicity of any epitope/peptide displayed in a highly organised manner on its surface.

In order to evaluate the potential of recombinant AHSV VP7 crystals as a vaccine delivery system it was first necessary to express and characterise the modified VP7 protein constructs *in vitro*. Chapter 5 will describe the generation of modified VP7/VP2 and VP7/BAP constructs, by inserting viral peptides of AHSV-4 VP2, or a biotin acceptor peptide (BAP), into different amino acid positions in the VP7 polypeptide chain. The resulting constructs were expressed by recombinant baculovirus and live viral vector MVA, to assess their antigenic reactivity and ability to aggregate and form crystals.

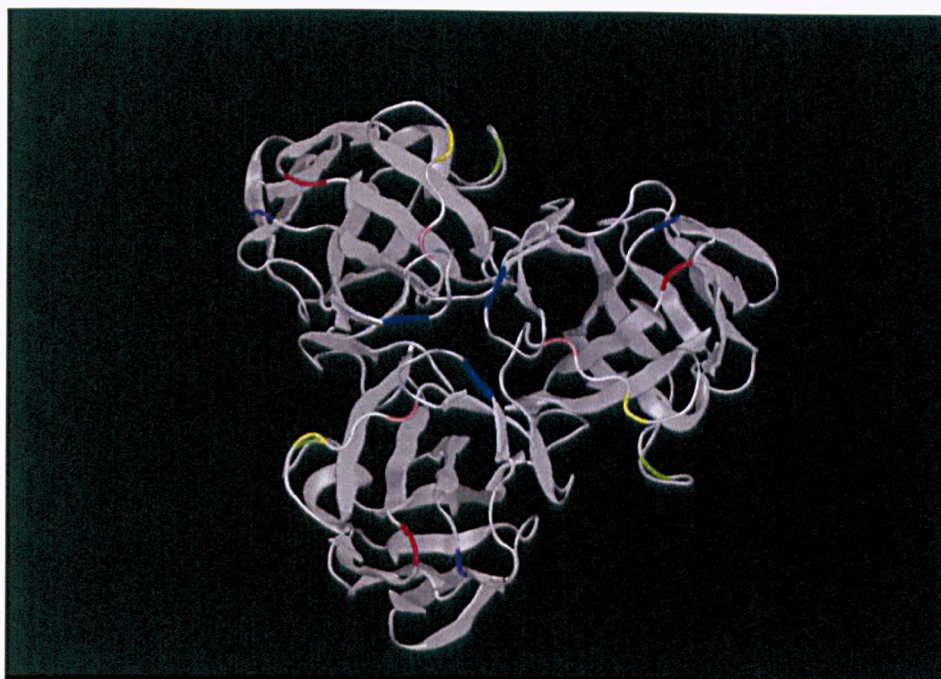
5.2. Results

5.2.1. Generation of modified VP7 gene constructs

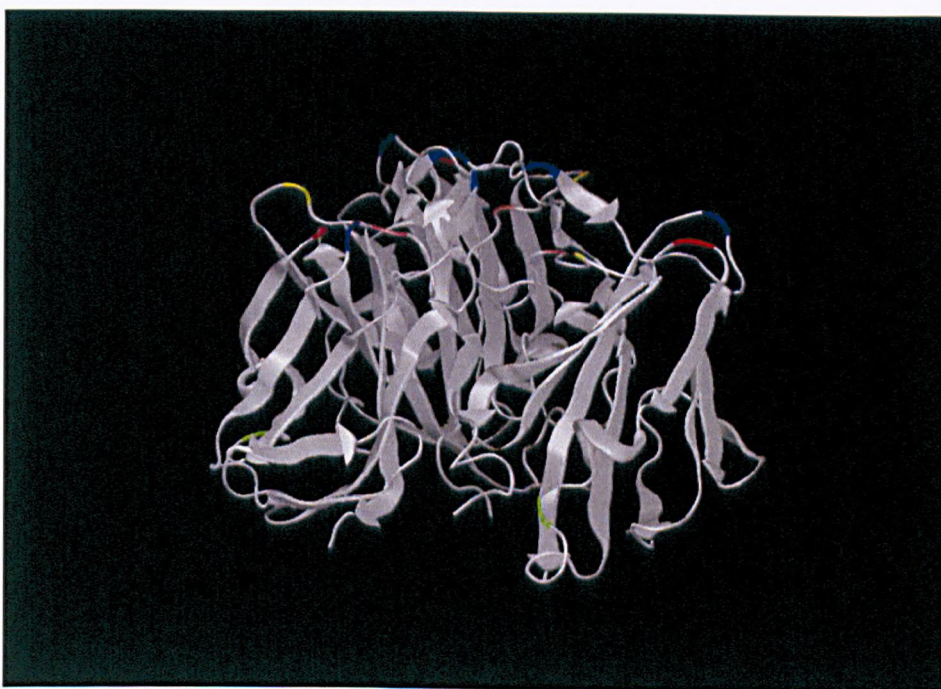
The modified gene constructs were generated by inserting the amino acid sequence of two AHSV-4 VP2 peptides (VP2a and VP2b), or a biotin acceptor peptide (BAP), into the VP7 polypeptide chain. The two VP2 peptides represent neutralising epitopes 'a' and 'b' previously described by Martinez-Torrecuadrada and co-workers (2001). Insertion was accomplished by incorporating the nucleotide sequence coding for the peptides directly into the VP7 gene sequence by site-specific mutagenesis, using splice-overlap extension (SOE) PCR techniques (Heckman & Pease, 2007).

a) Identification of insertion sites in the VP7 molecule

The sites for the insertion of foreign peptides into the head domain of the VP7 molecule were chosen according to residue topology. Resolution of the atomic structure of the top domain of the molecule (Basak *et al.*, 1996) facilitated identification of potential insertion sites located in the 'loops' on the more accessible areas of the trimer surface. Amino acid residues 141, 144, 164, 177, 200 and 238 were chosen as sites for foreign peptide insertion as shown in Figure 5.1.



A)



B)

Figure 5.1: A schematic representation of the structure of the AHSV VP7 trimer top domain; A) Vertical view; B) Lateral view:

VP7 monomers coloured in grey. Insertion sites on the head of the AHSV VP7 molecule indicated in pink (aa 141), yellow (aa 144), red (aa 164), green (aa 177), blue (aa 200) and purple (aa 238). Image created by Protean 3D. Sequence sourced from Basak *et al.* 1996.

b) Generation of the modified VP7 DNA constructs by splice overlap extension (SOE)

The modified VP7 constructs were generated by inserting the coding sequence of characterised peptides (Table 5.1) into the VP7 gene by splice overlap extension technique (as described in Chapter 2, Section 2.2.2.) using primers (Appendix I, Table AI) that were designed based on the coding sequence of the AHSV-4 VP7 gene (AHSV-4/Madrid 1987) (Appendix II, Figure A2.2) and the characterised peptides.

Table 5.1: The nucleotide and amino acid coding sequences introduced in the VP7 gene

To be inserted into the VP7 gene	Nucleotide (nt) and amino acid (aa) coding sequence 5'-3'
Peptide VP2a (amino acid 318 to 342)	nt: GTTCATGCGAAGAAGAAAGAAGAGGGTGAGGATG ATACTGCTCGACAGGAGATAAGAAAAGCATGGGTAA GGGG aa: VHAKKKEEGEDDTARQEIRKAWVKG
Peptide VP2b (amino acid 374 to 403)	nt: AAGAGGAACGGTGTAGATGTTGATCCGAATAAGG GTAAGTGGAAAGAACATATAAAGGAGGTAACCGAAA AATTGAAGAAAGCGCAAACCGAA aa: KRNGVDVDPNKGKWKEHIKEVTEKLKKAQTE
Biotin Acceptor Peptide (BAP) (Tanaka <i>et al.</i> , 2010)	nt: GGTCTCAACGACATCTTCGAAGCACAGAAAATTGA ATGGCAT aa: GLNDIFEAQKIEWH

Initially, modified VP7 constructs that incorporated characterised peptide VP2a and BAP into VP7, at amino acid position 141, were generated on the backbone of plasmid pVL1393 AHSV-4 VP7 - VP7 gene sequence I (Appendix II, Figure A2.1). The cDNA was derived from VP7 of the AHSV-4 Madrid 1987 strain and the plasmid provided by Dr Javier Castillo-Olivares. Expression of these constructs by

recombinant baculovirus did not generate crystalline structures. Subsequently, the VP7 gene was sequenced and analysed in detail, in comparison to the VP7 gene sequence of a recombinant baculovirus expressed VP7 (Chapter 3) and the VP7 gene sequence utilised by Chiam *et al.* 2009 during a MVA VP7 vaccination experiment in ponies. It was found that VP7 gene sequence I was 349 amino acids in length but contained two amino acid differences (aa 57, Gly – Glu; aa 119, Cys to Glu) relative to the VP7 gene utilised by Chiam *et al.* 2009 and wild-type VP7 crystal expression by recombinant baculovirus (Chapter 3) - VP7 gene sequence II. Following this result, the remaining constructs were based on the same VP7 gene sequence as described by Chiam *et al.* 2009 (VP7 gene sequence II) (Appendix II, Figure A2.2). The inability of modified constructs to form crystals will be discussed later in this chapter.

The SOE method involved three PCR reactions; the first two reactions generated overlapping PCR products as shown in Figure 5.2, which were then used as a template in a final PCR reaction to generate a full-length cDNA product (Figure 5.3). The properties of each construct generated are summarised in Table 5.2.

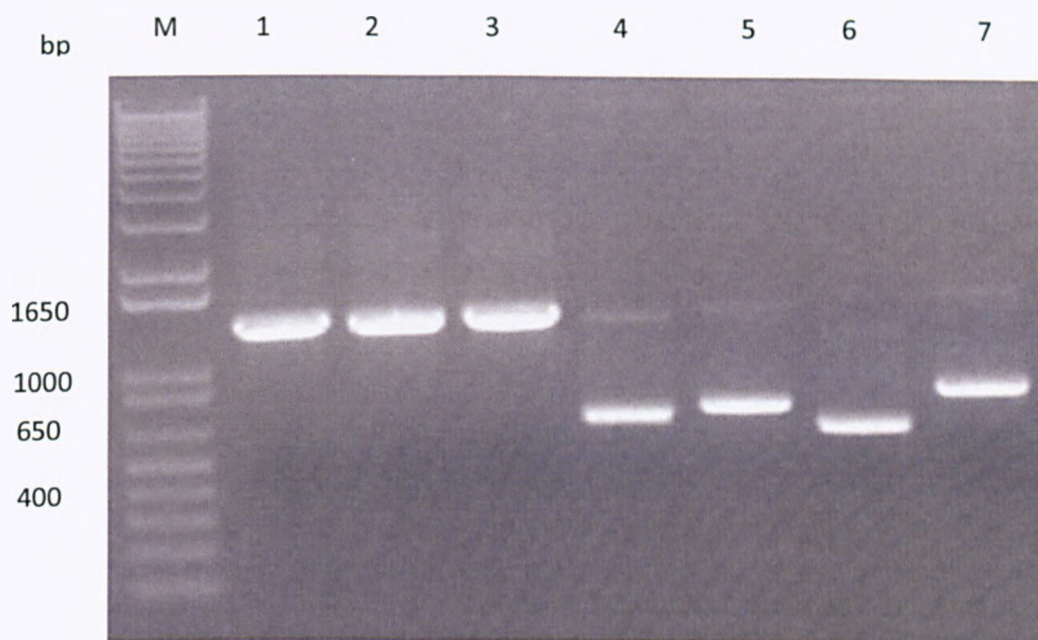


Figure 5.2: Splice-overlap extension intermediate ('A' and 'B') and full length ('C') SOE, PCR products.

AGE analyses of PCR products from the splice overlap extension method. M (molecular marker): TrackIt™ 1 Kb Plus DNA Ladder. Final SOE PCR products ('C') are shown in Lane 1: VP7/VP2a177; Lane 2: VP7/VP2a200; Lane 3: VP7/VP2a238. Intermediate SOE PCR products 'A' are shown in Lane 4: VP7/VP2a200; Lane 6: VP7/VP2a238. Intermediate SOE PCR products 'B' are shown in Lane 5: VP7/VP2a200; Lane 7: VP7/VP2a238. Details of each construct are given in Table 5.2.

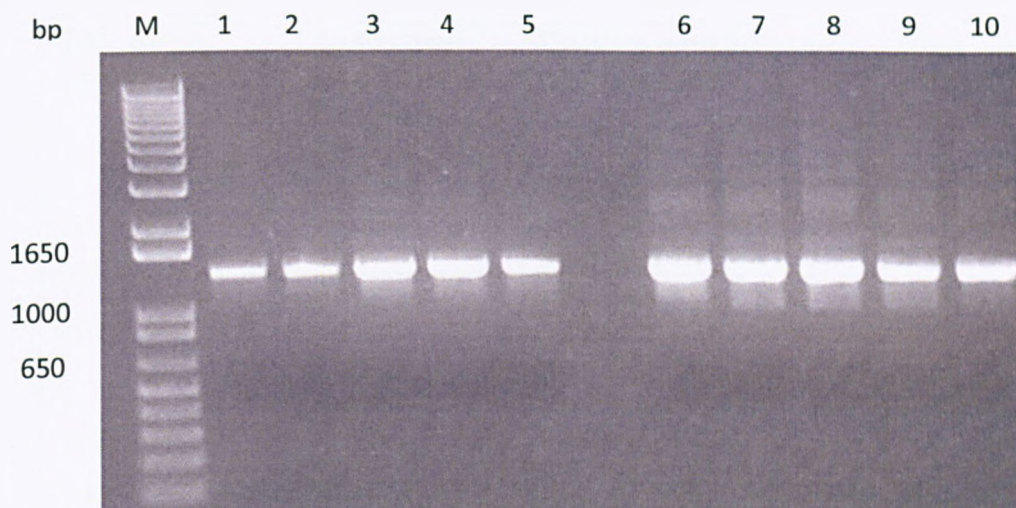


Figure 5.3: Splice overlap extension full length PCR products.

AGE analyses showing PCR products of the splice overlap extension method. M (molecular marker): TrackIt™ 1 Kb Plus DNA Ladder. Full length SOE products ('C') are shown in Lane 1: VP7/VP2a144; Lane 2: VP7/VP2a164; Lane 3: VP7/VP2a177; Lane 4: VP7/VP2a200; Lane 5: VP7/VP2a238; Lane 6: VP7/VP2b144; Lane 7: VP7/VP2b164; Lane 8: VP7/VP2b177; Lane 9: VP7/VP2b200; Lane 10: VP7/VP2b238. Details of each construct are given in Table 5.2.

Table 5.2: Summary of the modified VP7 gene constructs

VP7 gene construct	Insertion position in the VP7 sequence	Coding sequence to be inserted	Size: base pairs (bp)*
1 VP7/VP2a141	Before amino acid 141	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
2 VP7/VP2b141	Before amino acid 141	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143
3 VP7/BAP141	Before amino acid 141	Biotin Acceptor Peptide (BAP)	1092
4 VP7/VP2a144	After amino acid 144	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
5 VP7/VP2b144	After amino acid 144	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143
6 VP7/VP2a164	Before amino acid 164	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
7 VP7/VP2b164	Before amino acid 164	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143
8 VP7/VP2a177	After amino acid 177	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
9 VP7/VP2b177	After amino acid 177	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143
10 VP7/VP2a200	After amino acid 200	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
11 VP7/VP2b200	After amino acid 200	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143
12 VP7/BAP200	After amino acid 200	Biotin Acceptor Peptide	1092
13 VP7/VP2a238	After amino acid 238	VP2 neutralising epitope (a) (amino acid 318 to 342)	1125
14 VP7/VP2b238	After amino acid 238	VP2 neutralising epitope (b) (amino acid 374 to 403)	1143

*Including the stop codon

5.2.2. Generation and characterisation of modified VP7 constructs using baculovirus expression

a) Generation of recombinant baculovirus stocks

The modified VP7 gene constructs were cloned into the intermediate vector pGEM-T to facilitate cloning into the baculovirus transfer vector pVL1393. The intermediate vectors containing the gene of interest, as confirmed by colony PCR, were subjected to Not I digestion and the vector and the gene of interest separated by electrophoresis as shown in Figure 5.4).

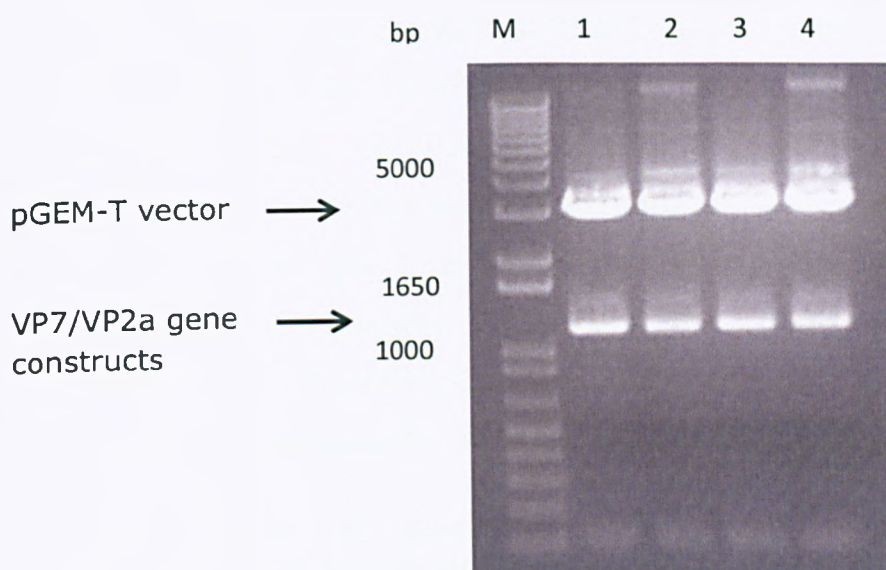


Figure 5.4: Digestion of the pGEM-T vector containing the modified VP7 gene constructs.

AGE analyses showing pGEM-T plasmid-gene constructs after digestion with Not I restriction enzyme. M (molecular marker): TrackIt™ 1 Kb plus DNA Ladder; Lane 1: VP7/VP2a141; Lane 2: VP7/VP2a177; Lane 3: VP7/VP2a200; Lane 4: VP7/VP2a238. Details of each construct are given in Table 5.2.

The modified VP7 genes were purified from the gel and cloned directly into the baculovirus transfer vector plasmid pVL1393. All of the constructs generated were sequenced to confirm that the open reading frame was intact and did not include any additional nucleotide changes. Modified VP7 constructs with

peptide insertions at VP7 amino acids 144 and 164, were found to contain multiple stop codons throughout the open reading frame. Adding glucose to the LB agar plates and the use of another source of competent cells were tried to overcome these cloning problems without success. The insertion of foreign peptides into the VP7 gene at VP7 amino acid positions 144 and 164 were therefore abandoned.

From the modified VP7 constructs that had been successfully generated and cloned in to pLV1393 plasmid vector, a number were chosen for further analysis. VP7 modified constructs VP7/VP2a141 and VP7/BAP141 were used to generate recombinant baculovirus stocks. Stocks of recombinant flashback (FB)-VP7/VP2a141 and FB-VP7/BAP141 viruses were successfully generated. Subsequently, recombinant FB-VP7/VP2a200 and FB-VP7/VP2a238 constructs were also generated successfully. Expression was confirmed by Western blot and immunofluorescence using polyclonal anti-AHSV-9 and anti-VP2a peptide antisera (Appendix III, Table A3.1).

b) Analysis of modified VP7 protein expressed by recombinant baculovirus using Western blot

The predicted molecular weights for these constructs are presented in Table 5.3. The antigenic reactivity of all four modified constructs with anti-AHSV-9 antisera was confirmed by Western blot (Figure 5.5). All four modified VP7 proteins migrated as distinct bands, slightly slower than wild-type VP7 (~37kDa) as expected due the addition of the insert. However, they migrated slightly faster than predicted from their molecular weights. Protein construct VP7/VP2a141 migrated slower than constructs VP7/VP2a200 and VP7/VP2a238 despite a marginally smaller predicted molecular weight of ~40.5kDa. A distinct band corresponding to the predicted molecular weight of modified VP7 (Table 5.3) was

absent from mock-infected cell lysates. However, extra bands were observed on the membrane but this was probably due to the non-specific reactivity of the polyclonal antibody with other proteins in the cell lysate preparation.

The antigenic reactivity of the VP7/VP2a141, VP7/VP2a200 and VP7/VP2a238 constructs with anti-VP2a peptide antiserum (Appendix III, Table A3.1) was confirmed by the presence of a distinct band on the blot membrane for each of the constructs and the absence of a distinct band for wild-type VP7 (Figure 5.6). The modified VP7 constructs continued to migrate slightly faster than expected, although construct VP7/VP2a141 still migrated marginally slower in comparison to the other constructs.

Table 5.3: Estimated molecular weights of AHSV VP7 and modified VP7 proteins (determined by SeqBuilder)

Protein construct	VP7 (1)	VP7 (2)	VP7/BAP 141	VP7/VP2a 141	VP7/VP2a 200	VP7/VP2a 238
Estimated MW (kDa)	~37.6	~37.7	~39.3	~40.5	~40.6	~40.6
Amino Acids No.	349	349	364	374	374	374

kDa M 1 2 3 4 5 6

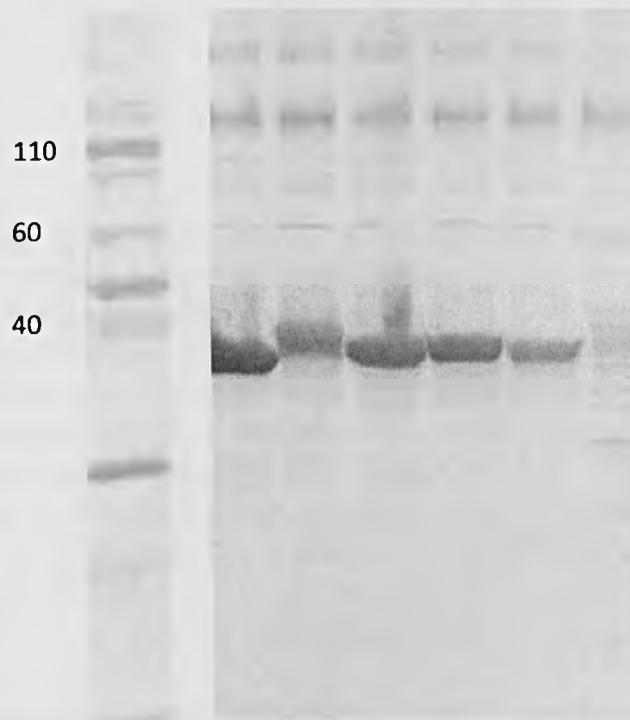


Figure 5.5: Detection of modified VP7 proteins in FB-VP7/VP2a and VP7/BAP infected cell lysates by Western blot using anti-AHSV-9 antiserum.

Sf9 cells were infected at MOI 1, harvested 72 hours post-infection and lysed with 0.25% NP40. The cellular lysates were separated by SDS PAGE and probed using anti-AHSV-9 antiserum. M (protein marker): Novex® Sharp Protein Standard; Lane 1: Wild-type VP7; Lane 2: VP7/VP2a141; Lane 3: VP7/BAP141; Lane 4: VP7/VP2a200; Lane 5: VP7/VP2a238; Lane 6: Mock infection.

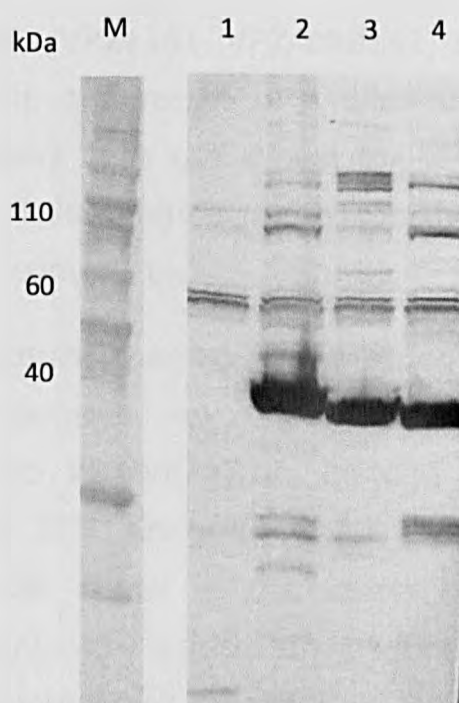


Figure 5.6: Detection of modified VP7 proteins in FB-VP7/VP2a infected cell lysates by Western blot using anti-VP2a peptide antiserum.

Sf9 cells were infected at MOI 1, harvested 72 hours post-infection and lysed with 0.25% NP40. The cellular lysates were separated by SDS PAGE and probed using anti- VP2a peptide antiserum (Appendix III, Table A3.1). M (protein marker): Novex® Sharp Protein Standard; Lane 1: Wild-type VP7; Lane 2: VP7/VP2a141; Lane 3: VP7/VP2a200; Lane 4: VP7/VP2a238.

c) Analysis of modified VP7 protein expression by recombinant baculovirus, using light microscopy and immunofluorescence

As described in Chapter 3, recombinant VP7 expression results in the generation of crystals that can appear as rod-like structures in the insect cell when visualised by light microscopy. The ability of the modified VP7 proteins to form crystals was first determined by the presence or absence of rod-like structure in the infected insect cell culture. Figure 5.7, Image A shows that VP7/VP2a200 expression resulted in the

generation of rod-like structures, mimicking those observed during wild-type VP7 infection in Sf9 insect cells. In contrast, expression of VP7/VP2a141, VP7/BAP141, and VP7/VPa238 did not result in the generation of similar structures inside the infected Sf9 insect cells (as shown for VP7/VPa238 in Figure 5.7, Image B). This image is representative of all of the non-crystal forming constructs.

Immunofluorescence imaging re-confirmed these results as no crystal-like structures were observed for VP7/VP2a144, VP7/BAP141 and VP7/VP2a238. Instead the distribution of these modified VP7 antigens was more evenly dispersed around the outer edges of the insect cell as detected by polyclonal anti-AHSV-9 antiserum (Figure 5.8, Images A, B and D). It was noted that detection of the non-crystal forming antigen by immunofluorescence was less evident than detection by Western blot. In contrast, modified crystals (approximately 1-4 per cell) ranging in size from $\sim 3\text{-}25\mu\text{m}$ in size were observed by immunofluorescence in Sf9 cells expressing construct VP7/VP2a200 (Figure 5.8, Image C).

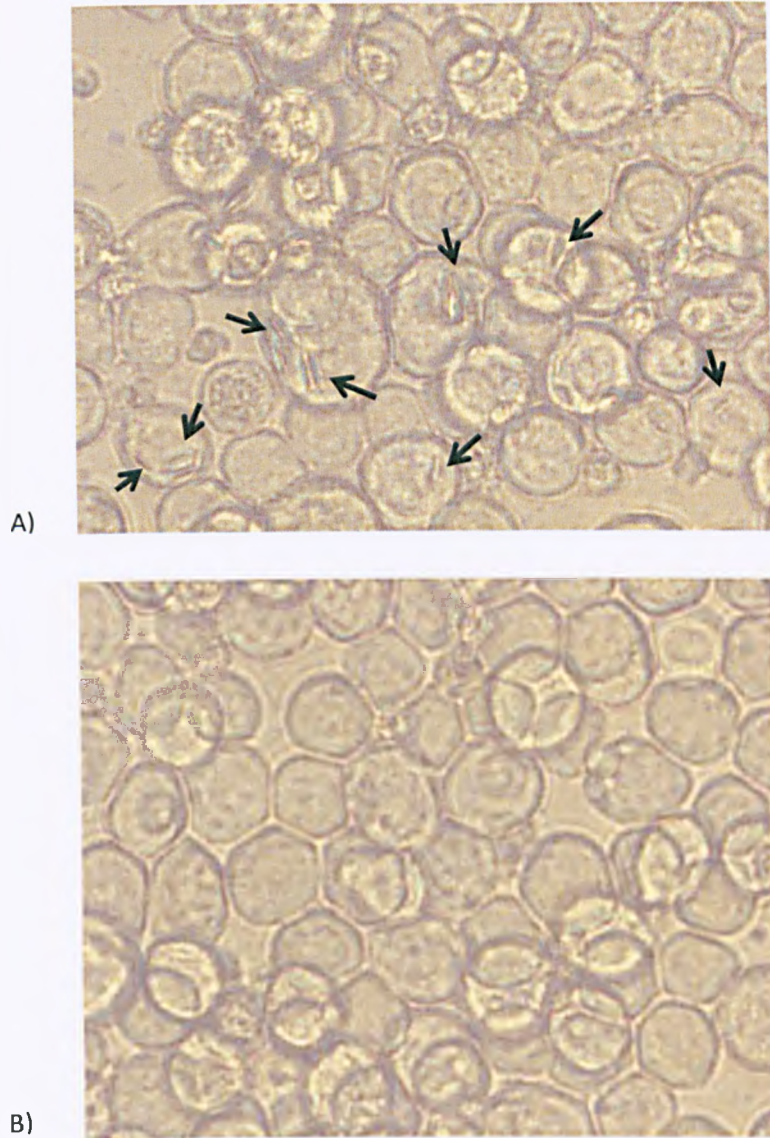
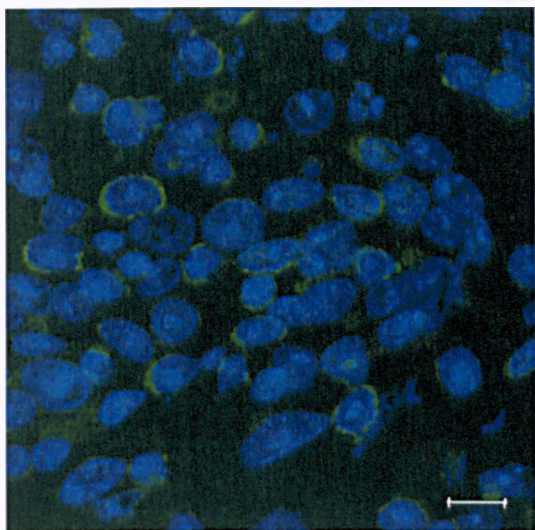
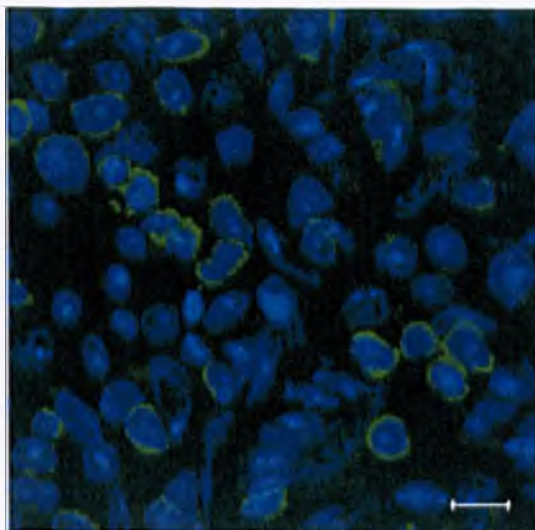


Figure 5.7: Detection of baculovirus expressed modified VP7 crystals in Sf9 insect cells, by light microscopy.

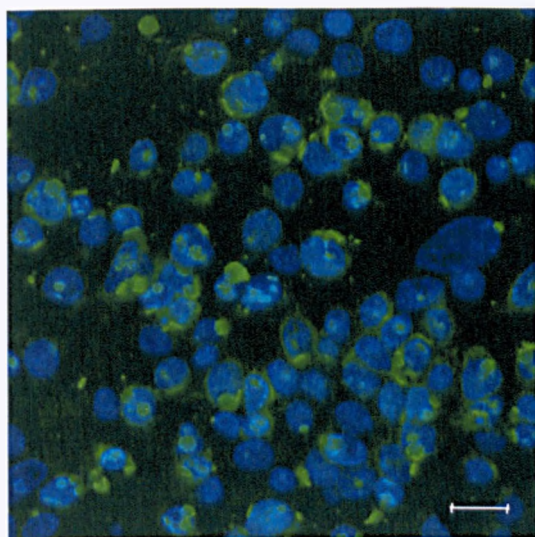
Sf9 cells were infected (MOI 1) with recombinant baculoviruses (FB-VP7/VP2a200 and FB-VP7/VP2a238) and imaged at 48 hours post-infection by light microscopy. Image A: insect cells infected with FB-VP7/VP2a200; Image B: insect cells infected with FB-VP7/VP2a238. Inside the living infected SF9 insect cell the VP7/VP2a200 crystals appear as rod-like structure as indicated by the black arrows.



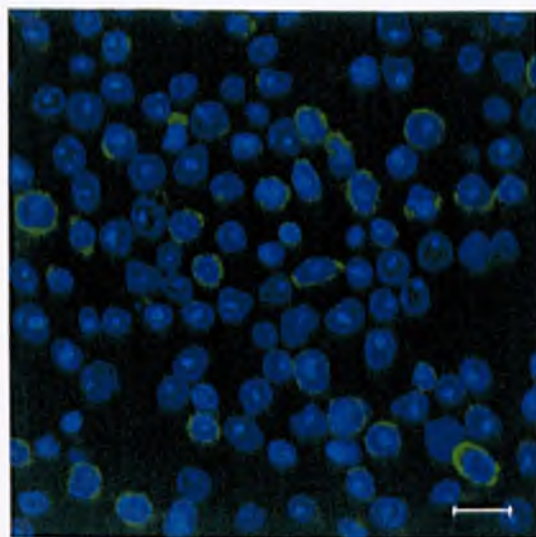
A)



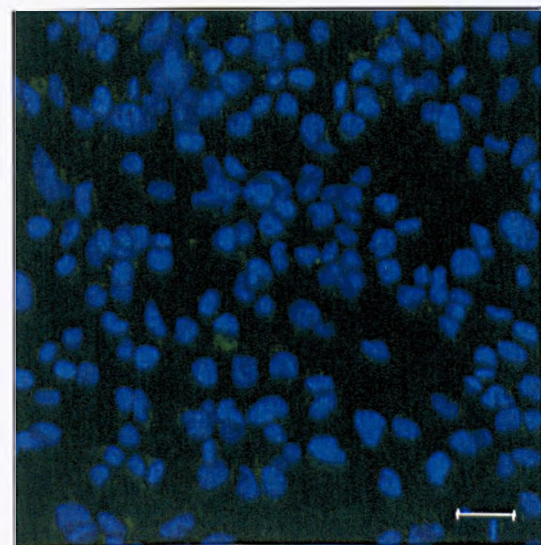
B)



C)



D)



E)

Figure 5.8: Detection of FB-VP7/VP2a and FB-VP7/BAP in Sf9 insect cells by immunofluorescence.

Sf9 cells were infected with recombinant baculoviruses expressing modified AHSV-4 VP7 proteins at high MOI (0.1-0.5), fixed at 72 hours post infection and probed with anti-AHSV-9 antiserum and an Alexa-fluor488 conjugated anti-rabbit secondary antibody. Image A: FB-VP7/VP2a141; Image B: FB-VP7/BAP141; Image C: FB-VP7/VP2a200; Image D: FB-VP7/VP2a238; Image E: mock infection. The cell nuclei are stained blue with DAPI. VP7 is stained green. Details of each construct are given in Table 5.2.

5.2.3. Analysis of purified modified VP7 crystals using DIC and Western blot

Expression of VP7/VP2a200 by recombinant baculovirus does results in the generation of modified VP7 crystals. As observed with wild-type VP7 crystals, the modified crystals can be purified using the same methods (Chapter 2, section 2.4.3). After purification, the presence of VP7/VP2a200 crystals in the preparation was confirmed by light and DIC microscopy. Figure 5.9 show that the basic crystalline structure mimics that of wild-type VP7 crystals. The antigenic reactivity of the purified VP7/VP2a200 crystals with anti-AHSV-9 and anti-VP2a peptide antiserum was confirmed by Western blot. Figure 5.10, Image A shows that a distinct band, migrating slower than wild-type VP7 (~37kDa) but corresponding to the predicted size of VP7/VP2a200 (40.6 kDa) was observed on the blot membrane after probing with anti-AHSV-9 antiserum. A band of the same characteristics was also observed on the blot membrane after probing with anti-VP2a peptide antiserum (Figure 5.10, Image B). There was no reactivity between the VP2a peptide antiserum and purified wild-type VP7 crystals, confirming the presence of the VP2a peptide in the VP7/VP2a200 crystals. Extra bands were observed on the blot membranes that are thought likely to represent cross-reactivity of contaminating proteins with the polyclonal antiserum.



Figure 5.9: Purified example of a modified, baculovirus expressed VP7/VP2a200 crystal.

Image obtained by differential interference contrast (DIC) microscopy.

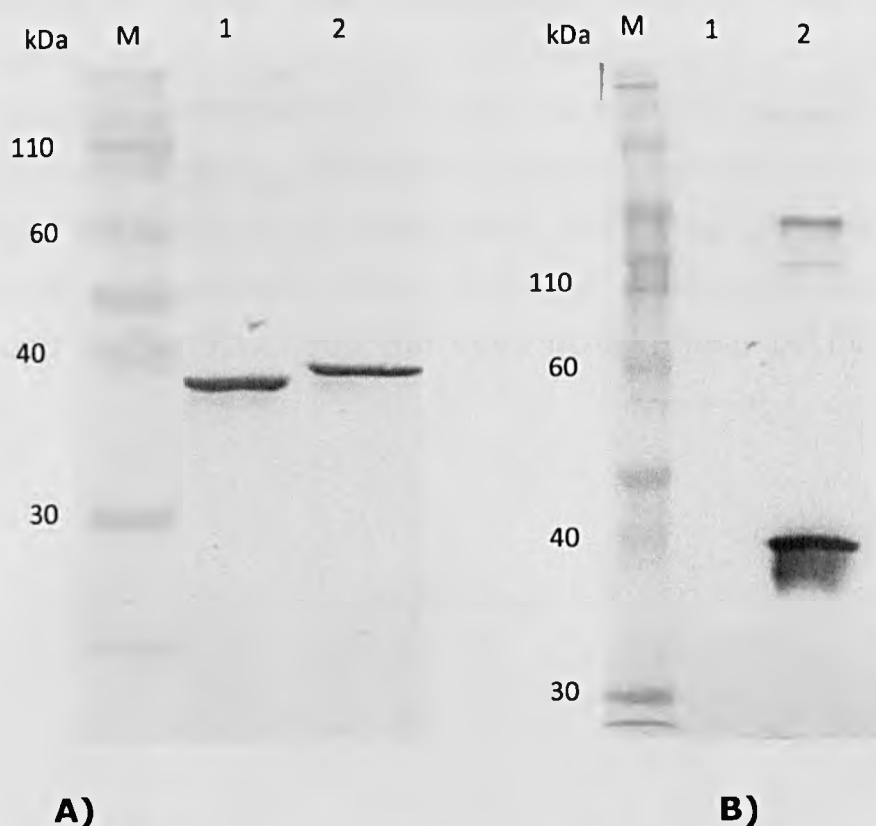


Figure 5.10: Detection of purified VP7 and VP7/VP2a200 crystals by Western blot.

Purified VP7 and VP7/VP2a200 crystals were separated by SDS PAGE and probed using anti-AHSV-9 antiserum (Image A) and anti-VP2a peptide antiserum (Image B). Image A) M (protein marker): Novex® Sharp Protein Standard; Lane 1: VP7 crystals; Lane 2: VP7/VP2a200 crystal. Image B) M (protein marker): Novex® Sharp Protein Standard; Lane 1: VP7 crystals; Lane 2) VP7/VP2a200 crystals.

5.2.4. MVA Expression of recombinant modified VP7

5.2.4.1. Generation of recombinant MVA stocks

The use of recombinant MVA to deliver the modified VP7 constructs within the host cells by inducing 'de novo' synthesis of these antigens would enable their delivery without the requirement for protein purification. Initially constructs VP7/VP2a200 and VP7/VP2a238 were used to generate recombinant MVAs. These two modified VP7 constructs were chosen as they represented one crystal forming construct

(VP7/VP2a200) and one non-crystal forming construct (VP7/VP2a238) for comparison. The modified gene constructs VP7/VP2a200 and VP7/VP2a238 were cloned into the vaccinia transfer vector pSC11, downstream of the vaccinia early/late promoter P7.5. Stocks of recombinant MVA were successfully generated after several rounds of plaque purification (Figure 5.11 and Figure 5.12) and the virus titre verified by plaque assay.

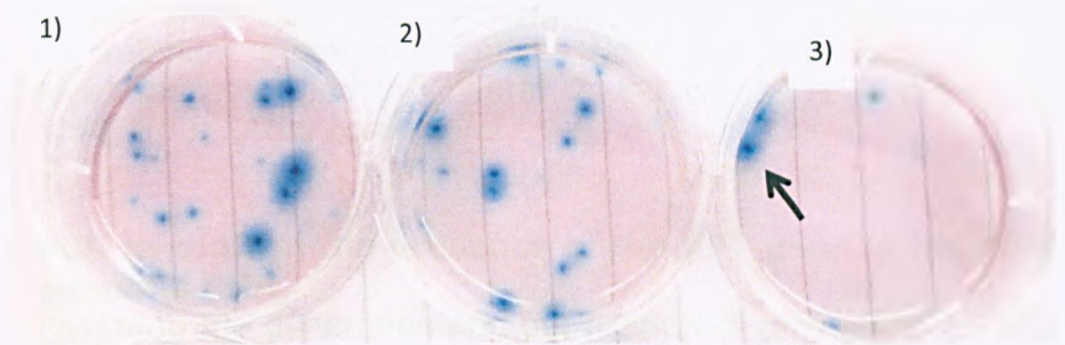


Figure 5.11: Selection of recombinant MVA expressing VP7/VP2a200 by plaque assay.

Visual selection of stained (X-gal positive) individual rMVA plaques. Isolated blue plaques (as indicated by the black arrow) were collected for further plaque purification steps/recombinant stock generation. Wells 1-3 represent plaque picks (as described in Chapter 2, section 2.5.1.2) diluted at 1/40 1/50, 1/200 respectively.

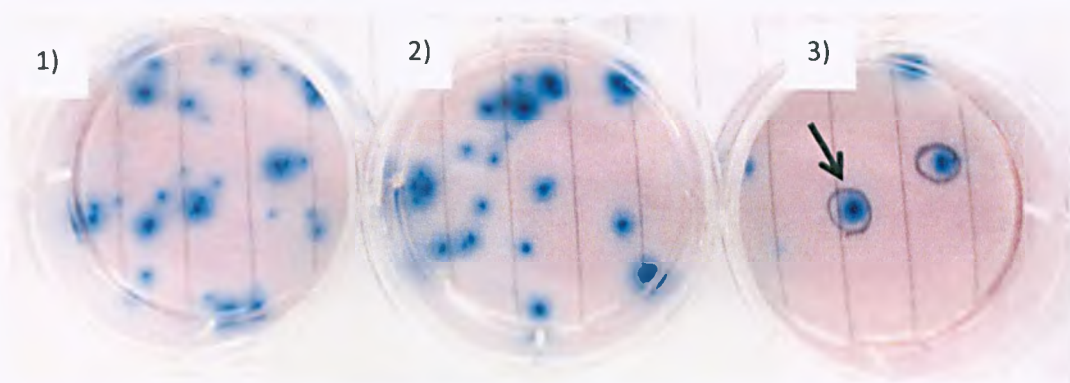


Figure 5.12: Selection of recombinant MVA expressing VP7/VP2a 238 by plaque assay.

A visual selection of stained (X-gal positive) individual rMVA plaques. Isolated blue plaques (as indicated by the black arrow) were collected for further plaque purification steps/recombinant stock generation. Wells 1-3 represent plaque picks (as described in Chapter 2, section 2.5.1.2) diluted at 1/40 1/50, 1/200 respectively.

PCR to confirm the presence of the VP7 gene in the MVA genomic DNA

Following the generation and purification of recombinant MVAs expressing VP7/VP2a200 and VP7/VP2a238 the presence of the modified VP7 genes in the MVA genomic DNA was confirmed by PCR using VP7-specific primers (Appendix I, Table A1, primer set 17) with template DNA extracted from DF-1 cells infected with either MVA VP7 (unmodified), MVA-VP7/VP2a200, MVA-VP7/VP2a238 or non-infected. As shown in Figure 5.13, each of the constructs gave a positive result whereby a PCR product corresponding to the expected size was observed for each construct (VP7, VP7/VP2a200 and VP7/VP2a238 - 1050bp, 1125bp and 1125bp respectively). As expected, addition of the insert caused the modified VP7 gene to migrate slower than the wild-type VP7 gene. The absence of a PCR product for DNA extracted from a mock infection and the presence of the PCR products for each of the VP7 based constructs indicates that the gene constructs were incorporated successfully in the MVA genome.

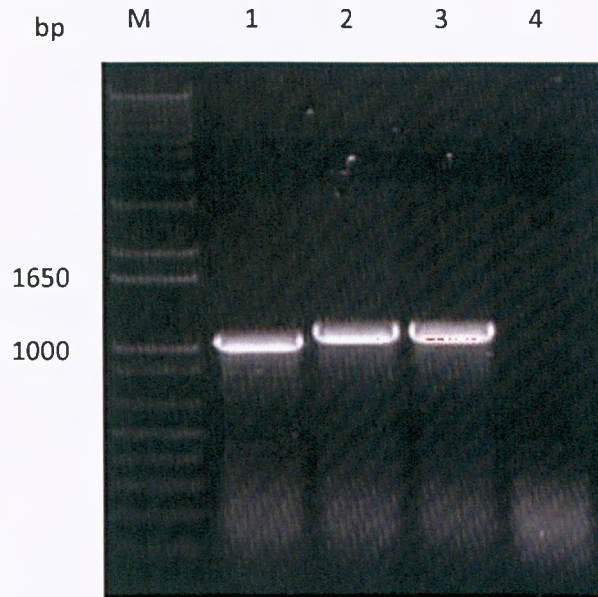


Figure 5.13: Detection of modified VP7 gene constructs in recombinant MVA genomic DNA by PCR.

AGE analyses showing PCR products from DNA extracted from DF-1 cells infected with MVA VP7 and MVA expressing VP7/VP2a, harvested at 24 hour post infection. M (molecular marker): TrackIt™ 1 Kb Plus DNA Ladder; Lane 1: Wild-type VP7; Lane 2: VP7/VP2a200; Lane 3: VP7/VP2a238; Lane 4: DNA extracted from mock infection.

Analysis of VP7 gene expression using Reverse Transcription (RT-PCR)

The functionality of the vaccinia P7.5 expression cassette present in the recombinant MVAs was evaluated by RT-PCR using RNA templates extracted from uninfected, MVA VP7 or MVA VP7/VP2a infected DF-1 cells at 24 hours post-infection. PCR products of the expected size were detected in each case (VP7: 1050bp, modified VP7/VP2a: 1125bp) demonstrating the synthesis of RNA transcripts from each of the constructs (Figure 5.14). To ensure that the PCR products observed represented amplification of RNA and not residual DNA in the RNA preparation, extracted RNA was pre-treated with DNase. The absence of a band by DNA PCR, after DNase treatment, confirmed the amplification of RNA by RT-PCR (Figure 5.14).

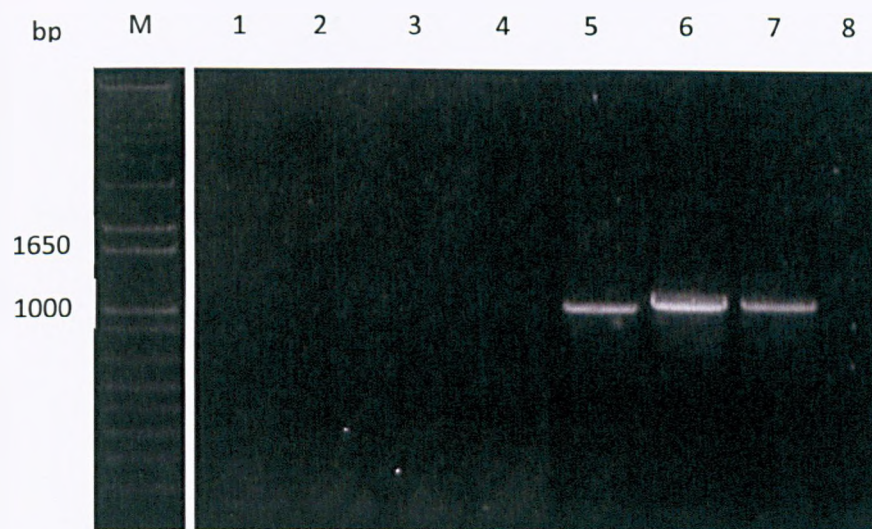


Figure 5.14: Detection of modified VP7/VP2 construct expression by recombinant MVA using RT-PCR.

AGE analyses showing the RT-PCR and DNA PCR products using RNA templates extracted from DF-1 cells infected with MVA VP7 or MVA VP7/VP2a, harvested at 24 hours post infection. M (molecular marker): TrackIt™ 1 Kb Plus DNA Ladder. Lanes 1-4 are products from DNA PCR post DNAase treatment. Lane 1: VP7; Lane 2: VP7/VP2a200; Lane 3: VP7/VP2a238; Lane 4: RNA extracted from mock infection. Lanes 5-8 are products from RT-PCR post DNAase treatment. Lane 5: VP7; Lane 6: VP7/VP2a200; Lane 7: VP7/VP2a238; Lane 8: RNA extracted from mock infection.

Analysis of modified VP7 protein expression by Western blot

Expression of modified VP7 constructs was evaluated by Western blot, probing the infected cellular lysates with anti-AHSV-9 or anti-VP2a peptide antiserum.

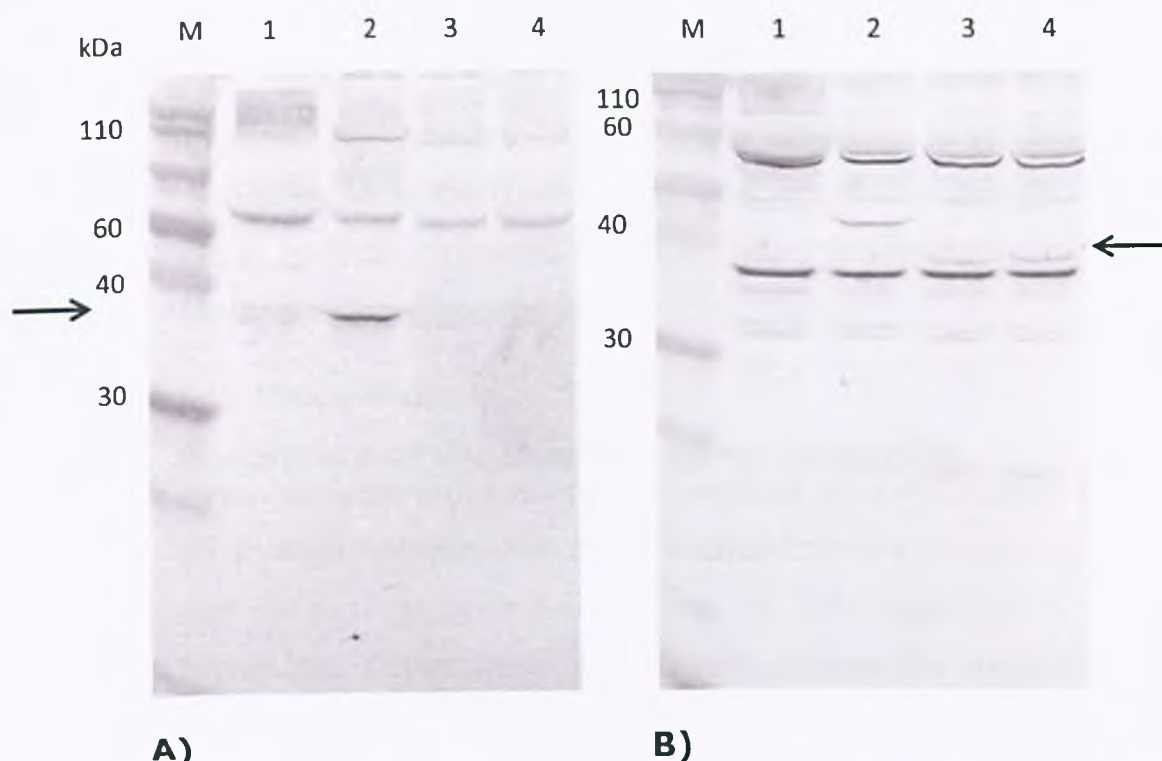


Figure 5.15: Detection of modified VP7 proteins in MVA expressing modified VP7/VP2a cell lysates by Western blot.

DF-1 cells were infected at MOI 1, harvested 24 hours post-infection and lysed with 0.25% NP40. The cellular lysates were separated by SDS PAGE and probed using anti-AHSV-9 (Image A) and anti-VP2a peptide (Image B) antiserum. Image A) M (protein marker): Novex® Sharp Protein Standard; Lane 1: Mock infection; Lane 2: Wild-type VP7, Lane 3: VP7/VP2a200; Lane 4: VP7/VP2a238. The black arrow depicts the molecular weight of VP7. Image B) M (protein marker): Novex® Sharp Protein Standard; Lane 1: Mock infection; Lane 2: Wild-type VP7, Lane 3: VP7/VP2a200; Lane 4: VP7/VP2a238. The black arrow indicates the bands corresponding to the modified VP7/VP2a constructs.

Neither of the MVA expressed modified VP7 protein constructs (VP7/VP2a200 and VP7/VP2a238) were detected using anti-AHSV-9 antiserum (Figure 5.15, Image A). In contrast, MVA expressed wild-type VP7 served as a positive control and was easily detected at the expected size of ~37kDa. When the membrane was probed with anti-VP2a peptide antiserum, a faint band was observed for both of the constructs at a slightly

lower molecular weight than predicted as shown in Figure 5.15, Image B. A band of this size was absent from the mock-infected cell lysates or the cells infected with MVA VP7, indicating that the band observed might be specific to the VP2a peptide, suggesting that the recombinant VP7/VP2a constructs were expressed.

5.3. Discussion

Generation of the modified gene constructs

When considering a particular antigen as a molecular scaffold for foreign antigen/epitope presentation, the position at which the foreign peptide/antigen is to be inserted is clearly important. It has been well described that the position of the foreign insert can influence the structure and interactions of the recipient construct, which could ultimately affect the overall immunogenicity of both the carrier protein and target peptide.

In considering the VP7 crystals as a molecular scaffold, it was first necessary to identify candidate sites that could accommodate insertion of a foreign peptide, without compromising the immunogenicity and ability of the modified VP7s to form crystals. Amino acid positions were selected in the external loops of the top domain of AHSV-4 VP7, for the insertion of foreign epitopes, based on the molecular structure of the protein.

Only residues in the top domain of VP7 were selected because firstly, only the top domain has been resolved at an atomic level (due to the insolubility of protein), which facilitates identification of candidate sites on the surface of the trimer head. Secondly, the top domain consists of the central portion of the VP7 polypeptide chain, 120-250 amino acids, and is

more hydrophilic than the bottom domain (N and C terminus), possessing alternating hydrophilic and hydrophobic stretches (Maree, 2000). Hydrophilic areas are typically a preferred location for foreign insertion because they are normally located on outer loop structures, on the external surface of the presenting antigen and are easily accessible to the aqueous environment and therefore presented optimally for B-cell activation (Barcena, 2013).

The AHSV-4 VP2 amino acid stretches that were chosen contained neutralising VP2 epitopes - sites 'a' and 'b' previously defined using Pepscan analysis by Martinez-Torrecuadrada and co-workers (2001). A hydrophobicity plot of AHSV-4 VP2 indicates that these defined sites are also in the most hydrophilic regions of the VP2 protein (Martinez-Torrecuadrada *et al.*, 2001). This suggests that after insertion of these peptides into external loops, they are more likely to be displayed efficiently and are less likely to disrupt the structure of VP7, in comparison to peptides of a hydrophobic nature.

Karpenko *et al.* 2000 describes how the insertion of amino acids with high hydrophobicity results in the inhibition of hepatitis core (HBcAg) particle assembly. The size of the insert can also influence whether or not the recipient molecule will tolerate an insertion. The sizes of VP2a/b peptides are within and around the peptide limits suggested for chimeric VLP particle generation (20-30 amino acids). Although this size range is not an absolute rule, insertions of >30 amino acids have been successfully incorporated into chimeric VLPs (Kratz *et al.*, 1999).

Expression and characterisation of modified VP7 expressed by recombinant baculovirus

The results presented here show that the generation of recombinant baculoviruses expressing a modified AHSV VP7 was successful. FB-VP7/VP2a141, FB-VP7/BAP141, FB-VP7/VP2a200 and FB-VP7/VP2a238 (recombinant baculoviruses) were successfully produced. Difficulties were experienced attempting to clone gene constructs VP7/VP2a144 and VP7/VP2a164 into the pVL1393 transfer vector. In every attempt stop codons presented in the open reading frame. A reason for this could be due to leaky expression of a toxic protein in the *E. coli*.

Western blot verified the expression of all of the modified VP7 proteins. However, there were mild discrepancies in the migration pattern of the constructs. Firstly, the chimeric protein constructs migrated slightly faster than predicted. Secondly, protein construct VP7/VP2a141 migrated unexpectedly slower than constructs VP7/VP2a200 and VP7/VP2a238. The migration rate of protein constructs by SDS PAGE does not show an exact correlation with their molecular weight and it is difficult to determine their size especially when using pre-stained protein standards that only offers an indication of an apparent molecular weight.

The addition of the dye carrying molecules may affect the overall charge of the standard proteins, potentially modifying their migration rate. It is also possible that because the infected cell lysates contain a huge amount of 'extra' cellular proteins, the proteins of interest (modified VP7s) may not have been fully denatured which would have ultimately affected the migration pattern. Equally, comparative SDS

PAGE assumes that each protein has adopted the equivalent shape after SDS treatment, although the amount of SDS that binds to a protein can vary from protein to protein. This might explain why construct VP7/VP2a141 migrated more slowly in comparison to the other constructs. It is important to recognise that this construct (plus VP7/BAP141) is generated from VP7 gene sequence I (Appendix II, Figure A2.1) that contained two amino acid differences (aa 57, Gly to Glu; aa 119, Cys to Glu) in comparison to VP7 gene sequence II (Appendix II, Figure A2.2) used in the control (VP7) and used to generate VP7/VP2a200 and VP7/VP2a238. These amino acid changes could have altered the residual conformation of the protein after treatment with SDS.

The ability of the modified VP7 proteins to form crystals was confirmed by immunofluorescence imaging and light microscopy. The results show that formation of the modified VP7 crystals was affected by the insertion of foreign peptides at VP7 amino acids residue sites 141 and 238. However, insertion of the peptide VP2a at VP7 amino acid position 200 had no marked effect on the ability for expressed VP7/VP2a200 to aggregate and form crystalline structures. This result is comparable with recombinant wild-type VP7 and re-confirms the findings described by Rutkowska and co-workers (2011) whereby insertion of a peptide at position 200 maintained crystal formation and did not affect solubility. Although the results indicate that the insertion of peptides at VP7 amino acid 141 does affect the ability for the modified VP7 molecule to form crystals, it is important to recognise that the 141 based constructs were generated on a different VP7 backbone. It will be important to express wild-type VP7 gene sequence I (Appendix II, Figure A2.1), that was utilised to

generate the VP7/BAP141 and VP7/VP2a141 constructs, and see if VP7 crystals are generated. This would determine if the inability for constructs VP7/VP2a141 or VP7/BAP141 to form crystals was associated with the peptide insert, or was a fundamental result of the amino acid changes in the VP7 template.

It is not completely unexpected that peptide insertion in the top domain of VP7 at specific sites can affect the ability for modified VP7 to form crystals. Although it was not investigated, insertion of peptides at position 141 and 238 may have affected the solubility of the constructs, especially since Monastyrskaya and co-workers (1997) described how the top domain of AHSV VP7 contributed to the insolubility of VP7. Insertions at specific sites may have influenced a change in the solubility of the construct. Rutkowska *et al.* 2011 described how insertion of foreign peptides at VP7 amino acid positions 144 and 177 increased the solubility of the protein in comparison with wild-type crystals.

It has previously been described that AHSV VP7 trimers comprise the insoluble VP7 crystals particles that are synthesised during AHSV replication (Burroughs *et al.*, 1994). Insertion at residue positions 141 and 238 may have disrupted the correct folding, assembly and ultimately trimer formation of VP7. Interestingly, insert position 200 is situated on the top of the VP7 head domain, in relatively close proximity to the two other molecules that would comprise a trimer. This construct might therefore be considered more likely to be affected by space constraints. However, VP7/VP2a200 was the only construct that formed modified VP7 crystal particles. Rutkowska and co-workers (2001) describes the instability of chimeric VP7 (with a small insertion at position 200), in

comparison to wild-type VP7, which might reflect a weakened monomer/monomer interaction within the VP7 trimers. Although intra and inter trimer interactions are likely to play an important part in crystal formation, they are unlikely to be the only factors involved in particle assembly. Monastyrskaya and co-workers (1997) describe how trimerisation of chimeric VP7 was not by itself a pre-requisite for CLP formation (Monastyrskaya *et al.*, 1997).

As well as visualisation by light microscopy, immunofluorescence imaging was used to determine whether modified VP7 constructs formed crystalline structures. Unexpectedly, expression of VP7/VP2a238, VP7/VP2a141 and VP7/BAP141 detected by immunofluorescence appeared to be impeded, in comparison to expression detected by Western blot. More VP7/VP2a200 expression was also observed despite using the same MOI for recombinant baculovirus infections. The Sf9 insect cells were clearly infected with recombinant baculoviruses FB-VP7/VP2a238, FB-VP7/VP2a141 and FB-VP7/BAP141, as demonstrated by the detection of characteristic enlarged cells, with a grainy appearance and a large distinct nucleus. Detection of high expression VP7 levels by western blotting of FB-VP7/VP2a238, FB-VP7/VP2a141 and FB-VP7/BAP141 infected cell lysates suggests that the reduced detection efficiency by immunofluorescence is unlikely to be associated with a reduced level of overall expression. It is possible that the reduced detection of some of the modified VP7 proteins or crystals was due to a reduced antigenic reactivity and modified protein structure for these specific constructs.

Expression and characterisation of modified VP7 expressed by recombinant live viral vector MVA

The results presented in this chapter show that the strategy used for the generation of recombinant MVA viruses containing modified VP7 genes was at least a partial success. The use of PSCII containing VP7/VP2a200 and VP7/VP2a238 allowed homologous recombination to occur facilitating the generation of MVA-VP7/VP2a200 and MVA-VP7/VP2a238, which were successfully plaque purified. However, protein expression for neither VP7/VP2a200 nor VP7/VP2a238 could not be confirmed by Western blot using polyclonal anti-AHSV-9 antiserum. A faint signal was detected using anti-VP2a peptide antiserum, indicating that protein expression had occurred, although the band was present at a smaller than predicted molecular weight. However, this concurs with results obtained by modified VP7 expressed using recombinant baculovirus. It was also observed by Western blot, that detection of modified VP7 from infected insect cell lysates resulted in the presence of a strong band that migrated slightly faster (smaller) than expected.

The lack/minimal level of protein expression detected could be due to instability of the modified protein; it is possible that the protein constructs were degraded, resulting in a much quicker turnover of protein than that observed by the control VP7. Expression from the constructs was driven using the same promoter as the MVA expressing the wild-type VP7 control, so it is unlikely that it is a timing issue. However, harvesting the infection earlier (for example 6-8 h) might have resulted in the detection of the proteins.

In conclusion, the work generated in this chapter demonstrates that insertion of the (AHSV VP2 VP2a) peptide into VP7 at amino acid position 200 does not affect the ability

of the VP7/VP2a200 antigen to assemble and form crystals. The availability of purified VP7/VP2a200 crystals provides a valuable resource for investigating the use of VP7 crystals as a vaccine delivery platform for the AHSV VP2a peptide (Chapter 6).

Chapter 6: The immunogenicity of carrier proteins and molecular assemblies displaying AHSV-4 peptide VP2a.

6.1. Introduction

The use of a carrier molecule / antigen to display and enhance the immunogenicity of an antigen / peptide attached or incorporated into its structure has been well described (Clarke *et al.*, 1987; Fehr *et al.*, 1998; Kaumaya *et al.*, 1992; Whitacre *et al.*, 2009). Small compounds such as peptides are often weak immunogens, most likely as a result of their small molecular weight and lack of structural complexity. However, B-cells can be efficiently activated and high titre peptide specific antibodies can be generated, by presentation in association with carrier antigens, such as VLPs (Middelberg *et al.*, 2011), capsomeres (Wibowo *et al.*, 2014), carrier molecules such as KLH (Coligan, 1996; Lateef *et al.*, 2007) and even soluble AHSV VP7 (Rutkowska *et al.*, 2011). Although there are some exceptions (e.g. some VLPs (Middelberg *et al.*, 2011), non-live vaccines are often administered with adjuvant, in order to stimulate an enhanced immune response. Aluminium salts have been widely used as an adjuvant, with great success, stimulating powerful antibody responses by inducing a pre-dominant type-2 inflammatory response (Brewer *et al.*, 1999; Sokolovska *et al.*, 2007).

The aim of this chapter was to characterise the immunogenicity of VP7/VP2a200 crystals (generated in Chapter 5) in the mouse model by analysing their ability to induce a VP7 and VP2a-peptide specific antibody response. The immunogenicity of urea treated VP7/VP2a crystals was also examined.

The AHSV-4 VP2a peptide was also 'delivered' to the murine immune system using murine polyomavirus (MPV) VP1 VLPs, MPV VP1 capsomeres, or keyhole limpet haemocyanin (KLH) carrier proteins, to explore their ability to generate an insert specific response and possible use as vaccine platforms.

6.2. Results

6.2.1 Production of vaccine platform candidates

Murine polyomavirus (MPV) VP1 based VLPs and MPV capsomeres expressing the AHSV-4 VP2a peptide (KEEGEDDTARQEIRKAWVK) were generated and provided by Dr Linda Lua, Protein Expression Facility (The University of Queensland, Australia). The MPV capsomere carrier (capsomere/VP2a), containing 3 copies of the peptide per VP1 molecule, were engineered to form capsomeres containing a total of 15 peptides. The MPV VP1 VLP carrier (VLP/VP2a), containing just two copies of the peptide per VP1 molecule, were engineered to form VLPs that consist of 72 capsomeres, containing a total of 720 peptides per particle. The AHSV-4 VP2a peptide was conjugated randomly at different sites on the KLH carrier (KLH/VP2a) (provided by Genscript) using primary amines. There are over 2500 amines on each KLH molecule.

The VP7/VP2a200 crystals used for these experiments were generated as described in Chapter 5.

6.2.2 Characterisation of 'vaccine platform' candidates *in vitro*

The ability of these potential 'vaccine platform' constructs to be recognised by VP2a peptide antiserum was tested *in vitro*, prior to vaccination studies. A Western blot was conducted, using the VLP/VP2a, capsomere/VP2a and VP7/VP2a200 crystal constructs as antigens (alongside wild-type VP7-as a negative control antigen), probing with VP2a peptide antiserum. All three constructs reacted well with the polyclonal anti-VP2a peptide antiserum (Figure 6.1), generating clear bands corresponding to the expected sizes of the modified VLPs, capsomeres and VP7 crystal constructs (48.2kDa, 40.3kDa and 40.6 kDa respectively). Several additional bands were observed on the blots that are probably due to non-specific reactivity of the polyclonal antiserum with other cellular proteins in the preparations. A band corresponding to the size of each construct was absent in the negative control. The results confirmed that the peptide was successfully incorporated into the VP7/VP2a200 crystals and the murine polyomavirus VP1 VLPs and capsomeres (VLP/VP2a and capsomere/VP2a respectively).

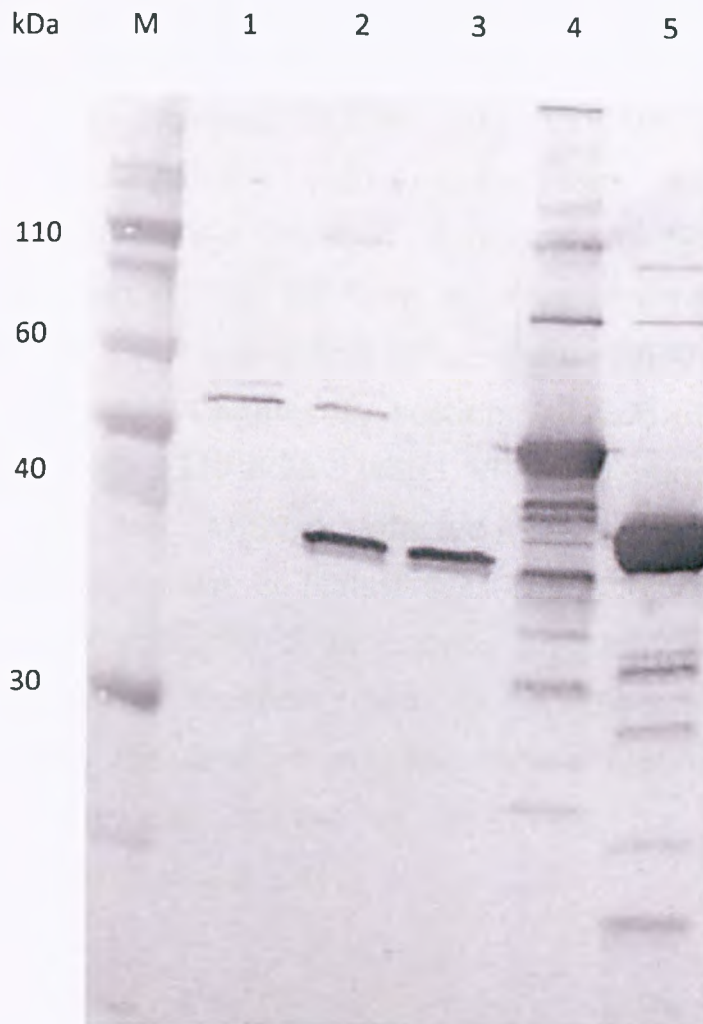


Figure 6.1: Detection by Western blot of AHSV-4 VP2a peptide in VP7/VP2a200 crystals, and in murine polyomavirus VP1 based VLPs and capsomeres expressing AHSV-4 VP2a.

AHSV-4 VP7/VP2a200 crystals and murine polyomavirus VP1 VLPs and capsomeres expressing AHSV-4 VP2a peptide were analysed by SDS page and Western blot using anti-VP2a peptide antiserum. M: Protein marker Novex® Sharp Protein Standard; Lane 1: Wild-type VP7 crystals; Lane 2: VP7/VP2a200 crystals (batch 1), Lane 3: VP7/VP2a200 crystals (batch 2); Lane 4: Murine polyomavirus VP1 VLP expressing AHSV-4 VP2a; Lane 5: Murine polyomavirus VP1 capsomeres expressing AHSV-4 VP2a.

The polyclonal anti-VP2a peptide antiserum used to confirm the antigenic reactivity of VP7/VP2a200 crystals and murine

polyomavirus (MPV) VP1 VLPs and capsomeres expressing VP2a (Figure 6.1) was raised against KLH bound VP2a. The antigenic reactivity of KLH/VP2a (used for vaccination) was confirmed by indirect ELISA using KLH/VP2a as coating antigen and probing with murine sera raised against experimental AHSV-4 infection (Appendix III, Table A3.1) and antisera taken on day 56 from mice vaccinated three times with MPV VP1 VLP expressing VP2a peptide (VLP/VP2a) (15µg) (as described in Chapter 2, section 2.7.1.7). The antigenic reactivity of KLH/VP2a with VP2a peptide antiserum (VLP/VP2a) and AHSV-4 infected mouse antiserum was confirmed as shown in Figure 6.2. The KLH/VP2a construct reacted well with the VP2a peptide antiserum. Additionally, there was a mild reaction (mean OD 0.375) with the coating antigen and the AHSV-4 infected mouse serum. The ELISA cut-off value was defined as 0.157 OD (as described in Chapter 2, section 2.7.6.4)

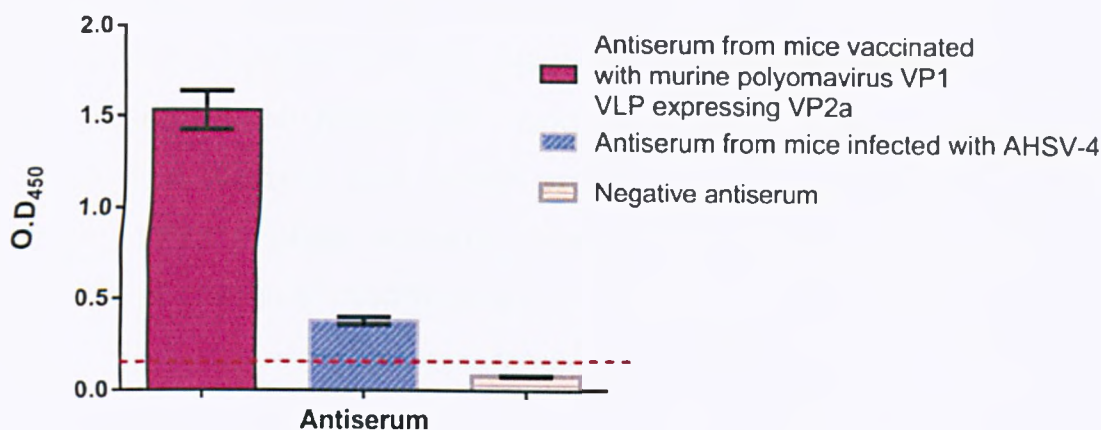


Figure 6.2: Detection of VP2a peptide specific antibodies using an indirect ELISA.

Serum samples taken from mice experimentally infected with AHSV-4 (Appendix III, Table A3.1) and from A129 mice (day 56) vaccinated three times with murine polyomavirus VP1 VLP expressing VP2a (15µg) (as described in Chapter 2, section 2.7.1.7) were screened using an indirect VP2a based ELISA with KLH/VP2a as coating antigen. The negative control serum was obtained from non-vaccinated A129 mice. The red line indicates the ELISA positive cut-off point (twice the mean OD value for the negative controls).

6.2.2 Treating VP7/VP2a200 crystals with 4M urea

Chapter 3, section 3.2.5 showed that urea treatment was able to solubilise wild-type VP7 crystals. Additionally, vaccination with urea treated VP7 crystals improved the generation of VP7 specific antibodies in the A129 mouse model (Chapter 4, section 4.2.7). It was therefore expected that treatment of VP7/VP2a200 crystals, would also increase their solubility, in a manner that was directly proportional to the urea concentration.

Since the 4M urea treated VP7/VP2a200 crystals were to be used for vaccination, it was important to check that the antigenic reactivity of 4M urea solubilised VP7/VP2a200 (at least for the denatured protein after Western blot) with polyclonal anti-AHSV-9 and anti-VP2a antisera had not been compromised in comparison with untreated VP7/VP2a200.

Figure 6.3, shows that after 4M urea treatment the VP7/VP2a200 protein antigen reacted in a similar manner to the untreated VP7/VP2a200 antigen (with similar band intensity) in Western blot, when probed with the anti-AHSV-9 and anti-VP2a peptide antisera, suggesting that there was no evidence of chemical modification.

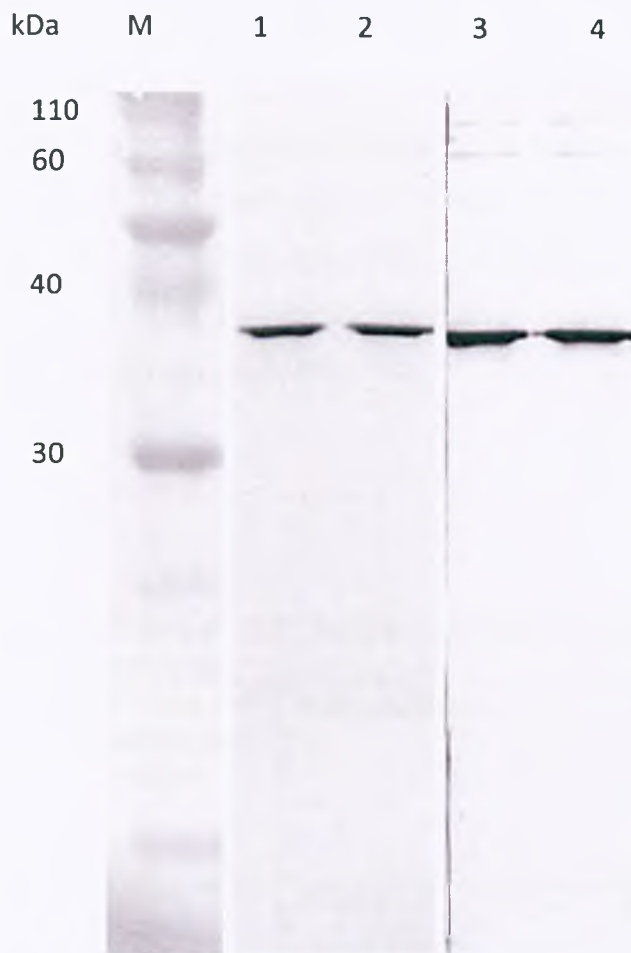


Figure 6.3: Detection of VP7/VP2a200 crystals before and after 4M urea treatment by Western blot.

Purified VP7/VP2a crystals were solubilised with 4M urea for one hour at room temperature. They were separated by SDS PAGE, alongside untreated VP7/VP2a200 crystals, then analysed by Western blot using polyclonal anti-AHSV-9 antiserum (Panel A) and anti-VP2a peptide antiserum (Panel B): M (Protein marker) Novex® Sharp Protein Standards. Panel A) Lane 1: VP7/VP2a200 crystals; Lane 2, Urea (4M) solubilised VP7/VP2a200 crystals. Panel B) Lane 3: VP7/VP2a200 crystals; Lane 4, Urea (4M) solubilised VP7/VP2a200 crystals.

6.2.3 The immunogenicity of delivery platforms expressing VP2a in A129 mice

This experiment was designed to investigate the effects that the size and structure of the different molecular assemblies, that were used as delivery platforms to display the AHSV-VP2a peptide, had on their immunogenicity in A129 mice. Twelve

groups of three A129 mice were vaccinated by intra-peritoneal injection on days 0, 21 and 42 as described in Table 6.1. Whole blood was collected and the sera that were obtained on day 35 and 56 (as described in Chapter 2, section 2.7.1.7) were analysed for VP2a-specific and VP7-specific antibodies using the OVA/VP2a based indirect ELISA and the INGENASA AHSV VP7 ELISA respectively.

Table 6.1: Groups of mice (A219) vaccinated with chimeric constructs

Group number (Mouse number)	Route of administration	Vaccination	
		Prime (Day 0)	Boost (Days 21, 42)
Group 1 (1.1-1.3)	IP (200µl)	5µg VP7/VP2a200 crystals with adjuvant*	5µg VP7/VP2a200 crystals with adjuvant*
Group 2 (2.1-2.3)		5µg of 4M urea solubilised VP7/VP2a200 crystals	5µg of 4M urea solubilised VP7/VP2a200 crystals
Group 3 (3.1-3.3)		5µg of 4M urea solubilised VP7/VP2a200 crystals with adjuvant*	5µg of 4M urea solubilised VP7/VP2a200 crystals with adjuvant*
Group 4 (4.1-4.3)		15µg polyomavirus VLP/VP2a with adjuvant*	15µg polyomavirus VLP/VP2a with adjuvant*
Group 5 (5.1-5.3)		5µg polyomavirus VLP/VP2a with adjuvant*	5µg polyomavirus VLP/VP2a with adjuvant*

Group 6 (6.1-6.3)		1µg polyomavirus VLP/VP2a with adjuvant*	1µg polyomavirus VLP/VP2a with adjuvant*
Group 7 (7.1-7.3)		15µg polyomavirus capsomere/VP2a with adjuvant*	15µg polyomavirus capsomere/VP2a with adjuvant*
Group 8 (8.1-8.2)		5µg polyomavirus capsomere/VP2a with adjuvant*	5µg polyomavirus capsomere/VP2a with adjuvant*
Group 9 (9.1-9.3)		1 µg polyomavirus capsomere/VP2a with adjuvant*	1 µg polyomavirus capsomere/VP2a with adjuvant*
Group 10 (10.1-10.3)		15µg KLH/VP2a with adjuvant*	15µg KLH/VP2a with adjuvant*
Group 11 (11.1-11.3)		5µg KLH/VP2a with adjuvant*	5µg KLH/VP2a with adjuvant*
Group 12 (12.1-12.3)		1 µg KLH/VP2a with adjuvant*	1 µg KLH/VP2a with adjuvant*

(*Administered with Imject Alum)

VP7 antibody analysis

The effects of the addition of VP2a peptide, or treatment with urea (used to solubilise VP7 crystals) on the VP7 crystal carriers, their immunogenicity and ability to generate an effective VP7 specific antibody response was assessed in the A129 mouse model (Figure 6.4,6.5 and 6.6). On day 35 (Figure 6.4), antibodies to VP7 were detected in Groups 1 and 3 (vaccinated with adjuvant, with untreated or 4M urea treated crystals of VP7/VP2a200 respectively) giving a mean blocking percentage > 50% (60.58 and 51.29% respectively). No VP7 antibodies were detected on day 35, in any of the mice vaccinated in Group 2 (vaccinated with 4M urea treated

crystals of VP7/VP2a200 – no adjuvant) giving a mean blocking percentage of 33.72%.

However, after a third vaccination, seroconversion was detected by ELISA in Group 2 (mean blocking percentage of 62.04% on day 56). At this stage, Groups 1, 2 and 3 had all developed similar mean blocking percentages (67.47, 62.04, and 64.35% respectively), although Group 1 was marginally higher than Groups 2 and 3 (Figure 6.5).

End-point titrations on day 56 (Figure 6.6) indicated a higher VP7 specific antibody titre in Group 1 (at 1/25) than in Group 2 (1/5) or Group 3 (1/5). Although at a dilution of 1/25, Group 3 was considered inconclusive in relation to the 50% positive ELISA cut off value. The end point was the last dilution at which the ELISA was considered positive (blocking percentage >50%).

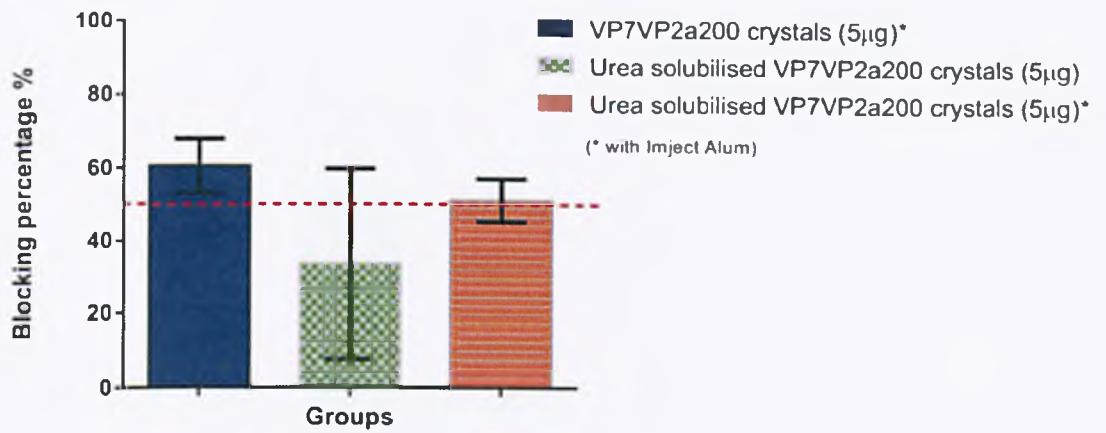


Figure 6.4: Generation of VP7 specific antibodies against VP7/VP2a200 in A129 mice on day 35 post-vaccination.

Serum samples taken on day 35 post-vaccination from mice vaccinated twice (see Chapter 2, section 2.7.1.7) with 5µg VP7/VP2a200 crystals (with Imject Alum) and 5µg urea solubilised VP7/VP2a200 crystals (with and without Imject Alum) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage).

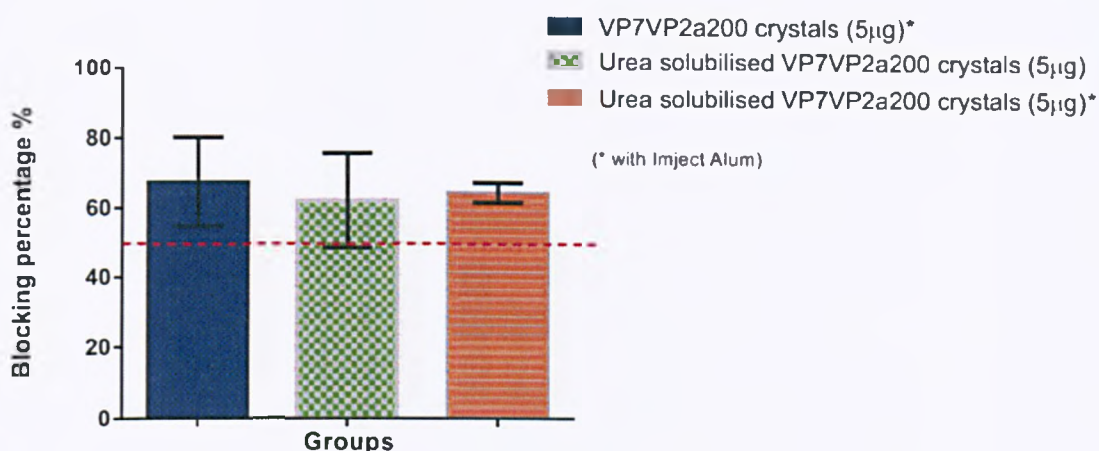


Figure 6.5: Generation of VP7 specific antibodies against VP7/VP2a200 in A129 mice on day 56 post-vaccination.

Serum samples taken on day 56 post-vaccination from mice vaccinated three times (see Chapter 2, section 2.7.1.7) with 5µg VP7/VP2a200 crystals (with Imject Alum) and 5µg urea solubilised VP7/VP2a200 crystals (with and without Imject Alum) were analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage).

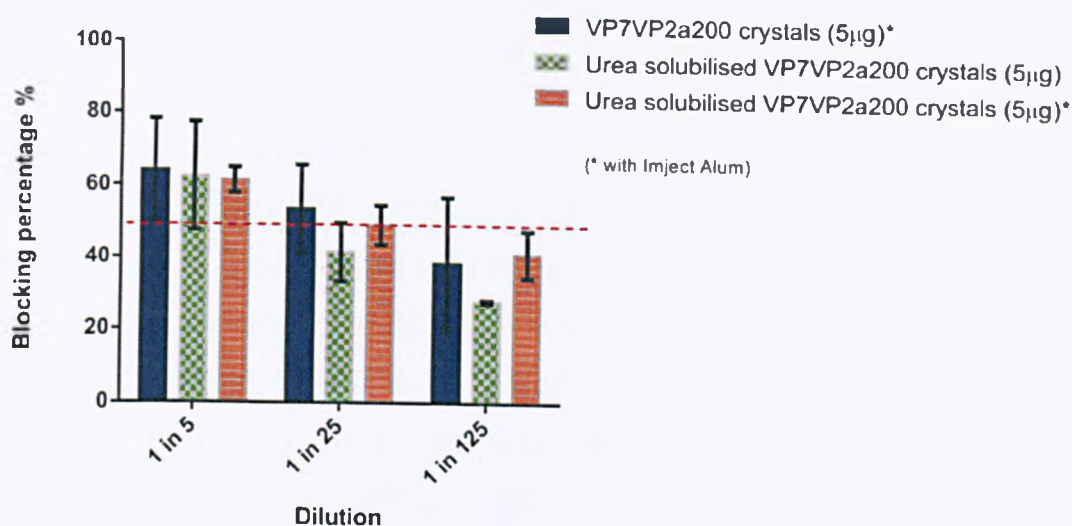


Figure 6.6: Dilution of VP7 specific antibodies raised against VP7/VP2a200 in A129 mice on day 56 post-vaccination.

Serum samples taken on day 56 from mice vaccinated three times (as described in Chapter 2, section 2.7.1.7) with 5µg VP7/VP2a200 crystals (with Imject Alum) and 5µg urea solubilised VP7/VP2a200 crystals (with and without Imject Alum) were diluted fivefold in diluent provided by INGENASA. The sera were then analysed for VP7 specific antibodies using the INGENASA AHSV VP7 ELISA. The red dotted line depicts the positive cut-off point (50% blocking percentage).

VP2a peptide specific antibody analysis

By day 35 all of the groups, except for Group 9 (vaccinated with 1 µg polyomavirus capsomere/VP2a), tested positive for VP2a-peptide specific antibodies (OD > 0.109) (Figure 6.7). The ELISA cut-off value (0.109 OD) was twice the mean of the OD value for negative sera.

Comparisons of the antisera raised against the VP7 based constructs on day 35, showed that Groups 1 and 3 (with adjuvant) (1.780, 1.540 OD respectively) had an OD value greater than Group 2 (solubilised VP7/VP2a200 crystals (without adjuvant) - 0.608 OD) (Figure 6.7). On day 56, Group 1 had a marginally higher mean OD value (2.000) than

Group 3 (1.798 OD), but both were much greater than Group 2 (0.463 OD) (Figure 6.8).

Comparisons on day 35 of the antisera raised against the polyomavirus VLP/VP2a constructs, showed a dose dependant response, with Groups 4 and 5 (15µg or 5µg of VLP/VP2a, with adjuvant) showing greater OD values (1.956, 2.064 OD respectively)than Group 6 (1 µg of VLP/VP2a - 1.065 OD) (Figure 6.7). By day 56 all of these groups gave consistently high mean OD value (2.210, 2.290, 2.100 OD respectively) (Figure 6.8).

Comparisons on day 35 of the antisera raised against the polyomavirus capsomere/VP2a constructs, also showed a dose dependant response. Group 8 (5µg polyomavirus capsomere/VP2a with adjuvant) gave a greater OD value (1.890 OD) than Group 7 (15µg polyomavirus capsomere/VP2a with adjuvant - 1.160 OD). Group 9 showed only a very low response on day 35 (vaccinated with 1µg polyomavirus capsomere/VP2a with adjuvant - 0.090 OD) (Figure 6.7). However, by day 56, the OD value of Groups 7 and 8 (1.463, 1.954 OD respectively) was much greater than Group 9 (0.185 OD), which was only considered marginally positive (>0.128 OD) (Figure 6.8).

Comparisons on day 35 of the antisera raised against the KLH/VP2a constructs, showed that each of the groups (Groups 10, 11, 12 - vaccinated with 15µg, 5µg, or 1µg, with adjuvant) had consistently high mean OD values (2.053, 1.974, 1.948 OD respectively) (Figure 6.7). By day 56, as observed for the VLP/VP2a constructs, the mean OD values for Groups 10, 11 and 12 were consistently high (1.974, 2.107, 2.043 respectively) (Figure 6.8).

The two groups with the greatest mean OD value (>2.000 OD) on day 35 were Groups 5 and 10 (vaccinated with $5\mu\text{g}$ polyomavirus VLP/VP2a with adjuvant, or $15\mu\text{g}$ KLH-VP2a with adjuvant respectively) (Figure 6.7). By day 56, the groups with the high OD values (>2.000 OD) were: Groups 1 ($5\mu\text{g}$ VP7VP2a crystals with adjuvant - 2.000 OD); Group 4 ($15\mu\text{g}$ polyomavirus VLP/VP2a with adjuvant - 2.210 OD); Group 5 ($5\mu\text{g}$ polyomavirus VLP/VP2a with adjuvant - 2.290 OD); Group 6 ($1\mu\text{g}$ polyomavirus VLP/VP2a with adjuvant - 2.100 OD); Group 11 ($5\mu\text{g}$ KLH/VP2a with adjuvant - 2.107 OD); and Group 12 ($1\mu\text{g}$ KLH/VP2a with adjuvant - 2.043 OD) (Figure 6.8). This demonstrates that VP2a specific antibodies were generated in response to all of the antigen constructs in the presence of adjuvant. The response to 4M urea solubilised VP7/VP2a200 crystals in the absence of adjuvant was much reduced.

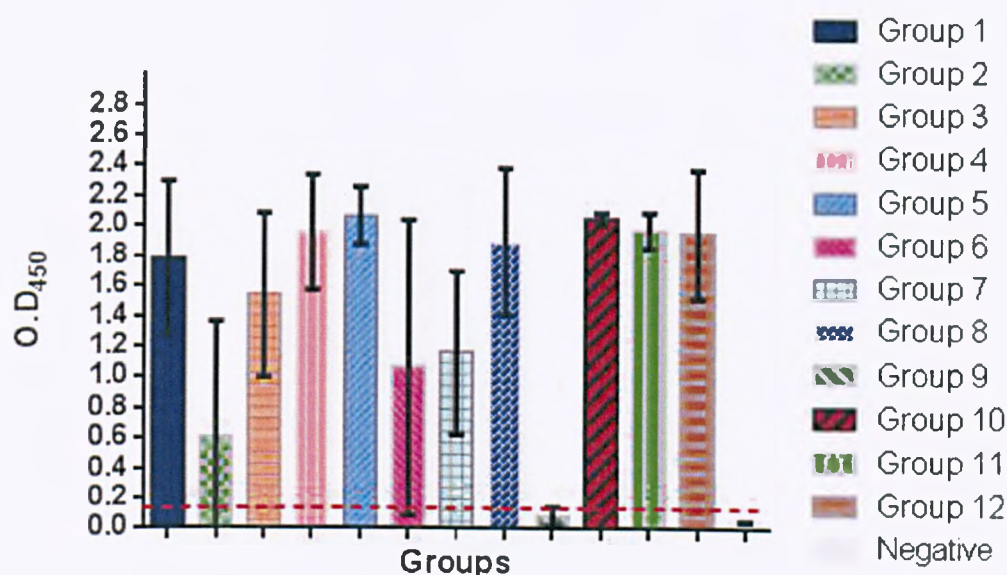


Figure 6.7: Detection of VP2a peptide specific antibodies in A129 mice on day 35 using an indirect ELISA.

Serum samples taken on D35 from mice vaccinated twice (as described in Chapter 2, section 2.7.1.7) with VP7/VP2a200 crystals (Groups 1, 2 & 3), murine polyomavirus VLPs expressing peptide VP2a (Groups 4, 5 & 6), murine polyomavirus capsomeres expressing peptide VP2a (Groups 7, 8 and 9) or KLH molecules conjugated to VP2a (Groups 10, 11 & 12) (see Table 6.1 for details of antigen amounts) were analysed for VP2a peptide specific antibodies using an OVA/VP2a based indirect ELISA. The red line indicates the ELISA positive cut-off point (twice the mean OD value for the negative controls). The negative control serum was obtained from non-vaccinated A129 mice.

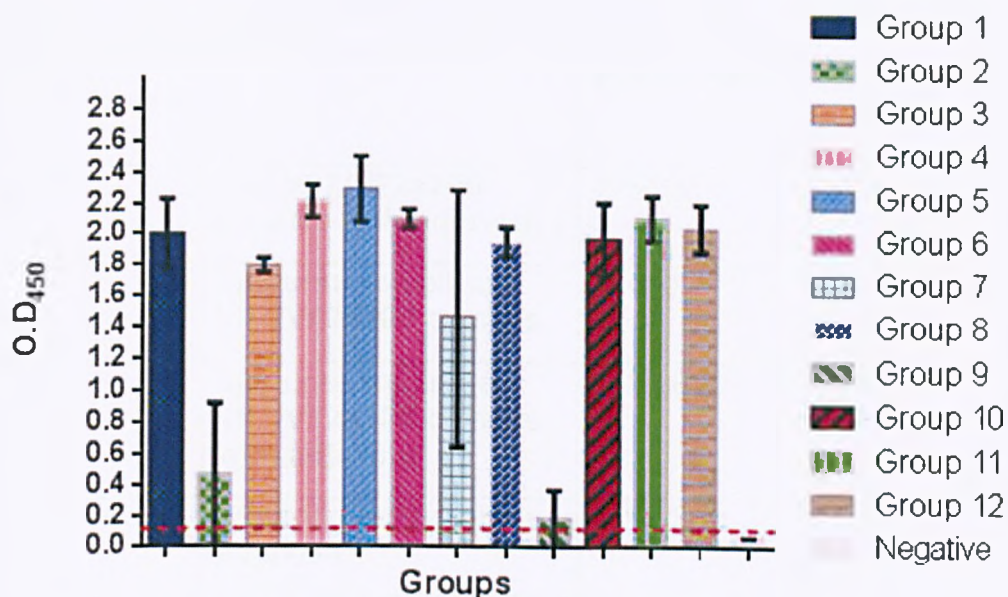


Figure 6.8: Detection of VP2a peptide specific antibodies in A129 mice on day 56 using an indirect ELISA.

Serum samples taken on D56 from mice vaccinated three times (as described in Chapter 2, section 2.7.1.7) with VP7/VP2a200 crystals (Groups 1, 2 &3), murine polyomavirus VLPs expressing peptide VP2a (Groups 4, 5 &6), murine polyomavirus capsomeres expressing peptide VP2a (Groups 7, 8 and 9) or KLH molecules conjugated to VP2a (Groups 10, 11 & 12) (see Table 6.1 for details of antigen amounts) were analysed for VP2a peptide specific antibodies using an OVA/VP2a based indirect ELISA. The red line indicates the ELISA positive cut-off point (twice the mean OD value for the negative controls). The negative control serum was obtained from non-vaccinated A129 mice.

In order to examine any differences within the groups in their responses to peptide VP2a, particularly those with a high OD values, an end-point ELISA was conducted using a twofold titration of the test sera. The ELISA utilised was the AHSV-4 peptide VP2a based indirect ELISA described in Chapter 2, section 2.7.6.4). Table 6.2 shows the ELISA titre and mean OD value for each group.

Table 6.2: End-point titration, ELISA values for vaccination groups

Groups		ELISA titre	Mean OD value
1	5µg VP7/VP2a200 crystals with adjuvant	1/6400	0.217
2	5µg urea solubilised VP7/VP2a200 crystals	1/400	0.196
3	5µg urea solubilised VP7/VP2a200 crystals with adjuvant	1/12,800	0.171
4	15µg VLP/VP2a with adjuvant	1/51,200	0.150
5	5µg VLP/VP2a with adjuvant	1/51,200	0.143
6	1µg VLP/VP2a with adjuvant	1/51,200	0.157
7	15µg capsomere/VP2a with adjuvant	1/12,800	0.237
8	5µg capsomere/VP2a with adjuvant	1/25,600	0.222
9	1µg capsomere/VP2a with adjuvant	1/200	0.195
10	15µg KLH/VP2a with adjuvant	Over 1/102,400	0.459 at 1/102,400
11	5µg KLH/VP2a with adjuvant	Over 1/102,400	0.469 at 1/102,400
12	1µg KLH/VP2a with adjuvant	Over 1/102,400	0.482 at 1/102,400

*ELISA cut-off 0.140 OD (twice the mean OD value of the negative controls).

Table 6.2 presents the ELISA titre and mean OD value for each group. The ELISA titre is defined as the end-point dilution before the ELISA value is considered negative. The results show that between the VP7 based constructs, Group 3 had the

highest end point ELISA titre (1/12,800) in comparison to Groups 1(1/6,400) and 2 (1/400). Between the VLP based constructs, irrespective of dose, the end point ELISA titre was consistent among the groups (1/51,200). Between the capsomere based constructs, there were differences between the groups. Group 8 (1/25,600) had the highest end point ELISA titre in comparison to Group 7 (1/12,800) and Group 9 (1/200). Between the KLH-based constructs, all the groups had a high endpoint ELISA titre ($> 1/102,400$). This titre was consistent among all three KLH-groups.

Virus neutralising (VNAb) antibodies

The capacity for the VP2a peptides to induce neutralising antibodies against AHSV-4 was assessed by plaque reduction neutralisation test (PRNT) (as described in Chapter 2, section 2.7.6.5). This assay was performed by Dr Eva Calvo-Pinilla. Due the limited availability of sera, only pooled sera from Groups 1, 3, 4, 7 and 10 were selected to test for neutralising antibodies. The results are shown in Table 6.3 and the values are presented as a plaque reduction percentage. Values over 50% were considered to be positive indicating the presence of neutralising antibodies. The results show that Group 3 (5 μ g of 4M urea solubilised VP7/VP2a200 crystals with adjuvant) and Group 7 (15 μ g polyomavirus capsomere/VP2a with adjuvant) showed a plaque reduction greater than 50% at dilution 1/5 and 1/10 respectively. Neutralisation was not evident in any other group. In conclusion, the results show that 4M urea solubilised VP7/VP2a200 crystals (with adjuvant) and capsomere/VP2a were capable of eliciting VNABs against the homologous AHSV-4, albeit at low levels.

Table 6.3: Plaque reduction percentages for vaccination Groups 1 , 3, 4, 7 and 10

Groups						
		Sera dilution				
		1/5	1/10	1/20	1/80	1/160
		Plaque reduction percentage %				
1	5µg VP7/ VP2a200 crystals with adjuvant	36.93	6.30	-16.21	-	--
		29.72	0	-12.61	-	-
3	5µg urea solubilised VP7/ VP2a200 crystals with adjuvant	83.78	27.02	-18.01	-	-
		84.68	33.33	*	-	-
4	15µg VLP/VP2a with adjuvant	6.30	-4.50	11.71	-	-
		*	*	*	-	-
7	15µg capsomere/ VP2a with adjuvant	82.88	61.26	-7.20	-	-
		83.78	*	-14.4	-	-
10	15µg KLH/VP2a with adjuvant	-0.90	-13.51	17.1	-	-
		7.20	-15.31	5.40	-	-
(+) control	Sera from mice infected with AHSV- 4 (Appendix III, Table A3.1)	-	-	-	98.19	94.59
		-	-	-	98.19	93.69

*- detached cells

6.3. Discussion

The use of a molecular assembly as a vaccine platform, to enhance the immunogenicity of otherwise poorly immunogenic antigens / peptides, has been widely explored (Fehr *et al.*, 1998; Middelberg *et al.*, 2011; Whitacre *et al.*, 2009; Wibowo *et al.*, 2014) and has provided a valuable alternative approach to vaccine development. In this chapter, the immunogenicity of VP7/VP2a200 crystals and different vaccine platforms (AHSV-VP7 crystals, murine polyomavirus VP1 VLP's or capsomeres and KLH carrier proteins) has been explored in relation to the generation of an insert (VP2a peptide) specific antibody response.

The results showed that all of the constructs were capable of delivering the VP2a peptide to the immune system, as demonstrated by the detection VP2a peptide specific antibodies. However, it is difficult to make a valid and direct comparison of the ability of each of these different constructs to induce an insert-specific antibody response. Firstly, the VLP and capsomeres were expressed in *E.coli* while the VP7/VP2a200 crystals were generated in a baculovirus / insect cell system. The proteins from the *E.coli* expression system may have been contaminated with endotoxins that could have an unintentional direct immunostimulatory effect on the immune system (Dobrovolskaia, 2013). Secondly, the molar and mass ratio of peptide to protein carrier was different between each of the constructs (VLP, capsomere, crystals and KLH). The vaccine dose was calculated according to total protein concentration instead of molar ratio. The experiments described here represent a pilot study, designed to confirm that each of the constructs, including the VP7VP2a200 crystals, were capable of generating a VP2a peptide specific

response. The primary aim of the study was to maximise the chances of success.

The results obtained indicated the total protein dose (for VLPs, capsomeres, and KLH carrier proteins) that was optimal to deliver the VP2a peptide to the immune system in the mouse model. The results showed that with both the VLPs and KLH constructs, there was little variation in the level of antibody production between different doses (1, 5, 15µg), with all three doses giving an end point titre of 1/51,200 (VLP/VP2a), or over 1/102,400 (KLH/VP2a). Even at the lowest dose (1µg per vaccination), the antibody response may have been saturated, so that the response was no longer enhanced as the antigen load increased. This was not completely unexpected as both VLPs and KLH are efficient B-cell response activators, generating a high yield antibody response.

VLPs (such as MPV VLPs) mimic the structural complexity and size of native virus particles, in the absence of replication (Liu *et al.*, 2012). At approximately 40nm in size, MPV VLPs are efficiently taken up by APCs. It has been documented that chimeric VLPs are capable of inducing powerful humoral immune responses against peptides presented on their surface, even in the absence of adjuvant (Middelberg *et al.*, 2011). Since VLPs are particulate in nature, they can induce T-cell dependent and T-cell independent antibody responses (Fehr *et al.*, 1998).

KLH is commonly also used as a carrier molecule for peptide presentation. KLH has been shown to induce high titres of peptide specific antibodies (Lateef *et al.*, 2007). The immunogenicity of KLH is most likely associated with its large

size and the high molar ratio between antigen and the attached peptide (Coligan, 1996).

There was a difference in the insert-specific antibody response generated by different capsomere doses: specifically, 1µg induced a low while 5µg achieved a higher antibody titre of insert-specific antibodies than 15µg. This suggests that the magnitude of the insert-specific antibody response is dose dependent. It is possible that 1µg was below the optimal antigen threshold necessary for the induction of a powerful antibody response. Typically, it is often the case that the magnitude of antibody response is directly related to the antigenic dose, until a plateau level is reached. Often, further increases in antigen dose above the optimum antigen threshold result in a decline in the generated antibody response (Murphy, 2008). This might explain why there was a reduced antibody titre after vaccinations with 15µg of capsomere antigen.

Unfortunately, due to the limited resources and lack of time, the crystals could only be administered at one dose (5µg). However, the results show that 5µg of VP7/VP2a200 crystals (including urea treated crystals, with and without adjuvant) were capable of inducing an insert-specific response, even with a molar ratio as low as 1:1 (VP7/VP2a200 molecule: peptide insert). This result supports the potential of the VP7/VP2a200 crystals as a vaccine delivery platform.

Unlike wild-type VP7 crystals, urea treatment of VP7/VP2a200 crystals did not enhance the VP7 specific antibody response. This may reflect a reduction in the stability of VP7/VP2a200 constructs, compared with the wild-type VP7. Rutkowska and co-workers (2011) reported that a chimeric VP7 construct,

with a foreign peptide inserted at position 200 was less stable than the wild-type VP7 crystals.

If the stability of the VP7/VP2a200 crystals was affected by the addition of the insert, it is possible that the urea treatment may have further disrupted the stability and potentially the structural integrity of VP7 epitopes. Due to limited resources and time restrictions, the effect of urea treatment on VP7/VP2a200 crystals was not fully investigated. For this pilot study, it was assumed that VP7/VP2a200 crystals would respond to the 4M-urea treatment in a similar manner to the wild-type VP7 crystals. The effect of urea treatment on VP7/VP2a200 crystals needs to be further explored in order to further explain the observed results.

However, in contrast to the results obtained for the VP7 specific response, the ability of urea treated crystals to deliver the VP2a peptide to the immune system was not compromised. The urea treated crystals (with adjuvant) induced a VP2a peptide-specific antibody response greater than the untreated VP7/VP2a200 crystals. This result concurs with the results of Rutkowska and co-workers (2011), which showed that the soluble chimeric VP7 antigen generated a better insert specific antibody response than the insoluble chimeric VP7.

To make sure that the differences observed in this study are significant, a repeat experiment should be conducted with a larger sample size allowing a more accurate and statistically valid comparison. Nevertheless, the results do show that the use of adjuvant promoted a greater VP2a specific antibody response for the urea treated crystals, compared with urea treated VP7/VP2a200 crystals administered without adjuvant.

The use of aluminium-based adjuvants to induce heightened immunogenicity is described in Chapter 4. Imject Alum is a mild adjuvant and is not considered to be as potent as some other aluminium based adjuvants (Cain *et al.*, 2013). However, the results indicate that it enhanced the antibody mediated immune responses and increased the concentration of antibodies generated against the target peptide. Even though VP7 specific antibodies were not generated in response to the wild-type VP7 crystals, in the absence of adjuvant (Chapter 4), it would be interesting to test if the VP7/VP2a200 crystals (no adjuvant) would generate a VP2a peptide specific response. As described earlier, if the addition of the insert does affect the stability of the crystals, the crystals may be more easily degraded after inoculation, which could facilitate the processing and the generation of an antibody response.

Martinez-Torrecuadrada and co-worker (2001) described the neutralising capacity of AHSV-4 VP2 fragment H (which contains the AHSV-4 VP2a peptide) and the capacity of antibodies generated against overlapping VP2a peptide fragments to induce neutralisation *in vitro*. The results in this chapter show that two constructs: 4M urea treated VP7/VP2a200 crystals (Group 3) and polyomavirus capsomere/VP2a (Group 7), were both capable of inducing neutralising antibodies, albeit at very low levels. Interestingly, neither the capsomeres nor the urea treated crystals were the constructs that induced the highest titre of VP2a peptide-specific antibodies. These results suggest that neutralisation is not directly dependent on antibody titre. However, it is difficult to draw further conclusions from this pilot experiment in terms of the neutralising capacity of the constructs. Interpretation is somewhat limited owing to the small sample size and limited

resources to repeat the study. Equally, the interpretation of a study of this type is speculative. Despite this, the results do support the capacity of linear VP2 peptides to induce neutralising antibodies and this aspect deserves further investigation.

Chapter 7: General Discussion

African horse sickness (AHS) is a lethal arthropod borne disease of *Equidae* that is caused by the African horse sickness virus (AHSV), of which there are nine serotypes. In the absence of treatment, disease control relies heavily on vaccination. The only vaccine currently available for commercial use is a live attenuated vaccine produced by Onderstepoort Biological Product (OBP) in South Africa. Problems associated with the use of a live attenuated vaccine including the ability to revert to virulence, DIVA incompatibility, transmission in the field and re-assortment with other vaccine and field strains make these 'live' vaccines unfit to be licensed for use in Europe. In recent years, there have been attempts to develop a new generation vaccine that will not inherit the disadvantages associated with the live attenuated vaccine. This research has been focused predominately on AHS antigens VP2, VP5 and VP7 (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marschat *et al.*, 1996; Wade-Evans *et al.*, 1997). AHSV VP2 is the dominant serotype specific antigen that is responsible for the induction of neutralising antibodies, while VP5 is also thought to play a role in virus neutralisation (Martinez-Torrecuadrada & Casal, 1995; Martinez-Torrecuadrada *et al.*, 1999). VP7 is a serogroup specific antigen that is highly immunogenic during AHSV infection, as demonstrated by its use in antibody based AHSV diagnostic assays (Bremer *et al.*, 1994; Maree & Paweska, 2005). However, antibodies to VP7 do not neutralise the virus (Mertens *et al.*, 1996; Wade-Evans *et al.*, 1997).

AHSV replication, or expression of VP7 in insect cells, results in the generation of crystalline structures composed entirely of VP7 that are approximately 5µm in diameter (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree & Paweska, 2005). The function of these crystals is unknown, but they may serve to modify (reduce) the immune response to VP7 (as discussed later in the chapter).

The use of multimeric antigens to display immunologically important peptide / antigens to the immune system has been well documented (Clarke *et al.*, 1987; Fehr *et al.*, 1998; Kaumaya *et al.*, 1992). The AHSV VP7 crystalline structure corresponds to other highly ordered antigens, capable of cross-linking B-cell receptors and inducing both T-cell independent and T-cell dependent antibody responses. The immunogenicity of a foreign antigen/ peptide could therefore be enhanced when displayed in a similarly highly organized manner on the VP7 crystal surface.

The aim of this project was to investigate the immunogenicity of AHSV-4 VP7 crystals and explore their use as a vaccine platform. In order to do this, recombinant VP7 based reagents were prepared for investigation *in vitro* and *in vivo*, including vaccination experiments. Two methods were used to express recombinant VP7; baculovirus and the live viral vector MVA. A recombinant baculovirus expressing AHSV VP7 has been previously described (Chiam *et al.*, 2009; Chuma *et al.*, 1992; Maree & Paweska, 2005) by which high levels of VP7 expression were observed resulting in the aggregation and generation of VP7 crystals, sized between ~3 and 25µm in diameter (Chuma *et al.*, 1992; Maree & Paweska, 2005). The results from this project concurred with these studies and demonstrated that over-expression of VP7 in insect cells

generated VP7 crystals averaging approximately 5µm in diameter (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree & Paweska, 2005).

VP7 expression using MVA has not yet been described *in vitro*, but vaccination with MVA VP7 has been shown to result in the generation of VP7 specific antibodies (Chiam *et al.*, 2009). MVA VP7 therefore provides a valuable source of endogenous VP7 antigen. The results from this project have demonstrated that during MVA VP7 infection of mammalian cells, VP7 crystals were similarly generated *in situ*. The availability of purified baculovirus expressed VP7 crystals and live viral vector MVA expressing AHSV-4 VP7 have provided the resources necessary for characterising the immune response to VP7 crystals *in vivo*, especially since both exogenously administered (Wade-Evans *et al.*, 1997) and 'in situ' synthesized VP7 (Chiam *et al.*, 2009) have both been shown to induce humoral immunity in equine and murine animal models.

The expression and purification of VP7 crystals has also provided a resource for *in vitro* immuno-assays. Vaccination with recombinant VP7 is compatible with the commercially available AHSV VP7 ELISA (INGENASA) to detect a response to the vaccine, and would be DIVA compatible, with the use of an ELISA based on an alternative AHSV antigen for example NS3 (Laviada *et al.*, 1995).

This project showed that in the absence of adjuvant but with up to four sequential vaccinations, purified VP7 crystals did not induce detectable VP7 specific antibodies in the mouse model or pony. VP7 specific antibodies were also not detected in mice after vaccination with MVA VP7. However, upon re-testing

sera obtained from a MVA VP7 vaccination study in ponies, VP7 specific antibodies were detected, albeit weakly.

Nevertheless, the immunogenicity of VP7 was improved when the crystals were treated with urea (4M); a process which was thought to partially solubilise the crystal preparation. This was particularly evident when urea-treated crystals that were administered without adjuvant, induced comparative antibody responses to VP7 crystals administered with adjuvant. Furthermore, vaccination with urea-treated crystals with adjuvant induced higher levels of VP7 specific antibodies after two vaccinations than the adjuvanted VP7 crystals after three vaccinations. These results suggest that the immunogenicity of VP7 is largely influenced by its aggregative state and solubility, indicating that antigen size is critical for the induction of a specific antibody response. This also suggests that the assembly of VP7 into these macromolecular assemblies *in vivo* may in some way offer a mechanism for immune evasion, at least as far as VP7 is concerned.

It is well known that antigen size can affect antigen uptake and processing by APCs and transport from the peripheral tissue to the secondary lymphoid organs (Bachmann & Jennings, 2010; Reddy *et al.*, 2007). It has been suggested that particulate antigens have a better interaction with APCs, leading to a more efficient antigen uptake (Bachmann & Jennings, 2010). However, APCs are adapted to preferentially uptake and process antigen particles which have pathogen-like dimensions (approximately 20nm-3µm) (Bachmann & Jennings, 2010). Dendritic cells optimally take up antigen particles that are approximately 40nm in diameter (Foged *et al.*, 2005), while macrophages preferably uptake antigen smaller than 5µm in size (Tabata & Ikada, 1988).

The size of the crystals averaged approximately 5µm but the upper limits have often been seen to be 25µm in diameter (Burroughs *et al.*, 1994; Chuma *et al.*, 1992; Maree & Paweska, 2005). In the absence of adjuvant (that may lead to tissue inflammation and enhanced recruitment of APCs – e.g. dendritic cells and macrophages), antigenic processing of the VP7 crystal may be inefficient. Consequently, the failure to induce a VP7-specific IgG antibody response may be the result of the threshold for T-cell priming not being adequate enough for its generation.

The capacity for APC uptake is one of the parameters, which can increase the immunogenicity of an antigen (Brewer *et al.*, 2004). The size of an antigen can also influence antigen trafficking from the peripheral tissue to the secondary lymphoid organs for B and T-cell activation (Bachmann & Jennings, 2010). Smaller particles (<200nm) do not require APC assistance and can freely drain to secondary lymphoid organs via the lymphatic system. Consequently the antigens arrive in their native form within hours of inoculation (Bachmann & Jennings, 2010). Arrival in a 'cell-free state' is particularly important for antigens that can efficiently cross-link B-cell receptors and induce both T-cell independent and T-cell dependent antibody responses. Because of the crystals' size and their potential inability to drain from the tissue to the lymph nodes in their native structure it could be expected, from the results so far, that VP7 would only have been presented to the B-cells in a cell-associated state with the help from APCs. This would ultimately have inhibited the overall immunogenicity of the antigen and specifically the induction of any potential T-cell independent antibody response. Additionally, in a cell-associated state, the transport of the antigen from the peripheral tissues to the lymphoid organs

may have taken at least 24 hours to occur (Bachmann & Jennings, 2010) further inhibiting the development of an immune response.

The results presented in this study do not explain the effect that treatment with 4M urea had on the molecular 'state' of the solubilized VP7 protein. Further investigation will be required to fully understand the mechanism behind the enhanced antibody response achieved by this process. For example it is considered to be important to determine if the urea treated 'solubilised' VP7 is a monomer, dimer, trimer, randomly re-aggregated multimer or fully denatured, as this is likely to affect its antigenicity. However, the results obtained so far suggest that the 'solubilised' portion of the urea treated crystals is responsible for the heightened immunogenicity observed. It is also important to recognise that treatment with 4M urea only partially solubilised the crystals and a portion of the crystal preparation was likely to be intact. Indeed a combination of 'soluble' and crystal VP7 together may be required for the heightened immune response demonstrated; further investigation is needed to further explore these possibilities.

The ability of 4M urea treated crystals to induce antibodies in the absence of adjuvant is similar to other antigens (such as VLPs that have been shown to be self-adjuvanting (Middelberg *et al.*, 2011)). Virus-like particles are highly immunogenic because they mimic the immunological and structural qualities of the viral pathogens (Liu *et al.*, 2012). VLPs induce both T-cell independent and T-cell dependent antibody responses (Fehr *et al.*, 1998). Their antigenic structure is particulate and repetitive in nature, and in these respects are similar to the structure of VP7 crystals. However, VP7 crystals were not

shown to be immunogenic in the absence of adjuvant. There is one key difference between VLPs and the crystals and that is antigen size. Once more this highlights the importance of both antigen size and solubility on the generation of VP7 specific antibody responses.

In contrast to vaccination with pre-formed purified VP7 crystals, vaccination using recombinant live viral vectors (such as rMVA) expressing VP7 induces the synthesis of VP7 *in situ*. Expression of MVA VP7 results in the generation of VP7 crystals that have assembled as early as 4 hours post-infection. The rapidity of crystal formation may inhibit the capacity of the immune system to respond to soluble VP7 / smaller aggregations and might help to explain why an antibody response was not generated post-MVA VP7 vaccination in mice. However, re-testing of sera from MVA VP7 vaccination in ponies, provided by Chiam and co-workers (2009) confirmed the generation of VP7-specific antibodies after MVA VP7 vaccination, although the response was weak with only one of two ponies seroconverting after two vaccinations. Vaccination with canarypox expressing BTV VP7 induced easily detectable VP7 antibodies post-vaccination (WadeEvans *et al.*, 1996). These results support the hypothesis that as with other antigens, particle size is critical for generation of an effective antibody response. BTV VP7 has not been shown to form crystalline structures. As with AHSV infection in equids, VP7-specific antibody is dominant during BTV infections of ruminants, or vaccinations of rabbits or guinea pigs with purified virus particles (Mertens, personal communication).

The protective capacity of AHSV VP7 has been explored in the literature. As described earlier, purified VP7 crystals induced

cross-protective immunity in mice against AHSV challenge (Wade-Evans *et al.*, 1997). Additionally, VP7 has been shown to induce protection in horses against AHSV challenge in combination with VP2 and VP5 (Martinez-Torrecuadrada *et al.*, 1996). The protective efficacy of VP7 was briefly explored in this project, however the exploration of VP7 protective immunity was treated as secondary to the generation of VP7 specific antibodies and sample sizes were too small to establish any significant differences between vaccinated and control mice. The results of challenge studies are therefore uncertain but suggest that MVA VP7 vaccination may afford partial protection against homologous challenge.

Small compounds such as peptides are often considered to lack immunogenicity due to their low molecular weight and lack of structural complexity. The immunogenicity of such compounds can be enhanced when incorporated/ attached to the surface of a macromolecular assembly or 'carrier molecule', such as KLH (Coligan, 1996; Lateef *et al.*, 2007), VLPs (Clarke *et al.*, 1987; Fehr *et al.*, 1998; Middelberg *et al.*, 2011) or capsomeres (Wibowo *et al.*, 2014). The use of soluble VP7 as a carrier molecule has also been described (Rutkowska *et al.*, 2011). Within this project, particulate VP7 crystals were investigated as a vaccine delivery platform, and in order to do this, modified VP7 gene constructs were generated. Characterized peptides (AHSV-4 VP2a, VP2b and a biotin acceptor peptide) were incorporated into the VP7 gene by splice overlap extension (SOE), which proved to be a versatile and simple method for the incorporation of a peptide by genetic insertion as previously described (Heckman & Pease, 2007).

Recombinant baculoviruses expressing selected VP7 constructs (VP7/VP2a141, VP7/BAP141, VP7/VP2a200 and VP7/VP2a238) were successfully generated, and their ability to form crystals was investigated (as described in Chapter 5, section 5.2.2). Insertions of foreign peptides, at specific amino acid residues of VP7, that disrupted the capacity for the chimeric VP7 to form crystals, have been previously described (Rutkowska *et al.*, 2011). The results from this project show that insertion of peptides into the VP7 gene at 141 and 238 affected the ability of the modified VP7 protein to form crystals. It is very difficult to predict whether or not a foreign insert and carrier will be compatible. Over half of the foreign peptides tested for incorporation into HBcAg affected the chimeric particle assembly (Billaud *et al.*, 2005; Jegerlehner *et al.*, 2002). Factors that may contribute to the inability of the modified VP7 to form crystals include: the ability of the modified protein to fold and assemble correctly, its solubility and its trimerisation and dimerisation domains.

The results from this project also showed that expression of VP7 with the VP2a insert at amino acid position 200 (VP7/VP2a200) did result in the generation of modified VP7 crystals, which could be purified as described for wild-type VP7. The availability of purified baculovirus expressed VP7/VP2a200 crystals (exogenous antigen) provided a valuable resource for investigating the use as vaccine delivery platform for AHSV-4 VP2a.

The availability of the live viral vector MVA expressing a crystal forming construct (VP7/VP2a200) and a non-crystal forming construct (VP7/VP2a238) would have provided a valuable resource to facilitate comparative investigations. These materials could be used to explore whether soluble or

crystal forming constructs were better at eliciting a peptide specific immune response, without the requirement for protein purification. During this project an unsuccessful attempt was made to generate such recombinant MVA constructs. However, the use of rMVA to deliver modified VP7 constructs deserves further investigation.

With the availability of purified VP7/VP2a200, the use of VP7 crystals as a vaccine delivery platform could be further explored in relation to the generation of a VP2a peptide specific antibody response. Thus, antibody production was determined in mice vaccinated with: VP7/VP2a200 crystals; 4M urea treated VP7/VP2a200 crystals; murine polyomavirus (MPV) VP1 VLP expressing AHSV VP2a;, MPV VP1 capsomere expressing AHSV VP2a; or AHSV VP2a bound to KLH .

The 4M urea treated VP7/VP2a200 crystals were used, because treatment of wild-type VP7 crystals with 4M urea induced a higher titre of VP7 antibodies. Both MPV VLP and capsomeres have been shown to deliver immunologically important peptides to the immune system in an effective manner (Middelberg *et al.*, 2011; Wibowo *et al.*, 2014) while KLH is a renowned commercial carrier molecule (Coligan, 1996; Lateef *et al.*, 2007). Although a direct comparison could not be made between all of the different constructs, due to differences in the molar ratio of carrier to peptide, the results showed that all of the constructs were able to generate a VP2a peptide specific antibody response. In particular, the KLH construct had a very high peptide to carrier molar ratio.

The MPV VLPs were effective for delivering the VP2a peptide, as demonstrated by the high titres of the peptide specific antibodies generated. As powerful immunogens in their own right, VLPs are often used as a vaccine delivery platform, to

present immunologically important peptides/ epitopes to the immune system (Chackerian, 2007a; Fehr *et al.*, 1998; Middelberg *et al.*, 2011; Whitacre *et al.*, 2009). Their size and structure reflects that of microbial pathogens (Chackerian, 2007a; Liu *et al.*, 2012). Even at a lower molar ratio, in comparison to the other constructs, VP7/VP2a200 crystals and specifically the urea treated VP7/VP2a200 crystals (with adjuvant) induced high titres of VP2a peptide specific antibodies. These data correspond to those described by Rutkowska *et al.* (2011) suggesting that soluble VP7 is better at inducing peptide specific antibodies in comparison with insoluble VP7. Nevertheless, the VP7/VP2a200 crystals were capable of inducing VP2a peptide specific antibodies when inoculated with adjuvant. This result indicates that the VP7/VP2a200 crystals effectively delivered the VP2a peptide to the immune system. Their use together with soluble VP7 as a vaccine delivery platform merit further investigation.

The AHSV-4 VP2a peptide was incorporated into the construct because it had previously been demonstrated to contain a linear AHSV neutralizing epitope (Martinez-Torrecuadrada *et al.*, 2001). During this project, the neutralizing capacity of VP2a peptide antibodies raised against selected AHSV-VP2a based constructs was investigated. A low level of neutralization was observed using the pooled sera obtained from mice vaccinated with capsomere/VP2a and urea treated VP7/VP2a200 crystals (administered with adjuvant). This suggests that the way in which the VP2a peptide was presented by the carriers was relevant to the neutralisation site present on native VP2. The absence of neutralising antibodies after vaccination with the other antigens carrying VP2a (even if they generated a higher VP2a specific response) may suggest that the peptide was not displayed correctly in

the context of the new molecule. However, due to the small sample sizes and the limited amounts of sera available, the neutralising capacity of linear peptide AHSV-4 VP2a requires further investigation, especially since the level of neutralisation was much lower than described by Martinez-Torrecuadrada *et al.* 2001.

AHSV VP2 is a highly variable protein (Sailleau *et al.*, 2000). However, the results indicate that there was not a mismatch of amino acid identity between the VP2a peptide and the corresponding AHSV-4 virus strain used in the PRNT. The AHSV-4 VP2a peptide was recognized by sera raised against the AHSV-4 strain, in the PRNT assay, and by indirect ELISA during screening of KLH/VP2a peptide with the AHSV-4 'infected mouse' sera.

Conclusions

The results from this project suggest that AHSV-4 VP7 crystals are not immunogenic in the absence of adjuvant even after multiple vaccinations. This indicates that during natural infection, the crystals are not solely responsible for the frequently reported dominance of antibody to AHSV VP7. The immunogenicity of the VP7 crystals can be improved by treatment with 4M urea, partially 'solubilising' the crystal preparation. This suggests that the heightened immunogenicity is associated with the 'soluble' VP7 fraction, that the VP7 crystals are too large to be efficiently processed by the immune system and that antigen size is critical for the efficient induction of a VP7 specific antibody response.

The use of VP7 crystals (or 4M urea treated VP7/VP2a200 crystals) as a vaccine delivery platform was explored. Insertion of AHSV VP2a peptide into the VP7 at amino acid

residue site 200 did not comprise the ability for the constructs to form crystals and both VP7/VP2a200 crystals and urea treated VP7/VP2a200 crystals induced VP2a-peptide specific antibodies. The VP2a peptide specific antibodies raised against urea treated crystals induced low levels of neutralisation against AHSV-4 *in vitro*.

Future work

Future work should include the expression of a soluble recombinant VP7 that does not form crystals. Amino acid residues have been identified that may contribute to VP7's insolubility, including Ala-167 (Basak *et al.*, 1996) and Leu-345 (Rutkowska *et al.*, 2011). Mutation of these residues may improve the solubility of VP7. A more detailed investigation into the effects of antigen size on the immunogenicity of VP7 could be made using soluble VP7. This would be a more accurate way of defining the immunological potential of soluble VP7 in more detail, avoiding concerns associated with the potentially immuno-stimulant effects of urea and differences in effective dose between the crystals and partially solubilized material.

The generation of recombinant MVA expressing soluble and modified VP7 constructs would avoid the need for protein purification and delivery of endogenous antigen, as well as exogenous antigen delivered in the rMVA infected cell lysate.

Although not thoroughly investigated, the results suggest that the protective capacity of VP7 at least against challenge with the homologous AHSV strain / serotype deserves further investigation. Specifically it would be interesting to compare soluble and crystal forms of VP7 in protection studies.

The results generated by this project suggest that VP7 may provide partial protection against homologous AHSV challenge. It has also been reported that AHSV VP7 crystals provide partial protection against heterologous challenge and further suggested that the protection afforded by VP7 crystals was associated with cell-mediated immune responses (Wade-Evans *et al.*, 1997). If AHSV VP7 contains protective CTL epitopes, as described for BTV (Andrew *et al.*, 1995; Rojas *et al.*, 2011) solubilisation may make VP7 more readily available to the immune system, in comparison to the protein contained within the crystals. This might ultimately result in an enhanced protective immunity against AHSV challenge. Future work should therefore include analyses of T-cell responses in the mouse model after vaccination with soluble and crystal VP7 and subsequent AHSV challenge.

If VP7 can generate a protective immune response against AHSV challenge, it is to the virus' benefit to reduce immune activation to the protein, especially since VP7 is a sero-group specific antigen and may be cross protective. VP7 crystals may therefore be generated to sequester 'free' VP7 reducing its exposure to the immune system. The effects of VP7 crystal formation on the immune system and its potential role as a mechanism to evade immunity deserves further investigation

The results from this project have demonstrated that both VP7/VP2a200 crystals and urea (4M) treated VP7/VP2a200 crystals can be used a vaccine platform to deliver foreign peptides to the immune system and elicit the generation of a peptide specific antibody response. Specifically urea (4M) treated VP7/VP2a200 crystals were shown to induce higher peptide specific antibodies than untreated VP7/VP2a200 crystals. Whilst VP2a peptide specific antibodies raised against

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urea treated crystals also induced low levels of neutralisation against AHSV-4 *in vitro*. Future work should include the further exploration into the capacity of soluble VP7 to deliver immunologically important epitopes / peptides to the immune system which should include the use of alternative AHSV VP2 peptides (such as AHSV-4 peptide VP2b) alone and in combination with AHSV-4 VP2 peptide VP2a. Co-vaccination may help to improve the neutralising antibody response especially since co-vaccination with two peptides containing VP2a and VP2b induced higher neutralising antibody titres in comparison to VP2a peptide administered alone (Martinez-Torrecuadrada *et al.*, 2001).

Furthermore, investigations should include the attachment of foreign peptides to VP7 by means of conjugation for example using BAP, which may facilitate the attachment of large and more complex antigens and peptides, maintaining more complex B-cell conformational epitopes and even the attachment of multiple epitopes.

In addition, future work should also include exploration into the use of alternative vaccine platforms for AHSV peptide display including orbivirus NS1 protein. Studies have already documented its potential as a delivery platform (Ghosh *et al.*, 2002; Murphy & Roy, 2008). NS1 protein forms tubule aggregates during infection and has been demonstrated to contain multiple CTL epitopes (Andrew *et al.*, 1995; de la Poza *et al.*, 2015). In addition, NS1 has helped to elicit cross-protection against heterologous AHSV and BTV challenge (Calvo-Pinilla *et al.*, 2012; de la Poza *et al.*, 2013).

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Use of reverse transcriptase-polymerase chain reaction (RT-PCR) and dot-blot hybridisation for the detection and identification of African horse sickness virus nucleic acids. *Archives of virology Supplementum* **14**, 317-327.

Appendices

Appendix I

Table A 1: List of primers

Primer set number (No.) 10 μ M	Primer nomenclature	Primer sequence 5'-3'
1	PLV1393 (Forward)	TGTAATAAAAAAACCTATAAATATTCC GGA
	PLV1393 (Reverse)	ACGGATTTCCTTGAAGAGAGTGAGT
2	VP7/VP2a141 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTTAAGG GGTATTACGTACCGCAAGGTC
	VP7/VP2(a)141 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACT CCGCCAGATTGTTGC
3	VP7/BAP141 (Forward)	ATCTTCGAAGCACAGAAAATTGAATGG CATTATTACGTACCGCAAGGTC
	VP7/BAP141 (Reverse)	CTGTGCTTCGAAGATGTCGTTGAGACC TCCGCCAGATTGTTGC
4	VP7/VP2a144 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTTAAGG GGCAAGGTCGAACACGTGGTGGG
	VP7/VP2a144 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACC GGTACGTAATATCCGCCAGATTG
5	VP7/VP2b144 (Forward)	GGTAAGTGGAAGAACATATAAAGGA GGTAACCGAAAAATTGAAGAAAGCGC AAACCGAACAAGGTCGAACACGTGGT GGG
	VP7/VP2b144 (Reverse)	TACCTCCTTTATATGTTCTTTCCACTTA CCCTTATTCGGATCAACATCTACACCG TTCCTCTTCGGTACGTAATATCCGCCA GATTG
6	VP7/VP2a164 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTTAAGG GGGCAGGCGCTGCGGGACAGGTC
	VP7/VP2a164 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACA TCCATACACACTTCAGCAAT
7	VP7/VP2b164 (Forward)	GGTAAGTGGAAGAACATATAAAGGA GGTAACCGAAAAATTGAAGAAAGCGC AAACCGAAGCAGGCGCTGCGGGACAG GTC
	VP7/VP2b164 (Reverse)	TACCTCCTTTATATGTTCTTTCCACTTA CCCTTATTCGGATCAACATCTACACCG TTCCTCTTATCCATACACACTTCAGCAA

		TATTTGA
8	VP7/VP2a177 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTAAAGG GGAGGGGGGACGCGAGTCATGATC
	VP7/VP2a177 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACC CTTGGGGCTAGCAGCGCATT
9	VP7/VP2b177 (Forward)	GGTAAGTGGAAGAACATATAAAGGA GGTAACCGAAAAATTGAAGAAAGCGC AAACCGAAAGGGGGGACGCGAGTCATG ATC
	VP7/VP2b177 (Reverse)	TACCTCCTTTATATGTTCTTTCCACTTA CCCTTATTCGGATCAACATCTACACCG TTCCTCTTCCTTGGGGCTAGCAGCGCA TT
10	VP7/VP2a200 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTAAAGG GGTCACTTGAAAGCGCTCCAGGG
	VP7/VP2a200 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACC GCACCTTGAGGATCACAAAA
11	VP7/VP2b200 (Forward)	GGTAAGTGGAAGAACATATAAAGGA GGTAACCGAAAAATTGAAGAAAGCGC AAACCGAATCACTTGAAAGCGCTCCAG GG
	VP7/VP2b200 (Reverse)	TACCTCCTTTATATGTTCTTTCCACTTA CCCTTATTCGGATCAACATCTACACCG TTCCTCTTCGCACCTTGAGGATCACAA AA
12	VP7/BAP200 (Forward)	ATCTTCGAAGCACAGAAAATTGAATGG CATTCACTTGAAAGCGCTCCAGGG
	VP7/BAP200 (Reverse)	CTGTGCTTCGAAGATGTCGTTGAGACC CGCACCTTGAGGATCACAAAA
13	VP7/VP2a238 (Forward)	GAGGGTGAGGATGATACTGCTCGACA GGAGATAAGAAAAGCATGGGTAAAGG GGCGCAGATCAATTTTACAGTTTG
	VP7/VP2a238 (Reverse)	TATCTCCTGTCGAGCAGTATCATCCTC ACCCTCTTCTTTCTTCTTCGCATGAACT GCCCCAGGATTTCACATTAC
14	VP7/VP2b238 (Forward)	GGTAAGTGGAAGAACATATAAAGGA GGTAACCGAAAAATTGAAGAAAGCGC AAACCGAACGCAGATCAATTTTACAGT TTG
	VP7/VP2b238 (Reverse)	TACCTCCTTTATATGTTCTTTCCACTTA CCCTTATTCGGATCAACATCTACACCG TTCCTCTTGCCCCAGGATTTCACAA TTCAC
15	VP7 with SMA I/XMA I sequence ends (Forward)	AAGTCCCGGGACCATGGACGCGATAG CAGCAAGAGCCTTGTC

	VP7 with SMA I/XMA I sequence ends (Reverse)	ATAACCCGGGCTAGTGGTAGGCTGCT AGCACAAGAGCT
16	VP7 with XhoI sequence ends (Forward)	AAGTCTCGAGACCATGGACGCGATAG CAGCAAGAGCCTT
	VP7 with XhoI sequence ends (Reverse)	ATAACTCGAGCTAGTGGTAGGCTGCTA GCACAAGAG
17	Flanking VP7 primers (Forward)	ATGGACGCGATAGCAGCAAGAGCCT
	Flanking VP7 primers (Reverse)	CTAGTGGTAGGCTGCTAGCACAAGAG
18	PSC11 (Forward)	TAATTTATTGCACGGTAAGG
	PSC11 (Reverse)	GAAATGTCCCATCGAGTG

Appendix II

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5' ATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCATGCGTCACAGTGACAGAT
0
0
60
5' GCGAGAGTTAGCTTGGATCCAGGAGTGATGAGACGTTAGGGATCGCAATTAATAGGTAC
0
0
120
5' AATGGTTTAACGAATCATTTCGGTATCGATGAGGCCACAAACCCAGCAGGACGAAATGAA
0
0
180
5' ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCATTGAACGTCCAAATTGGGAATATT
0
0
240
5' TCACCAGATTATGATCAGGCGTTGGCAACTGTGGGAGCTCTTGCAACGACTGAAATTCCA
0
0
300
5' TATAATGTTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAAATGCAAACATGCGGA
0
0
360
5' CCAAGCAAAGTACAGACGGGACCTTATGCAGGAGCAGTTGAGGTGCAACAATCTGGCGGA
0
0
420
5' TATTACGTACCGCAAGGTCGAACACGTGGTGGGTACATTAATTCAAATATTGCTGAAGTG
0
0
480
5' TGTATGGATGCAGGCGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGAC
0
0
540
5' GCAGTCATGATCTATTTTGTGGAGACCATTGCGTATATTTTGTGATCCTCAAGGTGCG
0
0
600
5' TCACTTGAAAGCGCTCCAGGGACTTTTGTACCCGTTGATGGAGTAAATGTGCGGCTGGA
0
0
660
5' GATGTCGTCGCATGGAAATACCATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGA
0
0
720
5' TCAATTTTACAGTTTGAAGTGTTATGGTATACGTCTTTGGATAGATCGCTAGACACGGTT
0
0
780
5' CCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTTTCTCCCACTTGGCACGCA
0
0
840
5' TTACGCGCTGTCAATTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCAATTTTCCA
0
0
900
5' CCGACTGAAAGGAATGAAATGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTAT
0
0
960
5' GCGGCTTTGAGGCCAGATTTCAGAAATGAATGGTGTGTTGCGCCAGTAGGCCAGATCAAC
0
0
1020
5' AGAGCTCTTGCTAGCAGCCTACCACTAG
0

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Figure A2.1: Nucleotide sequence of VP7 (AHSV-4/Madrid 1987) gene sequence I

5' ATGGACGCGATAGCAGCAAGAGCCTTGTCGGTTGTACGGGCATGCGTCACAGTGACAGAT 60
 0
 5' GCGAGAGTTAGCTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATTAATAGGTAC 120
 0
 5' AATGGTTTAACGAATCATTTCGGTATCGATGAGGCCACAAACCAAGCAGAACGAAATGAA 180
 0
 5' ATGTTTTTATGTGTACTGATATGGTTTTAGCGGCATTGAACGTCCAAATTGGGAATATT 240
 0
 5' TCACCAGATTATGATCAGGCGTTGGCAACTGTGGGAGCTCTTGCAACGACTGAAATTCCA 300
 0
 5' TATAATGTTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAAATGCAAACATTTCGGA 360
 0
 5' CCAAGCAAAGTACAGACGGGACCTTATGCAGGAGCAGTTGAGGTGCAACAATCTGGCGGA 420
 0
 5' TATTACGTACCGCAAGGTCGAACACGTGGTGGGTACATTAATTCAAATATTGCTGAAGTG 480
 0
 5' TGTATGGATGCAGGCGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGAC 540
 0
 5' GCAGTCATGATCTATTTTGTGTTGGAGACCATTGCGTATATTTTGTGATCCTCAAGGTGCG 600
 0
 5' TCACTTGAAAGCGCTCCAGGGACTTTTGTACCGTTGATGGAGTAAATGTTGCGGCTGGA 660
 0
 5' GATGTCGTCGCATGGAATACCATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGA 720
 0
 5' TCAATTTTACAGTTTGAAGTGTATGGTATACGTCTTTGGATAGATCGCTAGACACGGTT 780
 0
 5' CCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTTTCTCCCACTTGGCACGCA 840
 0
 5' TTACGCGCTGTCTATTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCAATTTTCCA 900
 0
 5' CCGACTGAAAGGAATGAAATGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTAT 960
 0
 5' GCGGCTTTGAGGCCAGATTTCAGAATGAATGGTGTGTTGCGCCAGTAGGCCAGATCAAC 1020
 0
 5' AGAGCTCTTGCTAGCAGCCTACCACTAG
 0

Figure A2.2: Nucleotide sequence of VP7 (AHSV-4/Madrid 1987) gene sequence II (Chiam *et al.*, 2009)

Appendix III

Table A3.1: A list of antisera (not generated in this project) used in this thesis

Nomenclature	Prepared against	Animal species	Time of collection (Post-inoculation)	Other details
Pony 5859, MVA VP7	MVA VP7 (Table 2.2) vaccination	Pony	Days 0, 42, 98	Generated as described by Chlam <i>et al.</i> 2009)
Pony 5717, MVA VP7, D42	MVA VP7 vaccination	Pony	Days 0, 42, 98	Generated as described by Chlam <i>et al.</i> 2009
AHSV-4 infected horse antiserum	AHSV-4 challenge	Horse (Horse 3)	Day 15	Obtained from The Pirbright Institute antiserum collection
AHSV-4 infected mouse antiserum	AHSV-4 (Table 2.2) challenge	Mouse (IFNAR -/- A129)	Day 7pi	Provided by Dr Eva Calvo-Pinilla
Anti-AHSV-9 antiserum (PM17)	Purified AHSV-9	Rabbit	Details not available	Obtained from The Pirbright Institute antiserum collection
Anti-VP2 peptide antiserum (Rabbit A)	AHSV-4 VP2 peptides; KKKEEGEDDT ARQEIRKAWC ; NKGKWKEHI KEVTEKLKKA C; DMNEKQKPYF EFEYDDFKPC, bound to KLH	Rabbit	-	Generated and provided by Genscript.
Normal rabbit antiserum	N/A	Rabbit	N/A	Provided by Sigma.

Table A3.2: Primary antibodies used for Western blot and immunofluorescence

Anti-sera (Appendix III, Table A3.1)	Dilutions western blot	for	Dilutions immunofluorescence	for
Pony 5859, MVA VP7	1/100		N/A	
Pony 5717, MVA VP7	1/100		N/A	
AHSV-4 infected horse antiserum	1/100		N/A	
AHSV-4 infected mouse antiserum	1/100		N/A	
Anti-AHSV-9 antiserum (PM17)	1/200		1/1000	
Anti-VP2 peptide antiserum (Rabbit A)	1/200		1/1000	
Normal rabbit antiserum	1/200		N/A	

Table A3. 3: Secondary antibodies used for Western blot and immunofluorescence

Number	Antibody label	Raised in	Source	Dilution for use
1	Anti-rabbit antibody conjugated to FITc.	Swine	Dako	1:50
2	Anti-horse IgG (whole molecule) Alkaline Phosphatase	Rabbit	Sigma	1:10,000
3	Anti-Mouse IgG (H + L)-HRP Conjugate	Goat	BioRad	1:2,000

