IMMUNE RESPONSES TO MODIFIED-LIVE AND RECOMBINANT AFRICAN HORSE SICKNESS VIRUS VACCINES

BY

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Declaration

I, *Jan Ernst Crafford*, do hereby declare that except where acknowledgements indicate otherwise and the normal advice from my supervisors, this dissertation is my own original work. I hereby acknowledge the contributions by the various co-authors in the published papers that form part of this dissertation. Neither the full dissertation nor any part of it has been, is being, or is to be submitted for another degree at this or any other University.

No conflict of interest has been declared.

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Signed:……

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Summary

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There have been numerous reports of vaccinated horses that contract fatal African horse sickness due to African horse sickness virus (AHSV) serotypes that are included in the current commercial vaccine used in southern Africa, which emphasizes the importance of thorough characterization of the equine immune response to AHSV. In particular, there are concerns about possible interference between vaccine strains in the polyvalent vaccine which led us to hypothesise that the administration of individual AHSV serotypes could induce a better immunity to individual serotypes than that achieved with the current polyvalent vaccines. There is also little published information describing the half-life of maternally derived neutralising antibody in foals to the nine AHSV serotypes. This is important for revising and developing vaccination protocols for foals of vaccinated mares.

Given the lethality of both natural and experimental AHSV infections in horses, several aspects of immunity induced by different types of AHSV vaccines were evaluated. The

neutralising antibody response of horses (foals) immunized with a commercial modified live virus (MLV) AHSV vaccine was evaluated and compared to the immune response elicited to monovalent MLV AHSV serotypes. Foals were immunized with either the polyvalent AHSV vaccine, or one of four monovalent vaccines containing individual AHSV serotypes 1, 4, 7 and 8. There were marked differences in the immunogenicity of individual virus serotypes contained in the vaccine. Foals more consistently seroconverted to AHSV 1 and responses to other serotypes were highly variable, and often weak or not detected. The serotype-specific responses of foals given the monovalent MLV vaccines were similar to those of foals given the polyvalent preparation suggesting that there is no apparent enhanced immune response through the administration of a monovalent vaccine as opposed to the polyvalent vaccine. Furthermore, the immunogenicity of individual AHSV serotypes contained in the commercial MLV vaccine varies remarkably.

Neutralising antibody titres to the 9 known serotypes of AHSV were determined in a cohort of brood mares that were regularly vaccinated with the MLV AHSV vaccine, and the passive transfer and rate of decay of maternal antibody to the individual virus serotypes in their foals were measured. Similar to the data obtained from immunized foals, there was marked variation in the neutralising antibody response of the mares to individual AHSV serotypes even after repeated vaccination. This was mirrored in the duration of maternally-derived antibodies in their respective foals.

In an effort to further characterize cellular immune (CMI) responses to AHSV, the immunity in horses induced by an experimental canarypox virus vectored recombinant (ALVAC®-AHSV4) vaccine was characterised. The detection of VP2/VP5 specific IFN-γ responses was assessed by enzyme-linked immune spot (ELISpot) assay and clearly demonstrated that all ALVAC®-AHSV4 vaccinated horses developed significant IFN-γ production compared to unvaccinated horses. Flow cytometry demonstrated that this vaccine induced mainly CD8+ T-cells, able to recognize multiple T-cell epitopes throughout all of VP2 and only the N-terminus portion of VP5.

In summary, the antibody and cellular response of horses to different AHSV vaccines was evaluated and compared. The results are relevant to the design of more efficacious AHSV vaccines and to identification of protective immunity in horses to this virus.

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List of Abbreviations

AHS	African horse sickness
AHSV	African horse sickness virus
ALVAC®	recombinant canarypox virus vector
ARC-OVI	Agricultural Research Council Onderstepoort Veterinary Institute
BTV	bluetongue virus
CMI	cell mediated immunity
CTL	cytotoxic T-lymphocyte
DOP	duration to onset of protection
ELISpot	enzyme-linked immune spot
IFN-γ	interferon gamma
$IFNAR^{(-/-)}$	IFN-α/β receptor knock-out mice
IgG	immunoglobulin G
MHC	major histocompatibility complex
MLV	modified live virus
MPD	minimum protective dose
MVA	modified vaccinia Ankara
NS1	non-structural protein
OBP	Onderstepoort Biological Products SOC Ltd, Onderstepoort, South Africa
OIE	Office International des Epizooties
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline – Tween-20
rMVA	recombinant modified vaccinia virus Ankara
RSA	Republic of South Africa
SD	standard deviation
SFC	spot forming cells
SNT	serum-virus microneutralisation test
$TCID_{50}$	tissue culture infective dose
TCR	T-cell antigen receptor
TH1	T-helper 1
VP2	virus protein 2
VP5	virus protein 5

Chapter 1

GENERAL INTRODUCTION

Introduction

African horse sickness (AHS) is typically a fatal disease in susceptible horses and vaccination is currently used to prevent the occurrence of disease in endemic areas. The recent changes in the disease patterns of arboviruses and the influence of climate change on the emergence and establishment of a transmission cycle of bluetongue virus (BTV) throughout much of Europe (Gould and Higgs 2009; MacLachlan and Guthrie 2010), has re-emphasised the need for integrated measures for the control of *Culicoides* transmitted viral diseases (MacLachlan and Mayo 2013). The complexity and challenges to effectively control future outbreaks of orbivirus diseases like BT and AHS was comprehensively described by MacLachlan and Mayo (2013). The presence of multiple virus serotypes within the individual species of orbiviruses and the opportunistic feeding behaviour of the midge vectors are some of the major obstacles in the control of these diseases. Vaccination will likely be central to the control of any future incursions of AHSV into previously unaffected areas and hence a renewed emphasis on the development of new generation vaccines against African horse sickness virus (AHSV).

Vaccination against AHSV is based on the assumption that neutralizing antibodies confer serotype-specific immunity to reinfection with the homologous virus serotype (Castillo-Olivares *et al*. 2011; Coetzer and Guthrie 2004; Lelli *et al*. 2013). Neutralizing antibodies are directed against specific epitopes on the VP2 outer capsid AHSV protein, and there is some limited cross-neutralization between virus serotypes e.g. serotypes 1 and 2, 3 and 7, 5 and 8, 6 and 9 (Coetzer *et al.* 2004). Similarly, cross immunity between AHSV serotypes

can be enhanced by repeated inoculation of the same virus (Alexander 1935a; Alexander 1935b; Alexander *et al*. 1936). The cellular immune response to AHSV is poorly described, although virus-specific $CD8^+$ T cells are present in the blood of horses previously vaccinated with live attenuated vaccine (Pretorius *et al*. 2012). The study of cellular responses to AHSV has been expedited by the recent development of an experimental model infection system using Type 1 interferon receptor deficient (IFNAR "knock – out") mice (Castillo-Olivares *et al.* 2011). Indeed, recent studies utilizing this experimental model showed that IFNAR mice were protected against lethal infection by homologous and heterologous serotypes of AHSV by prior immunization with recombinant non-structural AHSV protein NS1 expressed from naked DNA and vaccinia virus (de la Poza *et al*. 2013). Cross (heterotypic) immunity is also recognized in AHSV-vaccinated horses, suggesting that polyvalent vaccine strategies might be feasible although it is uncertain whether the mechanism of such immunity is similar in horses and IFNAR mice.

Several AHSV vaccines have been previously developed and used including: a polyvalent live-attenuated (modified-live virus [MLV]) vaccine of adult mouse brain origin, a polyvalent cell culture-adapted MLV, and an inactivated AHSV 4 vaccine. These vaccines and their potential limitations have been reviewed by others (House 1993; House 1998; MacLachlan *et al*. 2007). Although new generation vaccines have been described recently (Chiam *et al*. 2009; Guthrie *et al*. 2009; Martinez-Torrecuadrada *et al*. 1996; Roy *et al*. 1996), only a polyvalent cell culture-adapted MLV vaccine is currently commercially available.

Problem statement

Current vaccine strategies to prevent AHS among horses in endemic areas of Africa are reliant on a commercial MLV polyvalent vaccine. However, the immune status to all nine AHSV serotypes in a horse population that is regularly vaccinated in the field with the polyvalent cell culture-adapted MLV AHS vaccine produced by Onderstepoort Biological Products SOC Ltd, Onderstepoort, South Africa, as well as the transfer of maternal

antibody from the vaccinated mares to their foals have not been described adequately. There are, however, some published results of the neutralising antibody titres in horses vaccinated in a controlled experimental environment (von Teichman *et al*. 2010). There have been reports from the field and in the literature on repeatedly vaccinated horses that contract fatal AHS (Coetzer *et al.* 2004). These horses succumb to serotypes that are included in the vaccine (Erasmus 1978). There are therefore concerns about possible interference between vaccine strains in the polyvalent vaccine which led us to hypothesise that the administration of individual serotypes as monovalent vaccines could induce a better immunity to individual serotypes than the current polyvalent vaccines.

In the course of these studies it became clear that the poor immunogenicity of several MLV AHSV serotypes contained in the polyvalent MLV vaccine precluded investigation of potential heterotypic/cross reactive serological responses among/between individual AHSV serotypes. Thus, to further investigate other potential mechanisms that could contribute to protective immunity against AHSV infection in horses, cellular immunity (CMI) in particular, we sought to build on the promising results from studies with a canarypox virus-vectored recombinant vaccine (ALVAC®-AHSV4) described by Guthrie *et al.* (2009). It is to be emphasised that it is difficult to study immunity (humoral or CMI) in horses challenged with virulent AHSV either naturally or experimentally, because of the associated high mortality.

Hypotheses

In addressing the immune responses of horses to AHSV infection, the central research question was: what is the serological response to individual AHSV serotypes in horses in the field that are regularly vaccinated with the commercial MLV vaccine, and how does the serological status of individual mares impact on the maternal immunity conveyed to their foals. The following hypotheses were formulated to assist in answering this question.

1. Horses immunized with polyvalent cell culture-adapted MLV AHS vaccines do not respond equally to the various AHSV serotypes.
2. Horses sequentially immunized at 4 week intervals with monovalent components of the vaccine produce a better neutralizing antibody response to the individual serotypes than horses vaccinated with the standard two-dose polyvalent vaccine with a 4 week interval.
3. Horses sequentially immunized with monovalent cell culture-adapted MLV vaccines produce antibodies to serotypes of AHSV to which they were never exposed.
4. The decline of passively acquired neutralizing antibodies in foal sera occurs within 4 months of birth, similar to those against other viruses.

In light of the feeble serological response of horses to several of the MLV AHSV serotypes, regardless of whether they were delivered in monovalent or polyvalent formulations, additional studies were designed to further characterize the nature of CMI to AHSV. Specifically, does the ALVAC®-AHSV4 vaccine construct stimulate cellular responses in horses and were AHSV-specific T-cell epitopes present on the two virus proteins (VP2 and VP5) in the outer-capsid of AHSV. VP2 is important to the development of serotype specific neutralising antibody, and VP5 likely contributes through its conformational interactions with VP2. The following hypotheses were formulated to assist in answering this question:

5. The experimental ALVAC®-AHSV4 vaccine induces a humoral as well as a cell-mediated immune response in vaccinated horses.
6. There are multiple T-cell epitopes within the VP2 and VP5 outer-capsid proteins of AHSV.

Objectives

The objectives of this study were:

1. To determine antibody titres to each of the 9 serotypes of AHSV in a cohort of breeding mares that were regularly vaccinated with the polyvalent cell culture-adapted MLV vaccine.
2. To measure passive transfer and rate of decay of maternal antibody to the individual virus serotypes in their foals.
3. To evaluate the neutralising antibody response of naïve horses immunized with multivalent and monovalent components (i.e. individual AHSV serotypes) of the polyvalent cell culture-adapted MLV vaccine.
4. To characterise cell mediated immune responses induced in horses vaccinated with an experimental ALVAC®-AHSV4 vaccine previously described by Guthrie et al. (2009).

Application of results

The results and lessons learnt in this thesis will provide important baseline knowledge that can be used for the design of further studies on the immune response and pathogenesis of AHS in horses. Aspects of cellular and humoral immunity stimulated by the polyvalent cell culture-adapted MLV AHS vaccines and an experimental ALVAC®-AHSV4 vaccine are provided. The importance and need for the establishment of a reliable model for the challenge and protection of horses to the various AHSV serotypes is emphasised for the future development and testing of vaccines for AHS. The data in this study could also be expanded to assist in the development of more efficient vaccination protocols for the available AHS vaccine in adult horses and foals in AHSV endemic areas.

Chapter 2

REVIEW OF THE LITERATURE

Introduction

African horse sickness (AHS) is a non-contagious disease of horses and other solipeds caused by an arthropod-borne orbivirus of the family *Reoviridae* (Verwoerd *et al.* 1979). The disease is manifested by pyrexia and clinical signs and lesions compatible with impaired respiratory and circulatory functions that are characterized by oedema of subcutaneous and intermuscular tissues and of the lungs, transudation into the body cavities, and haemorrhages, particularly of the serosal surfaces (Coetzer and Erasmus 1994). Theiler (1921) distinguished 4 clinical forms of AHS, viz. the pulmonary form, the cardiac form, the mixed form and horse sickness fever. Knowledge of the role of the host immune response (either humoral or cell-mediated) in the outcome of African horse sickness (AHS) is limited at this point in time.

African horse sickness virus

African horse sickness virus (AHSV) is a collective term, referring to the entire serogroup or species, containing nine different serotypes (Calisher and Mertens 1998; Howell 1962). The morphology of AHSV is similar to that of BTV, the prototype for orbiviruses, and was described by Roy *et al.* (1994). The architecture for AHSV 4 was further described and confirmed using electron cryomicroscopy and three-dimensional image reconstruction recently (Manole *et al*. 2012). The typical icosahedral orbivirus core-particle consists of two protein layers, the inner sub-core consisting of the structural virus protein (VP) 3, and the outer core surface consisting of VP7. The core is encapsulated by the outer capsid layer consisting of two structural proteins, VP2 and VP5. These outer capsid proteins are

more variable than the other structural proteins and play a role in cell adhesion and entry. VP2, specifically, is the protein associated with the cell attachment and receptor-mediated endocytosis of virions (Hassan and Roy 1999) while VP5 causes destabilization of the virus containing endosomal vesicle membrane to allow the penetration of the uncoated core particles into the cytoplasm (Hassan *et al*. 2001). VP2 contains the most important serotype specific epitopes while VP3 and VP7 contain group-specific epitopes. VP1, VP4 and VP6 are smaller proteins which have RNA transcriptase- and RNA-modifying properties and are contained together with the 10 dsRNA segments within the centre of the virion (Grimes *et al*. 1998).

Apart from the 7 structural proteins the genome also codes for five distinct non-structural proteins (NS1, NS2, NS3/NS3A and NS4). NS1 is associated with the formation of intra-cellular tubules and is believed to play a role in the translocation of virus particles to the cell membrane (Huismans and Els 1979; Owens *et al*. 2004). NS2 is found as a matrix protein in virus inclusion bodies and seems to play a role in the selective recruitment of virus messenger RNAs from other RNA species within the infected cytosol during virus replication (Lymperopoulos *et al*. 2003)

More is known about the function of NS3 proteins (NS3/3A). They are profusely expressed in arthropod cells but much less in mammalian cells and they are believed to play a role in the budding (non-cytolytic) release of virus particles from insect cells (Hyatt *et al*. 1993). NS3 was shown to be a highly cytotoxic protein and both the hydrophobic domains of the protein increase membrane permeability (Huismans *et al*. 2004). In fact it seems that the ratio of NS1 to NS3 expression can contribute to the pathogenesis of orbiviruses where high levels of NS3 seem to favour budding release of virus (as seen in insect cells) and low levels (compared to NS1) are associated with more lytic release of virus particles as seen in mammalian cells (Owens *et al.* 2004).

The fifth orbivirus non-structural protein (NS4) was only recently described by Belhouchet and co-workers (2011). The specific role of NS4 is still uncertain but it may play a role alongside NS1 and NS3 during early virogenesis.

Factors that influence the outcome of African horse sickness

The literature regarding the immunity and pathogenesis of AHS was previously reviewed by Burrage and Laegried (1994) and they concluded that: 1) host factors have no influence on the susceptibility of horses to AHSV infection; 2) host genetics play an important role in the outcome of AHSV infection - horses are the only solipeds that demonstrate the more severe form of the disease while zebras and donkeys become infected with the AHSV and seroconvert but do not develop severe disease; 3) the role of the immune status of the horse in the pathogenesis of the various forms of the disease is unclear.

A high incidence of mortality in horses with high levels of neutralising antibody to various serotypes of AHSV but not to the serotypes that were isolated from dying horses was reported by Howell (1963), suggesting that heterotypic neutralising antibody had little effect in protecting these horses. Erasmus (1978) reported, while testing attenuated strains of AHSV, as possible vaccine candidates, that some strains did not induce neutralizing antibodies in vaccinated horses, in spite of prolonged viraemia. These horses were later highly susceptible to the severe form of the disease when challenged with homotypic strains of AHSV and showed high mortality rates, he suggested that weakly immunogenic AHSV may sensitise horses to homotypic infection, resulting in more rapid death. Erasmus also proposed that the virulence phenotype of a AHSV isolate influenced the form of the diseases expressed in naïve horses and that virulence of AHSV isolates appeared to be related to their ability to infect or injure endothelial cells of specific organs (Erasmus 1973).

Virus proteins can play an important role in determining pathogenicity, especially those involved in the interaction with cellular receptors as well as those involved in uncoating and the spread of virus particles in and between cells. Within the orbiviruses the two outer capsid proteins and the non-structural proteins are of particular importance, as they are involved in virus entry and the release of virus particles from a cell (Huismans *et al.* 2004).

Fulminant AHS shares many of the characteristic features of viral haemorrhagic fevers. Ultra structural studies on the capillaries of infected horses revealed similar lesions as described for orbiviruses which included: changes in permeability, alteration of intercellular junctions, hypertrophy, degenerative changes, loss of endothelium, sub endothelial deposition of cell debris and fibrin, and vascular repair. These lesions were associated with oedema, haemorrhages and microthromboses, particularly in the myocardium and lung (Gomez-Villamandos *et al*. 1999). These authors also proposed that there seems to be different levels of viral tropism for blood vessel endothelial cells in different organs.

More significant progress on the pathogenesis of BTV in ruminants was made in recent years paving the way for a better understanding of the virus-host interaction of orbivirus infections. Similarities between the pathogenesis of BT and viral haemorrhagic fevers (e.g. Ebola virus infections) of humans have been proposed in an attempt to explain the wide-spread vascular leakage caused by the relatively restricted and transient infection of vascular endothelium in ruminants with fulminant BT (MacLachlan *et al*. 2009). *In vitro* studies on the effect of BTV on bovine pulmonary artery endothelial cells have shown that the adherence junctions of infected endothelium cells remain intact during infection and that increased permeability was mainly due to cell death (Drew *et al*. 2010a). There is ample evidence that not only virus induced endothelial cell death but also virus induced host-derived inflammatory and vasoactive mediators that can act in a paracrine fashion is

responsible for endothelial cell injury and increased permeability (DeMaula *et al*. 2001; DeMaula *et al*. 2002a; DeMaula *et al*. 2002b; Drew *et al*. 2010b).

Humoral immunity

The humoral nature of protection against AHSV was demonstrated by the passive transfer of neutralizing antibodies from vaccinated mares to their foals via colostrum and by passive transfer of neutralizing monoclonal antibodies to neonatal mice (Blackburn and Swanepoel 1988b; Burrage *et al*. 1993).

Burrage and Laegried (1994) have reported a strong correlation between neutralizing antibody and protection to AHSV. These neutralizing antibodies were primarily directed towards epitopes on viral protein 2 (VP2) in the outer capsid of the virion. Further evidence of the protectivity of anti-VP2 antibody was provided when horses vaccinated with recombinant VP2 proteins were protected against virulent homologous virus challenge (Roy *et al.* 1996; Scanlen *et al*. 2002; Stone-Marschat *et al*. 1996). This is also further supported by the findings of Boone and co-workers (2007) who developed a canarypox virus vectored vaccine co-expressing VP2 and VP5 outer capsid proteins of BTV which conveyed high levels of protection against field challenge of BTV in sheep.

There is little published information on the neutralising antibody responses of horses to the historic polyvalent MLV vaccine of adult mouse brain origin. This vaccine constituted a cocktail of AHSV serotypes 1 to 6. Blackburn and Swanepoel (1988a; 1988b) reported that horses which received several annual immunisations had a broader response to the various serotypes in that vaccine and higher individual titres. Nevertheless some horses failed to respond to one or more virus serotypes despite receiving numerous immunisations.

After the 1987 outbreak of AHS in Spain, seed stock of the adult mouse brain adapted MLV, obtained from the Foreign Animal Disease Diagnostic Laboratory in New York, was tested in horses. House (1993) reported a wide spectrum of responses to the seed stock

of these individual AHSV serotypes, ranging from failure to induce any neutralising antibody response to induction of solid protective immunity. Notably in his report, two horses vaccinated with AHSV 6 and one horse vaccinated with serotype 7 did not seroconvert after vaccination, despite having prolonged fevers for 7-14 days. These horses did, however, survive challenge inoculation with homologous AHSV serotypes.

According to Erasmus (1978) several vaccinations with the previous mouse-brain attenuated polyvalent vaccine were needed to obtain polyvalent immunity and therefore annual vaccination was recommended. He also reported on observations that horses vaccinated (annually) ten or more times still contracted peracute horse sickness from virus strains that were present in the vaccine. He speculated that immunological unresponsiveness or even hypersensitivity could result due to over vaccination.

The MLV cell culture-adapted AHSV vaccine that is currently widely used in southern Africa was first introduced in the 1960s (Erasmus 1978). It is recommended that all horses in the AHSV-infected area in South Africa are vaccinated annually with this MLV vaccine, which includes a cocktail of serotypes 1, 3, 4 (“bottle 1”) and 2, 6, 7, 8 (“bottle 2”) delivered in two separate doses at least 21 days apart. African horse sickness virus serotype 9 is not included in the vaccine because of reported cross-protection from serotype 6, and serotype 5 was removed from the original vaccine due to severe adverse reactions in some immunized horses (von Teichman and Smit 2008). However, cross-protection between AHSV serotypes 8 and 5 was recently reported in horses immunized with this polyvalent MLV vaccine, as well as between serotypes 6 and 9 (von Teichman *et al*. 2010). There are no published studies describing seroconversion and serotype-specific antibody titres among horses vaccinated in the field with the commercially available MLV AHSV vaccine.

Recently an inactivated and adjuvanted AHS-9 vaccine was shown to protect horses against homologous challenge (Lelli *et al.* 2013). The AHS-9 virus was inactivated using BEI and adjuvanted with ISA27VG and saponin before use. This vaccine was able to induce

high levels of neutralising antibody that persisted for at least 365 days and was able to prevent virus replication in vaccinated horses.

Cell-mediated immunity

Stauber *et al.* (1992) showed that AHSV can infect equine leukocytes *in vitro* especially cells of the monocytic linage. This infection was successful in monocyte and young macrophage cultures, but as macrophages matured they became more refractory to infection. The effects of cytokines and inflammatory mediators released by monocyte derived macrophages have been shown to play a role in the pathogenesis of BTV infection in ruminants (Drew *et al.* 2010b). The same probably applies to AHSV but has not been specifically described. Further studies on the method of entry of the virus into monocytes and the involvement of immune complexes and Fc receptors could shed more light on the type of immune response and its role in pathogenesis and pathology.

Hassan and Roy (1999) have shown that VP2 in the outer capsid of BTV is responsible for binding to mammalian cells (mouse fibroblast cells L929) and receptor mediated internalisation of the virus. They have also shown that VP2 binds to sialoglycophorin A of erythrocytes and proposed that the receptor on the L929 cell surface could also be a sialic acid. Ultra structural studies of BTV association with bovine red blood cells revealed that viral particles were present within indentations or deep membrane-lined pits of the erythrocyte membrane, these virus particles were also shielded from binding to specific antibody (Brewer and MacLachlan 1992). More intensive studies are needed to characterise the cell receptors and interaction with AHSV.

The role of the CMI against AHSV infection is not well described in the literature. Since BTV is very similar to AHSV and more has been published on this topic some findings that could be relevant will be mentioned. The potential role of cytotoxic T lymphocytes (CTLs) in the partial protection of sheep against BTV infection was demonstrated through the adoptive transfer of primed lymphocytes between homozygous sheep (Jeggo *et al.* 1984).

Andrew and co-workers studied the antigen specificity of ovine cytotoxic T-lymphocytes to BTV and reported the following: VP2 and NS1 were recognized by CTLs whereas VP7 was not, and cross-reactivity of CTL responses to BTV 1 and BTV 20 was only against their respective NS1 proteins (Andrew *et al.* 1995).

In a study in mice by Jones *et al.* (1996) CTL populations recognized target cells expressing the non-structural BTV proteins better than those expressing the structural proteins. However, VP7 by itself, if expressed by the recombinant capripox virus within the target host animal, can generate a protective immune response in the absence of neutralising antibody, although this does not appear to prevent virus replication (Wade-Evans *et al.* 1996).

CTL epitopes for NS1, VP2, VP3, VP5 and VP7 with multiple epitopes on NS1 and VP2 have been described by Janardhana *et al.* (1999). The presence of multiple $CD4^+$ and $CD8^+$ T-cell epitopes was demonstrated on the structural protein VP7 of BTV 8 by Rojas *et al*. (2011). Several authors have reported that there seems to be an inhibition to CTL stimulation induced by BTV, which could possibly compromise the host's immunity to other pathogens (Ghalib *et al*. 1985; Odeon *et al*. 1997). Similarly white-tailed deer infected with either haemorrhagic disease virus or BTV showed decrease lymphocyte counts and proliferation indexes to concanavalin A between days six and ten post infection (Quist *et al*. 1997).

Studies using a VP2 DNA vaccine have indicated that a significant lympho-proliferative response as well as a cytotoxic cellular reaction was present in vaccinated horses (Romito *et al*. 1999). This correlates with findings that VP2 is one of the major antigens for CTLs in sheep vaccinated against BTV (Andrew *et al*. 1995). While investigating the immune response to live attenuated AHSV 4, Pretorius *et al*. (2012) were able to demonstrate an increase in virus specific $CD8^+$ T-cells and a reduced frequency of $CD4^+$ cells in vaccinated horses. These authors also reported an up regulation of circulating B-cells with a concomitant elevation in IL-4 mRNA expression.

Recently the efficacy of two different vaccination strategies *vs.* homologous and heterologous prime-boost of naked DNA and recombinant modified vaccinia virus Ankara (rMVA) expressing VP2 and NS1 proteins of AHSV 4 were evaluated in IFNAR$^{(-/-)}$ mice against challenge with AHSV 4 or AHSV 9 (de la Poza *et al.* 2013). These authors reported that a homologous prime-boost strategy using rMVA/rMVA was more efficient in protection than the heterologous prime-boost of naked DNA and rMVA. These mice developed neutralising antibody to AHSV 4 but not to AHSV 9 but, they were however protected against challenge with a less virulent AHSV 9. This was ascribed to the NS1 component included in the vaccine similar to what was described for BTV (Calvo-Pinilla *et al*. 2009b).

Methods for the assessment of host immunity to AHSV

The use of horses in challenge studies with virulent AHSV is tempered by ethical considerations and the requirement for containment facilities, the limited number of horses that can be used, and the high mortality in susceptible horses. A useful laboratory mouse model was recently developed to study the pathogenesis and immune responses of mice to several viruses e.g. poliovirus, measles virus and Rift valley fever virus (Bouloy *et al*. 2001; Ohka *et al*. 2007; Ohno *et al*. 2007). This model, which utilizes genetically targeted (knock-out) mice lacking the IFN-α/β receptor (IFNAR$^{(-/-)}$ mice), was successfully adopted for BTV infection (Calvo-Pinilla *et al*. 2009a). These mice lack a functional IFN system rendering them highly susceptible to many viral infections while keeping the rest of their immune system intact, therefore making them a valuable tool for studying BTV pathogenesis and immune responses (Calvo-Pinilla *et al*. 2010; Calvo-Pinilla *et al*. 2012; Jabbar *et al*. 2013; Rojas *et al*. 2011).

Recently IFNAR$^{(-/-)}$ mice were also shown to be susceptible to AHSV (Castillo-Olivares *et al*. 2011). Mice that were inoculated via the subcutaneous route developed clinical signs that included rough hair coat, a hunched posture, reduction of mobility, lethargy, lacrimation and swelling of the eyelids. Interestingly these mice also developed neurological signs

that are not typical for equids with AHS and which were also not reported in IFNAR$^{(-/-)}$ mice infected with BTV. A possible reason was that these mice were challenged with AHSV that was passaged in mouse brain and that the virus developed a tropism for brain tissue. The establishment of a repeatable laboratory animal models like the IFNAR$^{(-/-)}$ mice could assist with the evaluation of vaccines to all the AHSV serotypes before they are tested in horses, however there are inherent limitations of this model and its relevance to the pathogenesis of AHSV infection in horses remains to be fully established.

The development and refinement of functional assays for the evaluation of CMI in horses in recent years have provided invaluable tools for the evaluation of vaccines and immunity to viral diseases (El' Garch *et al.* 2008; O'Neill *et al.* 1999; Paillot *et al*. 2006a; Paillot *et al*. 2007; Shrestha *et al*. 2006; Shrestha and Diamond 2004). Immune or type II IFN (IFN-γ) is secreted by thymus-derived (T) cells under certain conditions of activation and by natural killer (NK) cells. Although originally defined as an agent with direct antiviral activity, the more important properties of IFN-γ include regulation of several aspects of the immune response e.g. stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, orchestration of leukocyte-endothelium interactions, as well as effects on cell proliferation and apoptosis (Boehm *et al*. 1997).

INF-γ synthesis by T cells as a surrogate marker of CMI is now well accepted and has been used in horses for the evaluation of recombinant vaccines to West Nile virus (El' Garch *et al.* 2008), equine influenza virus (Paillot *et al*. 2006b), and equine herpesvirus-1 (Paillot *et al.* 2006a). These methods include the detection of IFN-γ secretion in sensitised peripheral blood mononuclear cells (PBMCs) after *ex vivo* stimulation, using the ELISpot assay and the detection of IFN-γ production by different cell phenotypes using cytokine-specific flow cytometry analysis.

More advanced immunological studies are possible in mice and specific T cell responses to BTV (Rojas *et al.* 2011) and AHSV (de la Poza *et al.* 2013) were demonstrated in

IFNAR$^{(-/-)}$ mice. Sensitised splenocytes from these mice produced INF-γ in the presence of specific antigens as detected by ELISpot. Further characterisation of these responses was achieved by demonstrating BTV specific cell proliferation (using ^{3}H Thymidine). The phenotype of BTV-specific T cells was determined by flow cytometry and intracellular staining of INF-γ. The infection or vaccination with BTV of C57BL/6 and IFNAR$^{(-/-)}$ mice, results in the activation of anti-BTV CD4^{+} T cells and CD8^{+} T cells and are therefore suitable models to study anti-BTV T cell immunity (Rojas *et al.* 2011).

The identification of immunogenic proteins and their respective T cell epitopes is important when investigating the host immune response and for the development of efficacious vaccines. When protein antigens are used to stimulate PBMCs for the assessment of T cell responses, the host's antigen presenting cells must first process the protein via the exogenous antigen processing pathway and present specific peptides on the cell surface, in association with MHC class II molecules. These peptides are then recognised together with portions of the MHC molecule and are the first step in the activation of CD4^{+} T cells. The activation of CD8^{+} T cells also require specific peptides presented by MHC I molecules on the surface of infected cells. These peptides originate from the endogenous antigen processing pathway (Tizard 2013).

The use of mixtures of overlapping peptides as antigen to stimulate T cells during cytokine flow cytometry have been well documented (He *et al*. 2001; Hoffmeister *et al*. 2003; Kern *et al*. 1998; Kern *et al*. 2000; Maecker *et al*. 2001; Scheffold and Kern 2000). These peptides can (if they are of the appropriate length) bind to the MHC molecules on the cell membrane and efficiently stimulate T cells, bypassing the pathway for antigen processing. The ideal length of peptides for stimulation of CD8^{+} T cells is nine amino acids, however Kiecker *et al.* (2004) reported that sets of peptides of 15 amino acids with 11 amino acids overlap represent a good compromise for stimulating both CD8^{+} and CD4^{+} T cells.

The sequences for peptides that can interact with MHC molecules and that can act as potential T cell epitopes can be predicted in-silico (Nielsen *et al*. 2003; Nielsen and Lund

2009) and screened *in vitro* for their ability to bind to MHC molecules. This was described for BTV VP 7 specific T cell epitopes in mice by Rojas *et al.* (2011).

The characterization and quantification of cytokine production is another important tool for understanding the nature of the innate and adaptive immune responses (T helper 1 or T helper 2 responses) (Mosmann and Coffman 1989). Currently there are few optimized assays for quantification of cytokines in horses (Allen *et al*. 2007; Quinlivan *et al*. 2007). More recently a SYBR Green real-time RT-PCR assay for evaluation of cytokine gene expression in the horse was developed and applied to a horse infected with AHSV (Sanchez-Matamoros *et al*. 2013). This assay is capable of detecting the up-regulation of genes to a panel of the most common cytokines involved in the innate and adaptive immune responses of horses and includes: interleukin (IL)-1β, IL-2, IL-4, IL-10, IL-12, TNF-α, IFN-β and IFN-γ. These cytokines were selected for their roles in the inflammatory response (IL-1-β, IL-12, TNF-α and IFN-β) and in the activation of cellular immunity (IL-2 and IFN-γ) and humoral immunity (IL-4 and IL-10).

Several assays for the detection of specific antibody to AHSV have been described which include: complement fixation (McIntosh 1955); indirect immunofluorescence (Davies and Lund 1974); agar gel immunodiffusion (House *et al*. 1990); serum-virus neutralisation (House *et al.* 1990; Howell 1962); immune-blotting (Laviada *et al*. 1992); blocking ELISA (Hamblin *et al*. 1990; Wade-Evans *et al*. 1993) and indirect ELISA (Maree and Paweska 2005; Williams 1987). The indirect ELISA, blocking ELISA and the complement fixation tests are the three antibody detection assays that are prescribed for international trade by the World Organization for Animal Health (Office International des Epizooties [OIE]) (OIE Code Commission 2012a).

All the assays listed above, except for the serum-virus neutralisation test can only detect AHSV group-specific epitopes, which is useful for epidemiological studies of horse populations from non-endemic areas. However, when evaluating specific antibody responses to individual serotypes, for example when horses are vaccinated with a

polyvalent vaccine, the serum-virus neutralisation test still remains the only available assay. In fact the OIE recommends a neutralising antibody titre of at least 20 to at least three AHSV serotypes in each of the two bottles of the MLV AHSV vaccine (OIE Code Commission 2012a).

Specific antibody to the non-structural NS3 protein can be used as a marker to distinguish horses that were vaccinated with a MLV vaccine, or infected with AHSV, from those vaccinated with an inactivated vaccine (Laviada *et al*. 1995).

Summary

Vaccination will likely play an important role in the control of any future incursions of AHSV into previously unaffected areas. The development of new generation vaccines will be challenging due to the apparent serotype-specific nature of immunity to the various AHSV serotypes. There is only one commercial vaccine available, but because it is a polyvalent MLV vaccine, it is only suitable for use in AHS endemic areas.

There are some questions regarding the immune status of horses that are regularly vaccinated in the field with the commercial MLV AHSV vaccine and there are no published studies describing seroconversion and serotype-specific antibody titres amongst these horses. This also applies particularly to the level and duration of neutralising maternal antibody in foals born to mares vaccinated with this vaccine. We have therefore used serum-virus neutralisation tests to investigate the neutralizing antibody profile to each of the nine AHSV serotypes in vaccinated field horses.

It is also known that AHSV is capable of inducing CMI in vaccinated horses and in mouse models, but the specific epitopes involved, and their role in protection in horses still needs to be clarified. We have therefore used an experimental ALVAC®-AHSV4 vaccine and overlapping peptide pools to characterise INF-γ responses in horses.

Chapter 3

PASSIVE TRANSFER AND RATE OF DECAY OF MATERNAL ANTIBODY AGAINST AFRICAN HORSE SICKNESS VIRUS IN SOUTH AFRICAN THOROUGHBRED FOALS[1]

Abstract

Reasons for performing study: African horse sickness is an insect transmitted, non-contagious disease of equids caused by African horse sickness virus (AHSV). Mortality can exceed 90% in fully susceptible horse populations. A live-attenuated (modified-live) cell culture-adapted (MLV) polyvalent AHSV vaccine is widely used to control AHS in endemic areas in southern Africa. Field studies detailing antibody responses of vaccinated horses are lacking.

Objectives: To determine antibody titres to the 9 known serotypes of AHSV in a cohort of brood mares that were regularly vaccinated with the MLV AHSV vaccine, and to measure the passive transfer and rate of decay of maternal antibody to the individual virus serotypes in foals.

Methods: Serum was collected from 15 mares before foaling and from their foals after foaling and monthly thereafter for 6 months. Antibody titres to each of the 9 AHSV serotypes were determined by serum-virus neutralisation assay.

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Results: There was marked variation in the antibody response of the mares to individual AHSV serotypes even after repeated vaccination, with consistently higher titre responses to some virus serotypes. Similarly, duration of maternally-derived antibodies in foals differed among serotypes and the titres of the mare-foal pares were proportionate.

Conclusions: Data from this study confirm variation of the neutralising antibody response of individual mares to repeated vaccination with polyvalent AHSV vaccine. Virus strains of individual AHSV serotypes included in the vaccine may vary in their inherent immunogenicity. Passively-acquired maternal antibodies to AHSV vary markedly among foals born to vaccinated mares, with further variation in the duration of passive immunity to individual AHSV serotypes.

Potential relevance: These data are relevant to the effective utilization of live-attenuated AHSV vaccines in endemic regions, and potentially to the use of vaccines in response to future incursions of AHSV into previously free regions. Further studies involving a larger population will be required to determine the optimal time for vaccinating foals.

Keywords

passive transfer; maternal antibody; African horse sickness virus; neutralising antibody; antibody half life

Introduction

African horse sickness (AHS) is an insect-transmitted, non-contagious disease of equids caused by African horse sickness virus (AHSV) (Guthrie 2007; Theiler 1921). AHSV is spread by haematophagous *Culicoides* midges that serve as biological vectors of the virus; *C. imicola* and *C. bolitinos* are the most important vectors in South Africa (Meiswinkel and Paweska 2003). AHSV is a member of the family *Reoviridae* genus *Orbivirus* (Verwoerd *et al.* 1979), of which 9 serotypes have been described (Howell 1962; McIntosh 1958). AHSV is endemic in sub-Saharan Africa but several epidemics have occurred beyond this region (MacLachlan *et al.* 2010; Mellor and Boorman 1995). As a consequence of its severity and

because it is able to spread rapidly and without apparent warning from its historically endemic areas, AHS is listed by the World Organization for Animal Health (Office International des Epizooties [OIE]) as important to the international trade and movement of horses (OIE Code Commission 2010). There is a significant but undetermined risk that AHSV could spread in the future to Europe and beyond, as recently has occurred with related bluetongue virus that also is transmitted by *Culicoides* midges (MacLachlan *et al.* 2010; Purse *et al*. 2005).

Vaccination is currently central to the control of AHS in endemic areas, and vaccination would likely be used to control any future incursions of AHSV into historically free regions such as Europe. Several AHS vaccines have been developed and used in the past including: a polyvalent live-attenuated virus (modified-live [MLV]) vaccine of adult mouse brain origin, a polyvalent cell culture-adapted MLV, and an inactivated cell culture propagated AHSV 4 vaccine. The inherent limitations of each of these vaccine types have previously been reviewed (House 1993; House 1998; MacLachlan *et al.* 2007). There is clearly a need for safe and efficacious vaccines to facilitate the continued international movement of horses, but currently only a polyvalent cell-culture-adapted MLV AHSV vaccine is commercially available.

The MLV cell-culture-adapted polyvalent AHSV vaccine that is used widely throughout southern Africa was introduced in the 1960s (Erasmus 1978). Horses in the AHSV infected area in South Africa are vaccinated annually with this vaccine. The vaccine is administered in two doses, the first includes a cocktail of serotypes 1, 3, 4 (so-called “bottle 1”) and the second includes serotypes 2, 6, 7, 8 (“bottle 2”). Serotypes 5 and 9 are not included in this polyvalent vaccine because of reported cross-protection from serotypes 8 and 6, respectively (von Teichman *et al.* 2008). The two vaccine doses are administered at least 21 days apart. Despite the widespread use of this polyvalent MLV AHSV vaccine in southern Africa, there are few published data of antibody titres in horses that have been vaccinated in the field with this preparation and, further, there is no definitive

information on the level and duration of maternal antibody in foals (von Teichman *et al.* 2008).

Given the importance of vaccination to the control of AHS, the objectives of this study were to determine antibody titres to each of the 9 serotypes of AHSV in a cohort of breeding mares that were regularly vaccinated with the MLV cell-culture-adapted AHSV vaccine, and to measure passive transfer and rate of decay of maternal antibody to the individual virus serotypes in their foals.

Materials and methods

Study population and blood collection

Fifteen multiparous mares in late gestation (that foaled during the months of August and September, 2006) that were resident on a Thoroughbred stud farm in the surveillance zone of the AHS controlled area in the Western Cape Province of South Africa were evaluated. This comprised all the mares on the farm that were due to foal during August and September, which are the months most distant to the expected peak AHSV transmission season. This area is subject to an active AHSV surveillance programme that includes clinical surveillance and serological surveillance of negative sentinel horses. No cases of AHS have been detected within a radius of at least 30 km of this farm since the inception of the surveillance programme in 1996. All 15 mares had been annually vaccinated with the polyvalent AHSV vaccine®[2] and, during the 2006 foaling season, 12 were vaccinated during the last trimester of pregnancy (according to normal farm practice) and 3 were not (Table 3-1). Mares were vaccinated with AHSV serotypes 2, 6, 7, 8 (bottle 2) in mid-June and serotypes 1, 3, 4 (bottle 1) in mid-July. Blood for harvesting of serum was collected from each mare in mid-August (28 days after the last vaccination). Sample size of the study cohort was, therefore, determined by availability and

[2] Onderstepoort Biological Products, Onderstepoort, Gauteng, South Africa

practicality. Serum was collected from the foals of these mares within 9 days (range, 3 to 9 days) after foaling and monthly thereafter until the foals were 6 months of age. No colostrum or plasma supplementation was administered to any of the foals in the study. The study was performed with full owner consent and was approved by the Research Committee of the Faculty of Veterinary Science as well as the Animal Use and Care Committee of the University of Pretoria under protocol V052/07.

Serum neutralisation test

Antibody titres to each of the 9 AHSV serotypes were determined by serum-virus neutralisation assay, essentially as previously described (Howell 1962; Howell *et al*. 2002; McIntosh 1958). Briefly, each serum sample was inactivated at 56 °C for 30 min before testing and serum dilutions were made in Minimum Essential Medium[3] with 2 g/l $NaHCO_3$[4], gentamycin sulphate (Genta 50)[5] 0.05 mg/ml and 5% foetal calf serum[6]. An estimated 100 $TCID_{50}$ of cell-culture-adapted prototype strains of each AHSV serotype[7] were added to duplicate serial 2-fold serum dilutions (from 1:10 to 1:320), and plates were incubated for one hour at 37 °C prior to addition of a suspension of Vero (African green monkey kidney) ATCC CCL81 cells containing an estimated 480,000 cells/ml. The development of cytopathic effects was monitored daily for 4 to 5 days. Titres were determined as the reciprocal of the highest serum dilution that provided >50% protection of the cell monolayers.

Data analyses

A Microsoft Access® database was used and statistical analyses were performed with Microsoft Excel® (Microsoft), Kinetica® 5.1 (Thermo Scientific) and SigmaPlot® (Systat

[3] Highveld Biological, Modderfontein, Gauteng, South Africa
[4] Merck, Wadeville, Gauteng, South Africa
[5] Virbac Animal Health, Centurion, Gauteng, South Africa
[6] Sigma-Aldrich, Johannesburg, Gauteng, South Africa
[7] ARC Onderstepoort Veterinary Institute, Gauteng, South Africa

Software) software packages. The SNT titre distributions of the 9 AHSV serotypes in mare and foal sera were statistically described with box-and-whisker plots. Spearman rank order correlation was calculated between SNT titres of mare sera and those of the first serum collected from their respective foal. The biological half-lives in foals of maternal antibody to each of the 9 AHSV serotypes were estimated by the exponential decay equation $T_{1/2} = - (\ln 2) / \beta$ where $T_{1/2}$ is the decay time (days) and β is the regression coefficient. This analysis was performed on Ln transformed values from individual foals using least square means and mean antibody half-life for the cohort was calculated. Time until SNT titres became negative at a 1:10 serum dilution for each serotype was estimated using the Kaplan-Meier product-limit estimate of the survivor function (Morris *et al*. 1994).

Results

Although the mares that were evaluated in this study had been annually revaccinated with polyvalent MLV AHSV vaccine, there were marked differences in the neutralising antibody titres to each of the 9 AHSV serotypes (Table 3-1; Figure 3-1). The highest SNT titres were most consistently detected against AHSV serotypes 1, 4, 6 and 9, with a median titre value of at least 120. Titres were lower against other serotypes, specifically serotype 8 (median = 112), serotypes 2 and 3 (median = 56), serotype 7 (median = 28) and serotype 5 (median = 20). Neutralising antibodies were least consistently detected against serotype 5, with 5 mares (33.3%) testing negative to this virus serotype including 2 mares (2/12) that had recently been vaccinated.

Neutralising antibody titres against each of the 9 AHSV serotypes were determined on sera collected from 15 foals between 3 and 9 days after birth (Table 3-2; Figure 3-1). The highest titres were detected against serotype 1 (median titre = 224), followed by serotypes 7 (median = 80) 4 (median = 40), 2, 3, 6 and 9 (all with median values of 28), and serotypes 5 and 8 (median = 20). One foal had no detectable SNT antibodies to any of

the 9 AHSV serotypes. One foal had low titres to serotypes 1, 7 and 8 and was negative for the remainder.

The correlation between mare and foal SNT antibody titres was 0.50 ($P < 0.00001$), indicating that AHSV titres of each mare-foal pair were proportionate. Mare #15 was seronegative to both serotypes 2 and 5, and she also had only low SNT titres to the other virus serotypes (range, 20 to112). The foal from this mare also tested negative to all AHSV serotypes.

Analysis of the SNT titres to AHSV in 6 foals that were tested monthly for 6 months had an estimated mean half-live for neutralising antibodies to all 9 serotypes of 20.5 (± 2.6 SD) days, with a range of 15.4 days for serotype 8 to 22.6 days for serotype 3. The overall product-limit estimate for the mean time until the SNT became negative at a 1:10 dilution was 96 days for all 9 serotypes, with a range of 62 days for serotype 5 to 128 days for serotypes 3 and 4 (Table 3-3). The survival curves for antibody duration to the individual serotypes were significantly different ($P < 0.001$) (Figure 3-2).

Table 3-1 Serum-virus microneutralisation test titres to the 9 serotypes of AHSV of pregnant mares (n = 15) during the 2006 foaling season. Sera were collected on 15 August 2006. Animals identified in bold were not vaccinated during 2006 whereas the others were vaccinated in June and July of that year

Dam no.	AHSV 1	AHSV 2	AHSV 3	AHSV 4	AHSV 5	AHSV 6	AHSV 7	AHSV 8	AHSV 9
1	160	80	160	160	20	320	56	112	160
2	320	160	320	320	80	160	160	224	224
3	**112**	**28**	**56**	**80**	**Neg**	**56**	**20**	**Neg**	**80**
4	160	80	80	320	80	160	80	320	224
5	160	56	80	224	14	80	14	80	224
6	320	56	40	160	40	80	28	112	224
7	224	40	112	320	28	320	56	112	160
8	**20**	**10**	**40**	**80**	**Neg**	**56**	**28**	**40**	**112**
9	80	10	80	224	28	320	20	112	224
10	**80**	**80**	**80**	**160**	**20**	**160**	**40**	**80**	**80**
11	14	Neg	Neg	Neg	14	Neg	Neg	112	20
12	224	80	56	320	112	320	56	320	160
13	160	56	14	80	Neg	56	20	40	80
14	**160**	**20**	**14**	**40**	**Neg**	**120**	**Neg**	**20**	**112**
15	40	Neg	20	112	Neg	80	20	20	56
Mean	148.0	50.4	76.8	173.3	29.0	152.5	39.8	113.6	142.6
Median	160	56	56	160	20	120	28	112	160

Table 3-2 Serum-virus microneutralisation test titres to the 9 serotypes of AHSV from foals (n = 15) tested between 3 to 9 days after foaling.

Foal no.	AHSV 1	AHSV 2	AHSV 3	AHSV 4	AHSV 5	AHSV 6	AHSV 7	AHSV 8	AHSV 9
1	320	56	56	56	28	320	112	10	80
2	320	56	112	224	28	320	224	40	112
3	320	14	28	40	10	112	80	Neg	28
4	28	Neg	Neg	Neg	Neg	Neg	28	10	Neg
5	320	56	56	160	40	56	320	80	80
6	320	28	28	56	20	28	112	40	56
7	224	40	56	112	56	224	112	56	112
8	20	Neg	20	28	10	28	56	14	10
9	224	28	224	320	56	320	160	80	160
10	160	80	28	28	10	28	28	10	28
11	40	Neg	Neg	Neg	28	10	Neg	56	Neg
12	224	112	40	224	112	160	80	160	224
13	160	28	14	28	10	20	28	10	10
14	320	10	14	20	10	28	56	20	14
15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Mean	200	33.9	45.1	86.4	27.9	110.3	93.1	39.1	60.9
Median	224	28	28	40	20	28	80	20	28

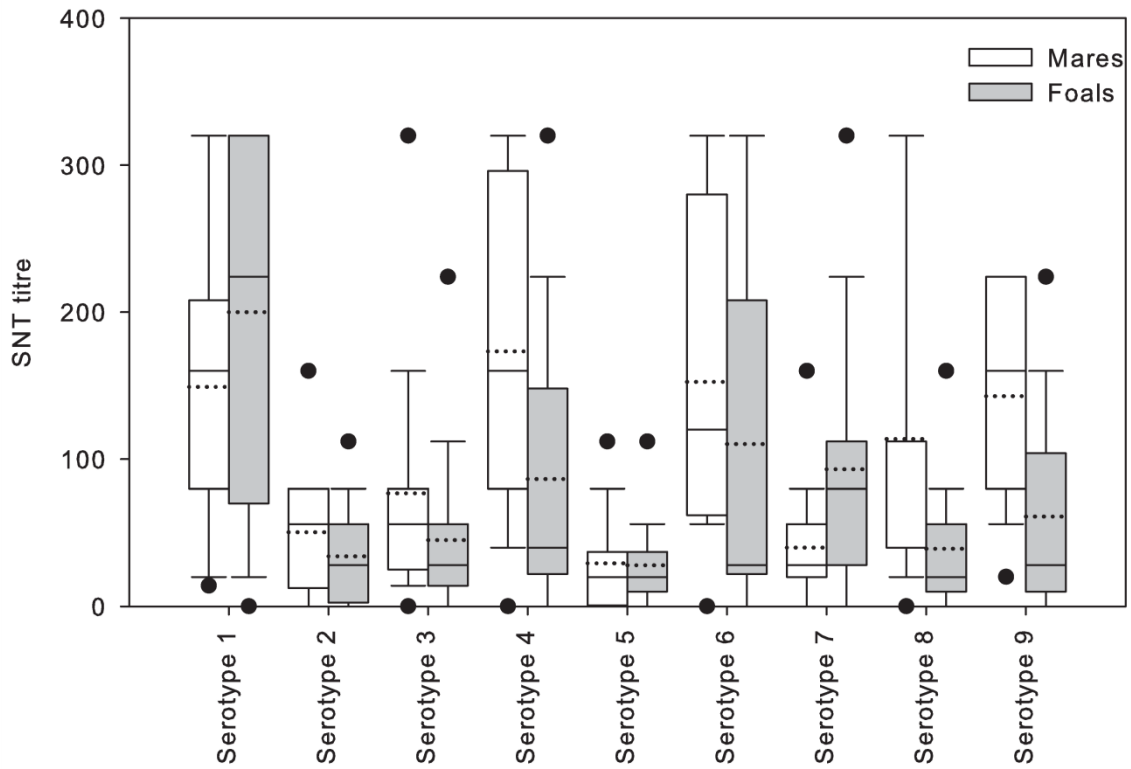

Figure 3-1 Box-and-whisker plot comparing SNT titres to the various AHSV serotypes from mares (n = 15) before foaling and their foals within 9 days after birth. The dotted lines represent mean titres.

Table 3-3 Half-life and duration of neutralising maternal antibody in foals (n = 6) to individual AHSV serotypes (1 – 9)

AHSV serotype	Mean biological half-life in days (median)	95% confidence intervals	Mean product limit estimate of antibody duration in days (median)	95% confidence intervals
1	21.5 (22.1)	26.0 – 16.8	121 (120)	111 - 132
2	18.4 (15.7)	41.7 to -9.8	66 (66)	52 - 81
3	22.6 (22.9)	29.5 – 15.2	128 (120)	119 - 137
4	23.3 (22.5)	28.4 – 18.0	128 (120)	120 - 137
5	23.3 (19.6)	58.6 to -21.7	62 (64)	49 - 74
6	20.6 (20.5)	29.9 – 10.5	115 (116)	103 - 126
7	21.1 (18.7)	37.5 – 2.8	96 (94)	84 - 108
8	15.4 (14.1)	19.4 – 11.1	75 (75)	59 - 106
9	18.7 (17.4)	28.1 – 8.5	76 (74)	61 - 90
Mean	20.5		96.3	

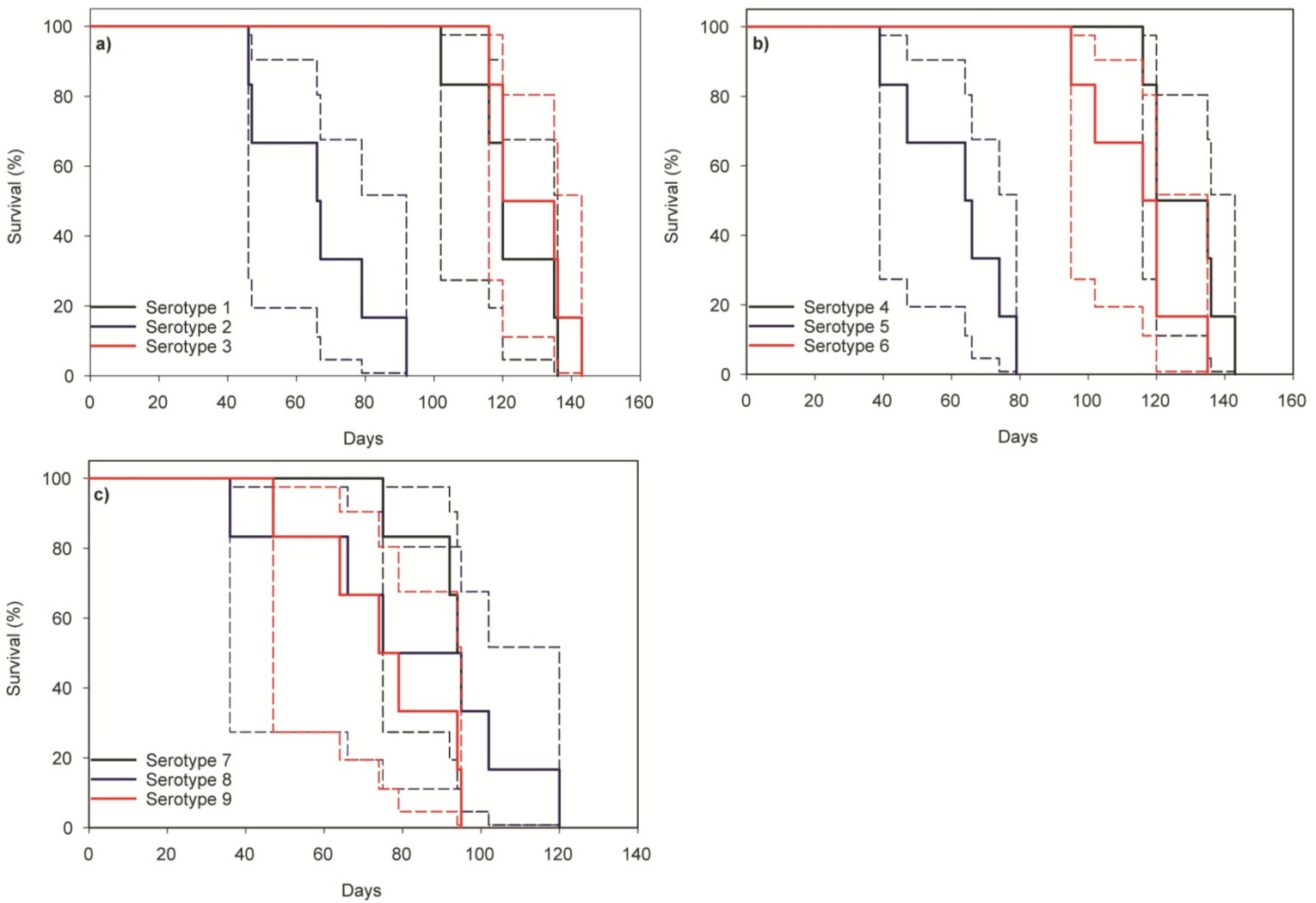

Figure 3-2 Survival curves: product limit estimates of persistence of maternal antibody to 9 AHSV serotypes in foals. Serotypes 1-3, 4-6 and 7-9 are represented in figures (a), (b) and (c), respectively. The dashed lines represent the 95% confidence intervals.

Discussion

In the current study, a cohort of 15 mare-foal pairs on a well-managed stud in the AHS surveillance zone was monitored to evaluate neutralising antibody titres in AHSV vaccinated horses. The mares were routinely vaccinated with a commercial polyvalent MLV AHSV vaccine, and the farm was shown to be free of AHSV infection since at least 1996.

There was marked variation in the SNT response of mares to the different AHSV serotypes contained in the vaccine. Specifically, serotypes 1, 4, 6 and 9 consistently induced the highest SNT titres in mares whereas the response to serotypes 5 and 8 was notably weaker and several horses had no demonstrable neutralising antibody to serotype 5. Serotype 5 is not included in this vaccine because of its purported cross-reactivity with serotype 8, however responses to serotype 8 also were consistently weak. In contrast, serotype 9 is also not included in the vaccine because of its cross-reactivity with serotype 6, yet high SNT titres were consistently detected against serotype 9. SNT titres to serotype 9 and serotype 6 were similar (usually within 1 dilution step). Cross-neutralisation between serotypes 6 and 9 as well as between several of the other serotypes were previously demonstrated (Howell 1962).

Considerable variation was evident in the antibody response of individual mares to the different AHSV serotypes. The study mares were all repeatedly vaccinated during prior seasons, suggesting that that repeated annual vaccination does not reliably induce an anamnestic response to all AHSV serotypes in every horse. Some mares developed high antibody titres to specific AHSV serotypes whereas titres to other serotypes remained low throughout the animal's life despite repeated vaccination. While this finding does not indicate that horses with weak SNT responses to individual virus serotypes are not immune to AHSV infection, it is consistent with field reports of severe AHS in some well-vaccinated horses (Guthrie and Quan 2009). Furthermore, the absent or low antibody titres to some serotypes in individual mares could impair passive protection of their foals.

Passive transfer of antibodies via colostrum is important for protection of foals against pathogens they encounter during the first few months of life. The survival of passively acquired antibodies against several equine pathogens has been documented (Hullinger *et al*. 1998; Wilson *et al*. 2001), but has not previously been determined for AHSV**.** Foals born to mares vaccinated with the neurotropic (mouse brain attenuated) MLV AHSV vaccine only had serum antibodies to AHSV after suckling colostrum from immune mares (Alexander and Mason 1941). Although pre-suckling foal sera and periparturient (pre-foaling) mare sera were not analysed in the current study, antibody titres detected in the post-suckling samples strongly suggest transfer of maternal antibody from the dam to the foal for all 9 virus serotypes. The same authors also reported higher antibody titres in some foals as compared to those of their dam, which was also identified in the current study with serotypes 1 and 7 in particular.

Alexander and Mason (1941) did not calculate the half-life of maternal antibody to AHSV but reported that the duration of demonstrable antibodies in foals was correlated with the mare's SNT titre at the time of foaling. They further reported that antibodies were generally not detectable by 6 months of age. Typical half-lives of maternal antibody to other pathogens include 27 and 39 days, respectively, for IgGa and IgGb to influenza virus; 28 and 34 days, respectively, for IgGa and IgGb to tetanus toxoid (Wilson *et al.* 2001) and 32 days for equine arteritis virus (Hullinger *et al.* 1998). The mean half-live of 20.5 days for passively-acquired maternal neutralising antibody to AHSV in the current study was calculated based on 6 foals and is therefore imprecise. However, the estimate is similar although somewhat shorter than for the other mentioned pathogens. There is no literature on the level of maternally derived antibody that is considered to be protective for AHSV in foals, but the vaccine manufacturer relies on a minimum titre of 16 for protection of vaccinated horses (Smit TK personal communication).

Data from this study confirm variation of the neutralising antibody response of individual Thoroughbred mares to repeated vaccination with an MLV polyvalent AHSV vaccine.

Furthermore, the data suggest that virus strains of individual AHSV serotypes included in the polyvalent vaccine vary in their inherent immunogenicity. Lastly, passively-acquired maternal antibodies to AHSV vary markedly among foals born to vaccinated mares, with further variation in the duration of passive immunity to individual AHSV serotypes. More data representative of a larger geographical distribution and study population are needed before definitive recommendations on the optimal timing of AHSV vaccination of foals in endemic areas can be made.

Chapter 4

SEROLOGICAL RESPONSE OF FOALS TO POLYVALENT AND MONOVALENT LIVE-ATTENUATED AFRICAN HORSE SICKNESS VIRUS VACCINES

Abstract

African horse sickness (AHS) is typically a highly fatal disease in susceptible horses and vaccination is currently used to prevent the occurrence of disease in endemic areas. Similarly, vaccination will likely be central to the control of any future incursions of AHS virus (AHSV) into previously unaffected areas. Horses in the AHSV-infected area in South Africa are vaccinated annually with a live-attenuated (modified-live virus [MLV]) vaccine, which includes a cocktail of serotypes 1, 3, 4 (bottle 1) and 2, 6, 7, 8 (bottle 2) delivered in two separate doses at least 21 days apart. In this study, the humoral immune (antibody) response of horses immunized with this polyvalent MLV AHSV vaccine was evaluated and compared to the immune response elicited to monovalent MLV AHSV serotypes. Naïve foals were immunized with either the polyvalent AHSV vaccine, or a combination of monovalent vaccines containing individual AHSV serotypes 1, 4, 7 or 8. There was a marked and consistent difference in the immunogenicity of individual virus serotypes contained in the vaccine. Specifically, foals most consistently seroconverted to AHSV 1 and responses to other serotypes were highly variable, and often weak or not detected. The serotype-specific responses of foals given the monovalent vaccines were similar to those of foals given the polyvalent preparation suggesting that there is no obvious enhanced immune response through the administration of a monovalent vaccine as opposed to the polyvalent vaccine. The polyvalent vaccine offers advantages in terms of

production lead time, production cost, and ease of use for the consumer with less "resting time" required post vaccination compared to multiple monovalent vaccines.

Key words:

African horse sickness virus; modified-live virus vaccine; vaccination; neutralizing antibody

Introduction

African horse sickness (AHS) is an insect-transmitted, non-contagious disease of equids caused by African horse sickness virus (AHSV) (Theiler 1921). African horse sickness virus is transmitted to horses by haematophagous *Culicoides* midges that serve as biological vectors of the virus (Meiswinkel *et al.* 2003). African horse sickness virus is a member of the family *Reoviridae* genus *Orbivirus* (Verwoerd *et al.* 1979), of which nine serotypes have been described (Howell 1962; McIntosh 1958). African horse sickness virus is listed by the World Organisation for Animal Health (OIE) as important to the international trade and movement of horses and disease freedom of a country or zone is officially recognised by the OIE (OIE Code Commission 2012b). African horse sickness virus is widespread in parts of sub-Saharan Africa and South Africa. All nine serotypes occur in South Africa where AHS is classified as a notifiable disease.

African horse sickness is typically a highly fatal disease in susceptible horses so vaccination is currently used to prevent the occurrence of disease in endemic areas (Guthrie 2007). Similarly, vaccination will be central to the control of any future incursions of AHSV into previously unaffected areas. Several AHSV vaccines have been previously developed and used including: a polyvalent live-attenuated (modified-live virus [MLV]) vaccine of adult mouse brain origin, a polyvalent cell culture-adapted MLV vaccine, and an inactivated AHSV 4 vaccine. These vaccines and their potential limitations have been reviewed previously (House 1993; MacLachlan *et al.* 2007). Although new generation vaccines have been described recently (Chiam *et al.* 2009; Guthrie *et al.* 2009; Martinez-Torrecuadrada *et al.* 1996; Roy *et al.* 1996), only MLV AHSV vaccines are currently

commercially available in South Africa (Erasmus 1978; von Teichman *et al.* 2010; von Teichman *et al.* 2008), Senegal (Diouf *et al*. 2013; van den Boom and Sloet van Oldruitenborgh-Oosterbaan 2013) and Ethiopia (Aklilu *et al*. 2012).

The MLV AHSV vaccine that is currently used widely in southern Africa was first introduced in the 1960s (Erasmus 1978) and has since undergone some modifications. AHSV 9 is not included in the vaccine because of the absence of serotype 9 circulating in South Africa (at least prior to 2005) and serotype 5 was removed from the original vaccine due to severe adverse reactions in some immunized horses (von Teichman *et al.* 2008). However, cross-protection between AHSV serotypes 5 and 8 was recently reported in horses immunized with this polyvalent MLV vaccine, as well as between serotypes 6 and 9 (von Teichman *et al.* 2010). The MLV vaccine currently used in southern Africa includes a cocktail of serotypes 1, 3, 4 (bottle 1) and 2, 6, 7, 8 (bottle 2) delivered in two separate doses at least 21 days apart.

Few published studies have evaluated seroconversion and serotype-specific antibody titres among horses vaccinated in the field with the commercially-available South African MLV AHSV vaccine. The goal of the present study, therefore, was to evaluate the humoral immune (antibody) response of horses immunized with the polyvalent MLV AHSV vaccine, and to compare it with that of horses immunized with selected individual monovalent MLV AHSV serotypes contained in the vaccine. Findings from the study are relevant to the control of AHS in endemic and potentially, future incursional areas.

Materials and methods

Study population

Three groups of Thoroughbred foals (n = 46) 7 to 10 months of age and confirmed to be seronegative by an AHSV-specific ELISA (Maree *et al.* 2005)were used to evaluate neutralizing antibody responses to MLV polyvalent and monovalent AHSV vaccines. Ethical approval was granted by the Animal Use and Care Committee of the University of

Pretoria (protocol V052/07). The foals were all resident on a commercial stud farm located near Wellington in the Western Cape, South Africa. This farm is located in an area that is subject to an active AHSV surveillance programme that includes clinical surveillance and the monitoring of seronegative sentinel horses. No cases of AHS have been detected within a radius of at least 30 km of this farm since the inception of the surveillance programme in 1996. The foals were segregated into groups by gender, and individual foals remained within the same group throughout the study. All foals were identified with a microchip and given the same routine veterinary care, including immunization against equine influenza, tetanus and botulism.

Study design

The 46 foals were randomly assigned to three groups (Group I, II and III) and immunized at specific intervals with either the polyvalent MLV AHS or monovalent serotypes of the vaccine according to the schedule listed in Table 4-1. Serotypes 1 and 4 were selected as representatives of the MLV strains contained in bottle 1, and serotypes 7 and 8 as representatives from bottle 2. As these were horses from a commercial stud it was not possible to give a second monovalent vaccination to horses in groups II and III because they were vaccinated with the polyvalent vaccine to comply with statutory requirements. Blood for harvesting of serum was collected from each foal and a blinded approach was used to determine the neutralising antibody titres to all nine AHSV serotypes on days, 56, 112, 196 and 238 after first vaccination.

Table 4-1 Treatment groups, vaccination intervals, and AHSV vaccine serotypes included in the vaccines administered to the foals in the different groups included in this study.

		AHSV serotype(s) and day of vaccination			
Group	Number of horses	Day 0 (Vaccination 1)	Day 28 (Vaccination 2)	Day 112 (Vaccination 3)	Day 168 (Vaccination 4)
I	16[†]	1*, 3*, 4*	2[#], 6[#], 7[#], 8[#]	1, 3, 4	2, 6, 7, 8
II	15[†]	1	8		
III	15[††]	4	7		

*AHSV serotypes included in "bottle 1" of commercial polyvalent MLV AHSV vaccine
[#]AHSV serotypes included in "bottle 2" of commercial polyvalent MLV AHSV vaccine
[†]Two foals were removed from the respective groups after Day 56
[††]One foal was removed from the group after Day 56

Vaccine

Foals of Group I were vaccinated subcutaneously with 2 ml of the commercially available polyvalent MLV cell culture-adapted AHSV vaccine (Onderstepoort Biological Products SOC Ltd, Onderstepoort, South Africa) given as two doses 28 days apart. Foals of Group II were initially vaccinated with monovalent AHSV vaccine serotype 1 followed by monovalent AHSV vaccine serotype 8, 28 days later. Similarly, foals of Group III were vaccinated at a 28 day interval, initially with monovalent AHSV serotype 4 and 28 days later with serotype 7. The monovalent AHSV vaccine serotypes 1, 4, 7 and 8 were prepared and supplied by the manufacturer to contain the same viral load and seed stock viruses as those used in the commercial polyvalent MLV vaccine (Table 4-1) and which meet the OIE requirements. For a live attenuated AHSV vaccine, the current OIE manual recommends a minimum immunizing dose of 1 x 10^3 pfu/ml for each serotype in the polyvalent vaccine and a minimum infectivity titre of the final product of 1 x 10^3 pfu/ml (OIE Code Commission 2012b).

All foals were vaccinated with an equine influenza-tetanus combination (ProteqFlu-Te® MERIAL) consisting of 2 equine influenza constructs in a canarypox vector and a tetanus

toxoid and formalinised aluminium hydroxide gel adsorbed toxoid of *Clostridium botulinum* types C and D (Onderstepoort Biological Products SOC Ltd) as separate vaccinations on Day 0 and repeated on Day 28.

Serum-virus microneutralisation test

The serum neutralising antibody titre in foal sera to each of the nine reference strains of AHSV (Howell 1962; McIntosh 1958) was determined by the serum neutralisation test (SNT) according to the procedure previously described for equine encephalosis virus (Howell *et al.* 2002). Briefly, each serum sample was inactivated at 56 °C for 30 min and serum dilutions were made in Minimum Essential Medium (Highveld Biological, Modderfontein, Gauteng, South Africa) with 2 g/l $NaHCO_3$ (Merck, Wadeville, Gauteng, South Africa), 0.05 mg/ml gentamycin sulphate (Genta 50, Virbac Animal Health, Centurion, Gauteng, South Africa), and 5% foetal calf serum (Sigma-Aldrich, Johannesburg, Gauteng, South Africa). An estimated 100 $TCID_{50}$ of cell-culture-adapted prototype strains of each AHSV serotype (courtesy of the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI), Gauteng, South Africa) were added to duplicate serial 2-fold serum dilutions (1:10 to 1:320), and plates were incubated for one hour at 37 °C prior to addition of a suspension of Vero (CCL81) (African green monkey kidney) cells containing an estimated 4.8 x 10^5 cells/ml. The development of any cytopathic effect was monitored daily for 4 to 5 days. Titres were determined as the reciprocal of the highest serum dilution that provided >50% protection of the cell monolayers. Titres of ≥10 were considered as positive.

Statistical analyses

Data were captured in Microsoft Access® database and statistical analysis was performed with Microsoft Excel® (Microsoft) and SigmaPlot® (Systat Software) software packages. The SNT titre distributions to the nine AHSV serotypes were statistically described with box-and-whisker plots. The median titre for all foals within each of the treatment groups was used to describe the antibody kinetics to individual serotypes of AHSV and the

Wilcoxon rank-sum test was used to compare the groups and a p value <0.05 was considered significant.

Results

Treatment group I (n=16)

Foals that were vaccinated with polyvalent MLV AHSV serotypes 1, 3, 4 (bottle 1) and serotypes 2, 6, 7, 8 (bottle 2) on Day 0 and 28 respectively, developed substantial neutralising antibody titres (median titre = 120) to AHSV 1 by Day 56, and had variable responses to AHSV 3 (median titre = 5). In contrast, all foals in Group I were seronegative to serotypes 4, 5, 6, 8 and 9 at Day 56 and only 44% (7/16) had titres to serotype 7 (range 10 – 80; median = 40). Two foals were removed from the cohort at this time for reasons unrelated to the study. The remaining 14 foals were re-vaccinated with the same vaccine in the same order on Day 112 (bottle 1) and Day 168 (bottle 2). Twenty-eight days after the last vaccination (Day 196) the median AHSV neutralising antibody titres increased to > 320 (serotype 1), 24 (serotype 3), 14 (serotypes 6 and 7), 12 (serotype 2) and 10 (serotype 9). Forty-two days later (Day 238) the median SNT titres in the group decreased to 160 (serotype 1), 20 (serotype 2) and were seronegative (< 10) to the remaining serotypes (Figure 4-1).

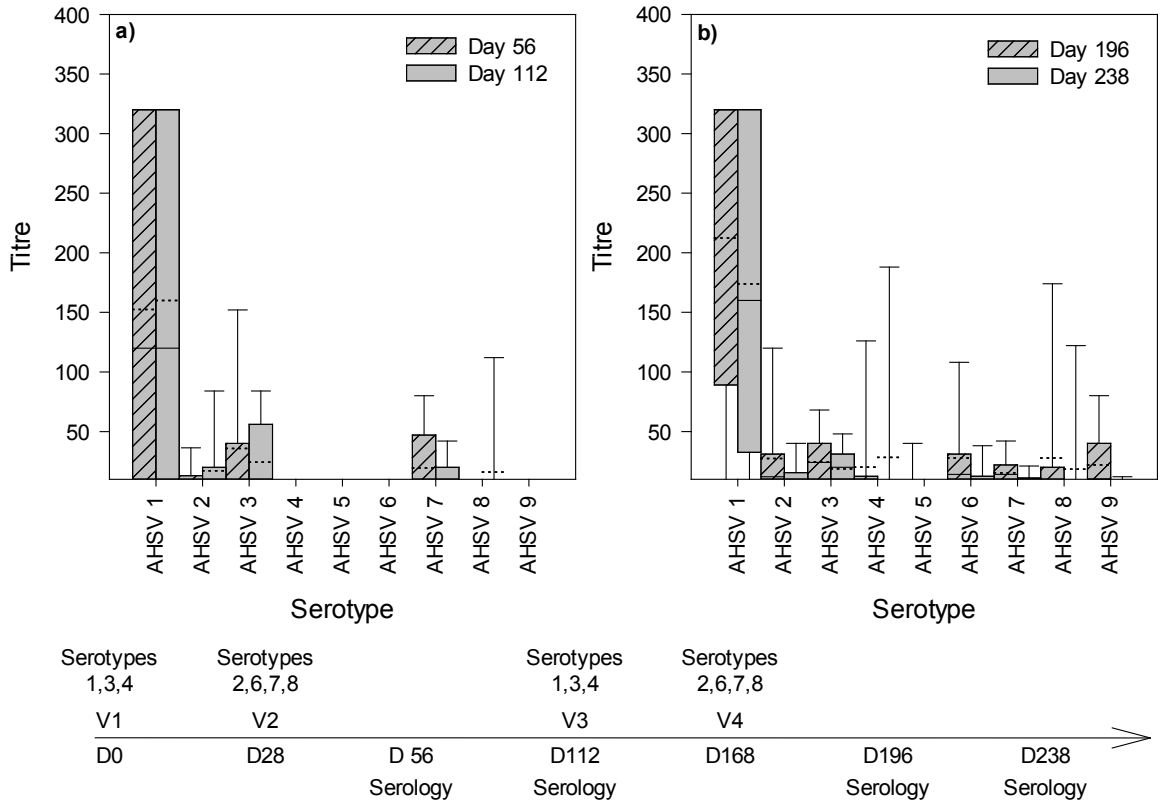

Figure 4-1 Box-and-whisker plots describing the SNT titre distributions of foals in Group I to the 9 AHSV serotypes after vaccination with the polyvalent MLV cell culture-adapted AHSV vaccine (Onderstepoort Biological Products SOC, Ltd, Onderstepoort, RSA).The dotted line represents the mean titres. (a) Represents the titres on days 56 and 112 after first vaccination with “bottle 1” and “bottle 2” including, respectively, serotypes 1, 3, 4 and 2, 6, 7, 8 . (b) Represents the titres after re-immunisation with the same vaccine preparations used in (a). The time-line for the various vaccinations and the respective vaccine serotypes used are presented below the graphs

The temporal occurrence of seroconversion of foals to individual AHSV serotypes following vaccination is represented in (Figure 4-2). A single foal in Group I developed an SNT titre (10) to AHSV 5 only at Day 112, however this same foal had a titre of 226 to AHSV 8 at the same time. Similarly, although the foals were all seronegative to AHSV 9 at Day 112, 8/14 foals (57%) were seropositive by Day 196 when 10/14 (71%) were also seropositive to AHSV 6.

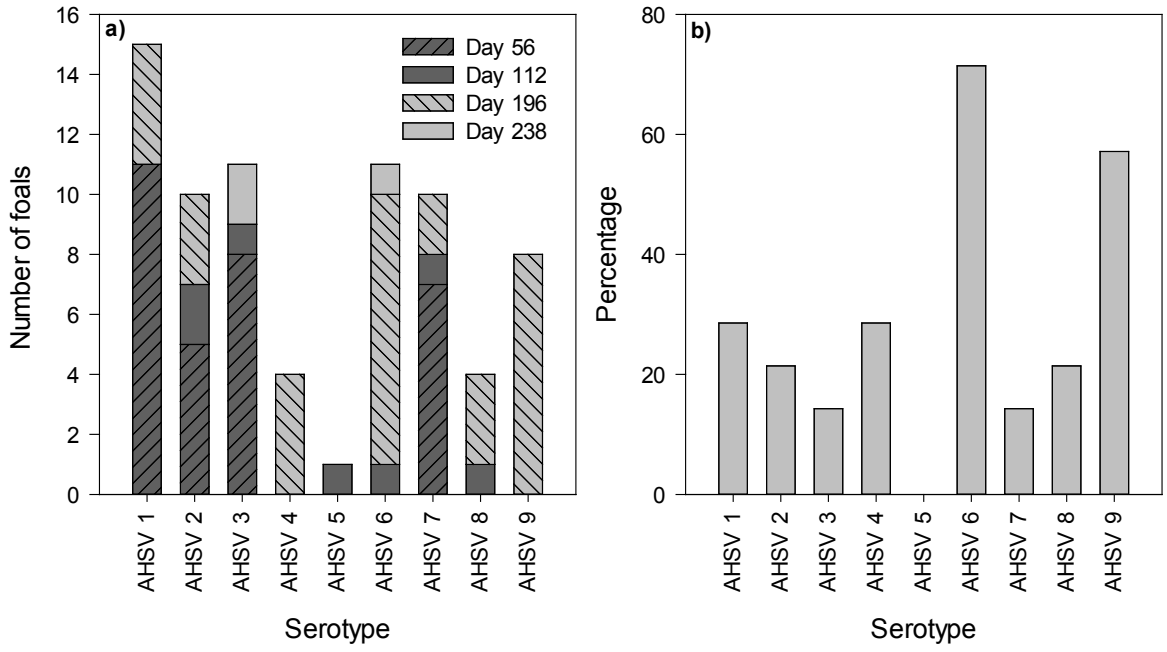

Figure 4-2 Serological response of foals in Group I following re-immunisation with AHSV serotypes 1, 3, 4 (bottle 1 at (Day 112) and AHSV serotypes 2, 6, 7, 8 (bottle 2 at Day 168). Figure (a) represents the cumulative number of foals that recorded titres ≥ 10 at days 56, 112, 196 and 238 after immunisation. Figure (b) represents the percentage of foals that recorded titres ≥ 10, 56 days after the second vaccination (re-immunisation)

Treatment group II (n=15)

Foals that were vaccinated with monovalent MLV AHSV serotype 1 and serotype 8 on Day 0 and 28 respectively, developed substantial neutralising antibody titres to AHSV 1 by Day 56 (median titre = 160), but not to other serotypes (median titres ≤ 10 to AHSV serotypes 2 – 9), however 5/15 (33.3%) of these foals seroconverted (median SNT titre 10 – 112) to AHSV 2. Two foals were removed from the cohort after Day 56 for reasons unrelated to the study.

All the foals were seronegative at Day 56 to AHSV serotypes 3, 4, 5, 6, 7 and 9, and one foal had a titre of 20 to AHSV 8. By Day 112, SNT titres increased to AHSV 1 (median titre = 224) whereas 6/13 (46%) foals had SNT titres to AHSV 2 (range 10 – 80) and all foals were seronegative to AHSV serotypes 3 to 9 (Figure 4-3a).

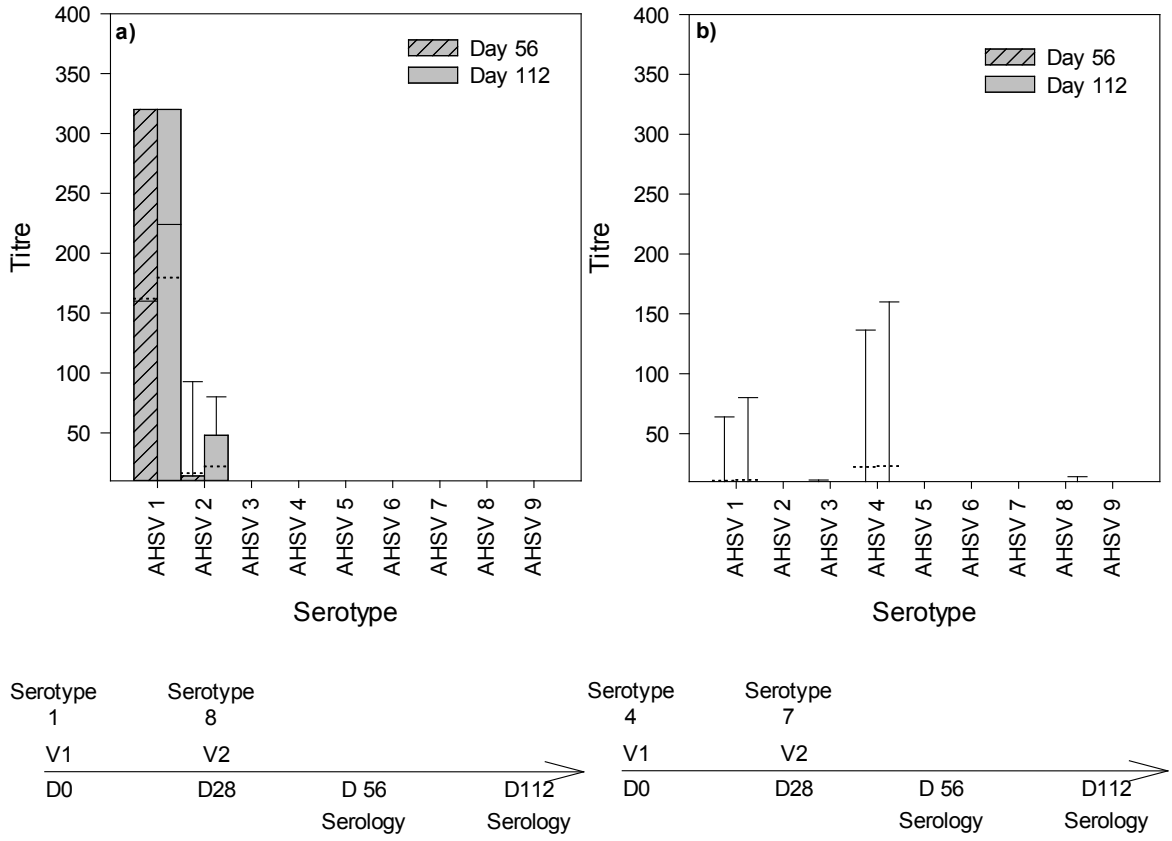

Figure 4-3 Box-and-whisker plots that describe the distribution of SNT titres against the 9 serotypes of AHSV at 56 and 112 days after vaccination of foals in groups II and III with monovalent AHSV serotypes. Figure (a) shows titres of foals in Group II after vaccination with monovalent MLV AHSV 1 (Day 0) and serotype 8 (Day 28), and figure (b) shows titres of foals in Group III after vaccination with monovalent MLV AHSV 4 (Day 0) and serotype 7 (Day 28). The dotted lines represent mean titres. The time-line for the various vaccinations and the respective AHSV vaccine serotypes used are represented below the graphs

Treatment group III (n=15)

The majority (12/15, 80%) foals vaccinated with monovalent MLV AHSV serotype 4 and serotype 7 on Day 0 and 28, respectively, remained seronegative to all 9 serotypes of AHSV at Day 56 (Figure 4-3b). One foal was removed from the cohort after Day 56 for reasons unrelated to the study. One foal seroconverted to AHSV serotypes 2, 4 and 8 (SNT titres 10, 320, 28 respectively), another foal seroconverted to AHSV serotypes 1, 2, 3, 7 (SNT titres of 160, 10, 20 and 14 respectively), and a third foal seroconverted to only AHSV 4 (SNT titre 14).

SNT titres of foals that received polyvalent vaccine (n=16) compared to those that received monovalent vaccines (n=30)

There were no significant differences between titres on Day 56 to AHSV-1 (p=0.852), AHSV-4 (p=0.150) and AHSV-8 (p=0.333) respectively, when comparing foals that received the polyvalent vaccine to those that received the monovalent components. Foals that received the polyvalent vaccine had a significantly greater response to AHSV-7 (p=0.021) than those that received serotype 7 as a monovalent vaccine (Figure 4-4)

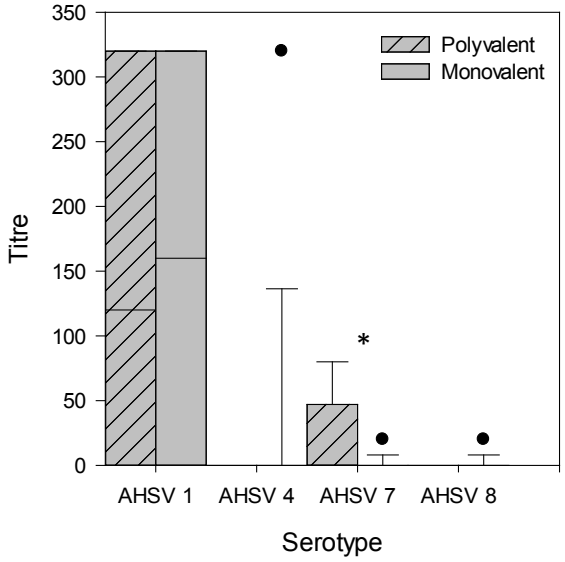

Figure 4-4 Box-and-whisker plots comparing the distribution of SNT titres against serotypes 1, 4, 7 and 8 of AHSV at 56 days after vaccination of foals vaccinated with the polyvalent vaccine (Group I) and foals vaccinated with monovalent vaccines (groups II and III).Significant differences (P <= 0.05) between groups using the Wilcoxon rank-sum test are indicated with *.

Discussion

The objective of this study was to compare the neutralising antibody responses of foals to each of the nine serotypes of AHSV following vaccination with a commercial polyvalent MLV vaccine with that to immunization with selected monovalent AHSV serotypes. There was a marked and consistent difference in the immunogenicity of individual virus serotypes contained in the vaccine, thus foals more consistently seroconverted to AHSV 1 than any other virus serotype. Responses to other serotypes were highly variable, and often weak or not detected. Furthermore, the presence of neutralising antibody to individual AHSV serotypes was often transient and quickly waned after vaccination. Of potential significance is the fact that a second round of vaccination generally did not increase the antibody titres persisting from the initial immunization more than four-fold, indicating that there was a poor anamnestic response to re-vaccination. However, some animals that were seronegative after initial immunization did transiently seroconvert

after re-vaccination. Unpublished data by the manufacturer, (Smit TK personal communication) indicate a similar pattern in that a detectable antibody response is not evident in all animals to all serotypes and increased antibody titres to most or all serotypes develop only after repeated vaccination.

To assess whether or not there was interference between the individual virus serotypes contained in the polyvalent MLV preparations, we also immunized foals with monovalent MLV strains (those contained in bottles 1 and 2 of the commercial vaccine). These data confirm that the serotype-specific responses of foals that received the monovalent vaccines were similar to those of foals that received the polyvalent preparation, with the notable exception of AHSV 7 where the foals that received the polyvalent vaccine had significantly higher titres than those receiving the monovalent vaccine. This finding was somewhat paradoxical in that it could be speculated that without interference between viruses contained in the polyvalent preparation, monovalent vaccines would be expected to induce higher antibody titres. These results suggest that there is no obviously enhanced immune response through the administration of a monovalent vaccine as opposed to the polyvalent vaccine. In fact, the polyvalent vaccine offers advantages in terms of production lead time, production cost, and ease of use for the consumer with less “resting time” required post vaccination.

The data from these studies are consistent with results of our recent study (Chapter 3) on passive transfer of AHSV neutralising antibody from repeatedly vaccinated mares to their foals (Crafford *et al*. 2012). Specifically in that study we also showed that neutralizing antibody responses to individual AHSV serotypes were highly variable, even after repeated immunisation of horses. Similar data were also reported by other investigators using the same MLV AHSV vaccine, however in contrast to the present study where AHSV 1 was most immunogenic, data from that study indicated that AHSV 4 was most immunogenic (von Teichman *et al.* 2010). The possibility of vaccine batch variation was not investigated.

This and other studies also indicate variability in responses of individual foals/horses to AHSV vaccination. Although only a limited or poor neutralising antibody response is elicited by the polyvalent vaccine, which could suggest limited immunogenicity of some of the serotypes, horses might still be immunologically primed. Recently Pretorius *et al. (2012)* have confirmed that the major immune response after AHSV vaccination was humoral, but they also describe, similar as for BTV immunity (Andrew *et al.* 1995; Jeggo *et al.* 1984; Umeshappa *et al*. 2010), a significant $CD8^+$ response in horses vaccinated with modified live AHSV serotype 4. Although the protective role of cell-mediated immunity in the absence of neutralising antibody was shown for BTV in sheep (Jeggo *et al.* 1984), it has not been shown for the protection of horses against AHSV.

Challenge studies were not done to confirm immune status of the vaccinated foals. Although a cut-off titre of ≥10 was assumed as evidence of seropositivity, the actual SNT titre required for protection of individual horses is likely to be variable. The manufacturer relies on a minimum titre of 16 as a protective dose, based on extensive internal testing and establishment of the minimum quality requirements of the vaccine. Furthermore, other potential correlates of protective immunity were not assessed in the immunized foals, notably cellular immune mechanisms.

It is uncertain why most virus strains contained in the MLV polyvalent AHSV vaccine are so poorly immunogenic in horses, at least as assessed by their ability to induce seroconversion in naïve and previously vaccinated horses. Possible explanations could include: i) antigenic competition between the multiple serotypes included in the polyvalent MLV preparations, although data from horses immunized in this study with monovalent vaccines suggests this is not the case; ii) epitope masking by circulating antibody when a second vaccination is administered; iii) direct effects of AHSV on immune cells, or the effect on virus replication by vaccine-induced antiviral mediators such as interferon; and iv) possible attenuation of antigenic determinants of VP2, VP5 and or VP7 affecting binding of neutralising antibodies and thus antibody titres elicited.

Existing antibody can certainly suppress or enhance markedly the antibody response of an individual to a specific antigen, notably when antigens are administered as physiological preparations that are linked to large particles like red blood cells (Heyman 2000; Hjelm *et al*. 2006). We were also not able to determine to what extent if any, the concurrent vaccination with equine influenza, tetanus and botulism influenced the immune response in the foals.

MLV AHSV vaccines continue to play a vital role in the control of AHSV infection of horses in endemic regions and have been used successfully in incursional areas. Whilst the basis of the efficacy of these vaccines has not been fully described the results of our studies emphasize the need for on-going studies to enhance the understanding and knowledge of the immune response to AHS. Such information will contribute significantly to the development of more effective or new generation AHSV vaccines.

Chapter 5

AN AFRICAN HORSE SICKNESS VIRUS SEROTYPE 4 RECOMBINANT CANARYPOX VIRUS VACCINE ELICITS SPECIFIC CELL-MEDIATED IMMUNE RESPONSES IN HORSES[1]

Abstract

A recombinant canarypox virus vectored vaccine co-expressing synthetic genes encoding outer capsid proteins, VP2 and VP5, of African horse sickness virus (AHSV) serotype 4 (ALVAC®-AHSV4) has been demonstrated to fully protect horses against homologous challenge with virulent field virus. Guthrie *et al.* (2009) detected weak and variable titres of neutralizing antibody (ranging from <10 to 40) 8 weeks after vaccination leading us to hypothesize that there could be a participation of cell mediated immunity (CMI) in protection against AHSV 4. The present study aimed at characterizing the CMI induced by the experimental ALVAC®-AHSV4 vaccine. Six horses received two vaccine injections twenty eight days apart and three horses remained unvaccinated. The detection of VP2/VP5-specific IFN-γ responses was assessed by ELISpot assay and clearly demonstrated that all ALVAC®-AHSV4 vaccinated horses developed significant IFN-γ production compared to unvaccinated horses. More detailed immune responses obtained by flow cytometry demonstrated that ALVAC®-AHSV4 vaccinations induced immune cells, mainly $CD8^+$ T-cells, able to recognize multiple T-epitopes through all VP2 and only the N-terminus sequence of VP5. Neither VP2 nor VP5 specific IFN-γ responses were detected in unvaccinated horses. Overall, our data demonstrated that an experimental recombinant

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canarypox based vaccine induced significant CMI specific for both VP2 and VP5 proteins of AHSV 4.

Keywords:

African horse sickness virus serotype 4 (AHSV 4); Recombinant canarypox based vaccine (ALVAC®); Cell mediated immunity (CMI)

Introduction

The recent changes in the disease patterns of arboviruses and the influence of climate change on the emergence and establishment of a transmission cycle of bluetongue virus (BTV) in northern Europe (Gould *et al.* 2009; MacLachlan *et al.* 2010), has re-emphasised the need for a new generation of vaccines against African horse sickness virus (AHSV), a very similar virus to BTV, which are safe and effective to prevent AHS in equids. The traditional live attenuated vaccines against AHSV serotypes and the risks involved with their use in non-endemic areas have been reviewed (House 1993; House 1998).

Over the last few years new generation recombinant vaccines against equine Influenza virus and West Nile virus have been developed and commercialized using the canarypox vector platform (Poulet *et al*. 2007). This technology was recently used to create experimental vaccines against AHSV 4 (Guthrie *et al.* 2009) and BTV 17 (Boone *et al*. 2007). Both vaccine constructs express VP2 and VP5 outer capsid proteins of the respective viruses and vaccinated animals (horse and sheep) successfully developed serotype-specific neutralizing antibodies and were fully protected against challenge with virulent field strains of AHSV 4 and BTV 17, respectively.

The humoral nature of protective immunity to AHSV has been clearly demonstrated and linked to neutralizing antibodies directed towards VP2 and VP5 outer core proteins (Burrage *et al.* 1993; Chiam *et al.* 2009; Martinez-Torrecuadrada *et al*. 1994; Martinez-Torrecuadrada *et al.* 1996; Martinez-Torrecuadrada *et al*. 1999; Martinez-Torrecuadrada *et al*. 2001; Scanlen *et al.* 2002; Stone-Marschat *et al.* 1996). However, reports on BTV

and AHSV infections have underlined that there is no direct correlation between neutralization *in vitro* and protection *in vivo* as animals can be protected without neutralizing antibodies and animals with high neutralizing antibody titres are not necessarily protected (Martinez-Torrecuadrada *et al.* 1996; Osburn 1992).

The hypothesis that antibody dependant cell mediated cytotoxicity (ADCC), which is another humoral immune mechanism, could be involved against viral infection was investigated but could not be demonstrated for BTV infection (Jeggo *et al*. 1983). Another immune mechanism such as cellular mediated immunity (CMI) has been suggested to be involved somehow in the process of protection. The importance of BTV-specific cytotoxic T-lymphocytes (CTL) in protection in sheep has been well demonstrated by various groups (Andrew *et al.* 1995; Janardhana *et al*. 1999; Jeggo *et al*. 1984; 1985; Jones *et al*. 1996; 1997; Lobato *et al*. 1997; Wade-Evans *et al*. 1996). Overall they reported that multiple CTL epitopes are present on non-structural proteins NS1 and NS3 and on structural proteins VP2 and VP5. Additionally and more recently, Rojas *et al*. (2011) have demonstrated the presence of multiple $CD4^+$ and $CD8^+$ T-cell epitopes on the structural protein VP7 of BTV 8

The role of the CMI against AHSV infection is not very well described in the literature. One paper reported the presence of a specific lymphoproliferative response as well as specific $CD8^+$ T-cells with cytotoxic activity in a single horse vaccinated with DNA encoding for VP2 of AHSV 3 (Romito *et al.* 1999). The development and refinement of functional assays for the evaluation of CMI in horses in recent years have provided invaluable tools for the evaluation of modern vaccines to viral diseases (El' Garch *et al*. 2008; O'Neill *et al*. 1999; Paillot *et al*. 2005; 2006a; 2006b). These methods include the detection of IFN-γ secretion by ELISpot assays and flow cytometry.

In the present study we characterized cellular mediated immune responses induced in horses vaccinated with an experimental ALVAC®-AHSV4 vaccine previously described (Guthrie *et al.* 2009). In summary, we were able to detect neutralizing antibodies against AHSV 4 and for the first time reported the detection of AHSV 4-specific IFN-γ responses in

vaccinated horses. We demonstrate that an experimental ALVAC®-AHSV 4 vaccine efficiently induced mainly VP2- and VP5-specific $CD8^+$ IFN-γ responses after *ex vivo* stimulation of PBMCs with peptide pools.

Materials and methods

Vaccine

The construction of the recombinant canarypox virus has been described previously (Guthrie *et al.* 2009). Each vaccinated horse received a dose of vaccine in liquid format containing 7.1 $\log_{10}$ $TCID_{50}$ in 8 mg Carbomer.

Animals and vaccination schedule

Nine horses (Welsh mountain ponies and mixed breed) of both sexes were used in this study. They were approximately 3-4 years of age at the time of first vaccination. The horses were kept at an appropriate farm for this type of work for the duration of the study under a protocol approved by MERIAL Institutional Animal Care and MERIAL Ethical Review Committee. All horses were negative for virus neutralization antibody to AHSV 4 before vaccinations (data not shown). They were randomly allocated into two groups, one group of six horses was vaccinated with ALVAC®-AHSV4 and the remaining three horses were not vaccinated and served as negative controls. Vaccinations were administered on days 0 and 28 by deep intra-muscular injection in the neck.

Antibody detection

Approximately 2 mL of blood for processing into serum were collected from the jugular vein on days 0, 28, 42 and 56. Samples from days 0 and 28 were taken prior to vaccination. Sera were prepared and stored frozen at -20 °C until analysis. Serotype-specific neutralizing antibodies to AHSV were detected by SNT using AHSV 4 as previously described (Howell *et al.* 2002). Antibody titres were recorded as the reciprocal of the

highest final dilution of serum that provided at least 50% protection of the BHK-21 cell monolayer.

Cell isolation and antigen stimulation

Whole blood was collected on days 28, 42 and 56 in heparinised tubes (BD Biosciences, Le pont de claix, France) and centrifuged for 10 min at 400 × g. The buffy coat was collected and re-suspended in 15 ml PBS without Ca^{2+} and Mg^{2+}. PBMCs were isolated through density centrifugation by using Pancoll (D. Dutcher, Issy-les-moulineaux, France). The ring of PBMCs was collected and washed twice in PBS^{-} and subsequently counted using an automated ABX Pentra 120 cell counter (Horiba medical, Montpellier, France). Cells were then resuspended at a concentration of 1.0 x 10^7 cells/ml in RPMI complete medium (500 mL of RPMI 1640 (Life Technologies SAS, Saint Aubin, France), 10% irradiated foetal calf serum (PAA GmbH, Pasching, Austria), 25 000 units/mL penicillin – 25 000 µg/mL streptomycin (Life Technologies SAS, Saint Aubin, France) and 0.01% 0.5 M β mercapto-ethanol (Saint-Quentin Fallavier, France).

Detection and characterization of AHSV 4-specific IFN-γ producing cells were performed by ELISpot and flow cytometry, respectively.

For ELISpot assays, 125 000 purified PBMCs per horse were transferred in ELISpot wells (Millipore SAS, Molsheim, France) and infected directly within the plate either with ALVAC®-AHSV4 vector or parental ALVAC® vector as negative control. Infections were performed at multiplicity of infection of 5 for 24 hours at 37 °C and 5% CO_2.

For flow cytometry assays, 500 000 purified PBMCs per well were ex-vivo stimulated in 96-well plates for 2 hours at 37 °C and 5% CO_2 with pools of synthetic overlapping peptides encoding for VP2 and VP5 outer capsid proteins (Eurogentec S.A., Seraing, Belgium). These peptides were 15-mers overlapping by 10 amino acids. VP2 and VP5 peptides were pooled into 13 and 6 pools respectively. Each peptide pool contained 15 to 18 peptides per pool. An irrelevant peptide pool produced and concentrated as for the

others was used as negative control. The peptide pools were used at a concentration of 5 µg/ml in RPMI complete medium. Positive control cells (500 000 cells/well) were stimulated with PMA/Ionomycin (PMA (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 100 ng/ml; Ionomycin (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 12.5 µM) and negative control cells (500 000 cell/well) only with RPMI complete medium. After the incubation time, the extracellular protein transport was inhibited by the addition of brefeldin A (GolgiPlug, BD Biosciences, Le pont de claix, France) at a dilution of 1/1 000 in RPMI complete medium. Cells were further incubated overnight at 37 °C and 5% CO_2.

ELISpot assay for the detection of IFN-γ producing cells

ELISpot assays were performed in 96-well plates (Multiscreen$_{HTS}$-IP Filter Plate with hydrophobic PVDF membrane, Millipore SAS, Molsheim, France) using the Bovine/Ovine/Equine IFN-γ ELISpot-ALP kit (Mabtech AB, Sophia Antipolis, France). Plates were activated with 50 µl 70% ethanol per well for 90 seconds, washed five times in PBS and then coated overnight at 4 °C using 0.75 µg/well of purified anti-IFN-γ antibody provided in the kit. Plates were washed five times using PBS and blocked with 200 µl/well of RPMI complete medium at ±22 °C for at least 2 hours. Purified PBMCs were stimulated as described above for 24 hours at 37 °C and 5% CO_2. Cells were then removed and 200 µl/well of cold distilled water was added for 5 min to allow red blood cell lysis if necessary. Plates were washed five times in PBS containing 0.05% Tween-20 (PBS-T). One hundred µl/well of diluted anti-IFN-γ monoclonal antibody conjugated to biotin was added per well and plates were incubated overnight at 4 °C. They were washed five times with PBS-T and 100 µl/well of diluted streptavidin-ALP was added per well. After 1 hour incubation at 22 °C in the dark, plates were finally washed five times with PBS-T and 100 µl/well of BCIP/NBT chromogen (R&D Systems Europe, Lille, France) was added per well. Spots were developed for 30 to 40 min at 22 °C in the dark and under agitation. Plates were washed thoroughly with tap water and dried before counting with a CCD camera system (Microvision Instruments, Evry, France). One spot corresponds to one IFN-γ producing cell. The spot count of antigen-specific cells were calculated as the spot count

difference between ALVAC®-AHSV4 infected cells and ALVAC® parental construct infected cells.

Flow cytometry for intracellular IFN-γ detection

For CD8α extracellular staining, activated PBMCs were washed twice in FACS buffer (PBS, 0.5% BSA, 0.01% sodium azide) at 1 400 rpm for 5 min at 4 °C. Cells were stained by using mouse anti-equine CD8α (VMRD, Pullman, USA) and FITC conjugated goat anti-mouse IgG3 (Southern Biotech, Alabama, USA) antibodies. Cell membranes were fixed and permeabilised with *Cytofix/Cytoperm*® solution (BD Biosciences, Le pont de claix, France) and washed twice in permeabilisation buffer (BD Biosciences, Le pont de claix, France). Mouse IgG3 (Southern biotech, Alabama, USA) was used as negative control for CD8α staining.

Cells were then stained by using mouse anti-bovine IFN-γ conjugated to biotin (AbD Serotec Ltd, France) followed by PerCP conjugated streptavidin (BD Biosciences, Le pont de claix, France). Biotinylated mouse IgG1 (Southern biotech, Alabama, USA) was used as negative control for IFN-γ staining. Stained cells were washed twice in permeabilisation buffer and resuspended in FACS buffer plus 1% formaldehyde. The acquisition and analysis was performed with a *FACSCalibur*® flow cytometer (BD Biosciences, Le pont de claix, France) and *CellQuest Pro*® software, respectively. Data were obtained by gating 100 000 lymphocytes on the basis of forward (FSC) and right-angle side (SSC) scatter parameters. The percentage of antigen-specific IFN-γ producing cells was calculated according to the following formula: (percentage of IFN-γ producing cells obtained with each peptide pool) – (percentage of IFN-γ producing cells obtained with irrelevant peptide pool).

Statistical analysis

Statistical analyses were performed using *STATGRAPHICS*® Plus program for *Windows*® (Manugistics, Rockville, MD). Analysis of variance (ANOVA) was used for the graphic

representations. Statistical significance was based on two-tailed tests of the null hypothesis resulting in a p-value of 0.05 or less. The detection of neutralizing antibodies to AHSV 4 was statistically assessed at each time point by using the signed ranked test. The number of responders (IFN-γ ELISpot value greater than the cut-off point) was compared between vaccinated and control animals by using the Fisher's exact test. The mean percentage of VP2- and VP5-specific IFN-γ producing cells was compared with the null value (no response for controls) by using the Student t-test, for each of the thirteen peptides pools for VP2 antigen, and six peptides pools for VP5 antigens.

Results

Detection of neutralizing antibodies to AHSV 4

All horses were seronegative as demonstrated by AHSV 4 SNT prior to vaccination (Figure 5-1). Twenty eight days after the first immunization with ALVAC®–AHSV4, only one horse out of six developed neutralizing antibodies. Two weeks after the second immunization the remaining five vaccinated horses developed neutralizing antibodies (*Signed rank test: p = 0.035*). The titres slightly decreased on Day 56 but remained positive in all ALVAC®-AHSV4 vaccinated horses (*Signed rank test: p = 0.034*). No neutralizing antibodies were detected in the unvaccinated animals at any of the time points.

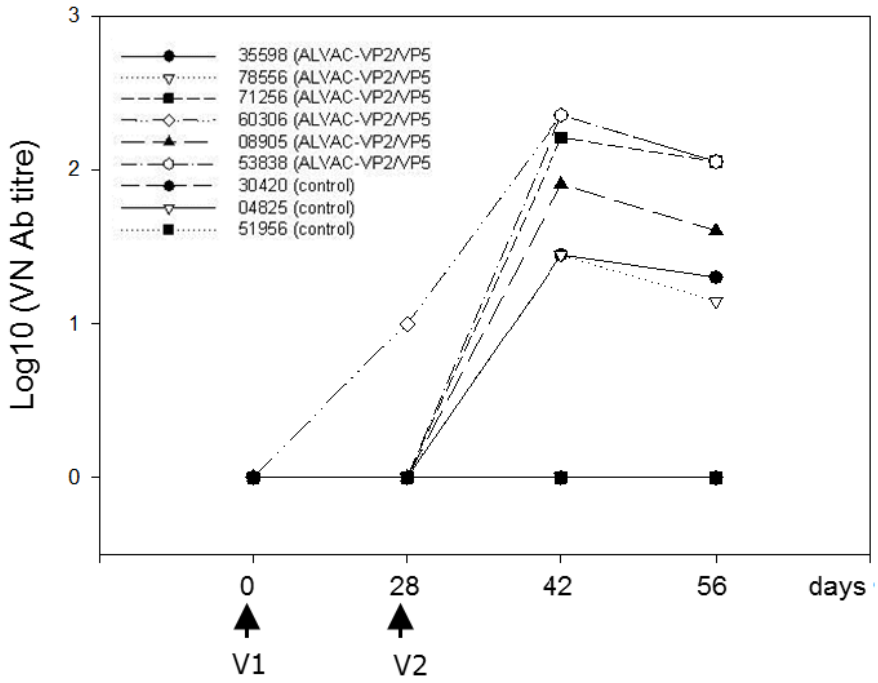

Figure 5-1 Kinetics of individual AHSV 4 neutralizing antibody titres expressed as the reciprocal of the highest dilution that provided >50% protection of the BHK-21 cell monolayer (log_{10} scale). Mean titres detected in all ALVAC®–AHSV4 vaccinated horses were significantly higher (*) than those obtained in control horses after the second injection (Signed rank test: p = 0.035 and 0.034 on days 42 and 56, respectively).

Detection of global VP2/VP5-specific IFN-γ response by ELISpot

ELISpot results for the detection of IFN-γ are represented in Table 5-1. Non-specific responses of all horses were below the baseline at the start of the study (less than 10 spots). Twenty eight days after the first immunization two horses developed detectable VP2/VP5-specific IFN-γ producing cells. Fourteen days after the booster injection (Day 42), more horses responded with 4/6 displaying a higher frequency of VP2/VP5-specific IFN-γ producing cells when compared to the control group. The horse # 53838 which did not show a response on Day 42 was one of the two earliest responders observed at the previous time point (Day 28). The means of VP2/VP5-specific IFN-γ responses on Day 56

were of a similar magnitude as on Day 42. Overall we were able to demonstrate a significant difference in VP2/VP5-specific IFN-γ response between vaccinated and control horses (Fisher's exact test, p=0.012: 6/6 in vaccinates versus 0/3 in Controls).

Detection of IFN-γ producing cells after *ex vivo* stimulation with VP2 overlapping peptide pools.

Flow cytometry results confirmed those obtained by ELISpot and led us to phenotype VP2-specific IFN-γ producing cells induced *in vivo.* A representative dot plot of PBMCs from one ALVAC®-AHSV4 vaccinated horse and labelled for CD8α and intracellular IFN-γ is presented in Figure 5-2.

Table 5-1 Detection of AHSV 4-specific IFN-γ producing cells by ELISpot. AHSV 4-specific IFN-γ responses expressed as spot forming cells (SFC) and detected by ELISpot. Values <10 were considered as negative (mean value on Day 0 plus 3 standard deviations)

	ALVAC®-VP2/VP5 re-stimulation				ALVAC®-empty re-stimulation				VP2/VP5-specific responses			
	D0	D28	D42	D56	D0	D28	D42	D56	D0	D28	D42	D56
Vaccinated horses												
#35598	0	0	27	42	0	2	19	1	<10	<10	<10	41
#78556	0	32	96	51	1	34	57	75	<10	<10	39	<10
#71256	7	46	62	42	4	52	23	14	<10	<10	39	28
#60306	2	10	91	33	0	11	18	0	<10	<10	73	33
#08905	8	31	97	40	2	15	59	0	<10	16	38	40
#53838	5	48	43	13	0	8	70	15	<10	40	<10	<10
							Means of responders		–	28	47	36
								SD	–	17	17	6
Control horses												
#30420	0	4	5	2	0	0	3	4	<10	<10	<10	<10
#04825	0	0	0	1	0	0	0	1	<10	<10	<10	<10
#51956	3	0	0	1	1	0	2	1	<10	<10	<10	<10
							Means of responders		–	–	–	–
								SD	–	–	–	–

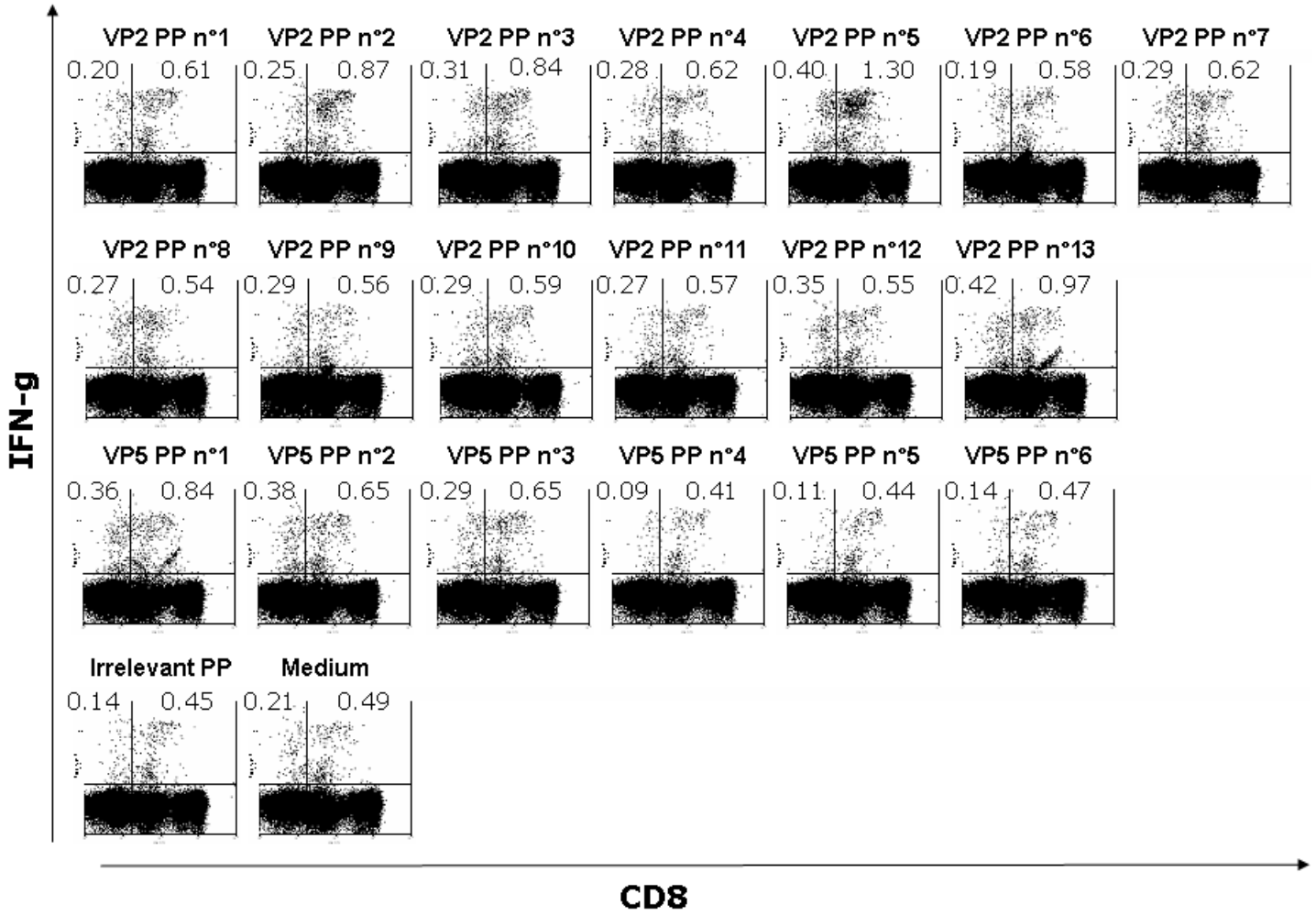

Figure 5-2 Flow cytometry dot plots depicting $CD8^+$ and IFN-γ expressing cells from one representative ALVAC®–AHSV4 vaccinated horse on Day 42 after stimulation with thirteen VP2 peptide pools, six VP5 peptide pools, one irrelevant peptide pool and culture medium.

The frequency of VP2-specific IFN-γ producing cells in vaccinated and unvaccinated horses were analysed on days 28, 42 and 56 (Figure 5-3A and B, respectively). No VP2-specific IFN-γ producing cells were detected in unvaccinated horses at any of the examined time points (Figure 5-3B). Weak VP2-specific IFN-γ responses were observed with some peptide pools on Day 28 in ALVAC®-AHSV4 vaccinated horses (Figure 5-3A).

These responses were boosted after the second injection and by days 42 and 56 all the vaccinated horses responded significantly to each of the VP2 peptide pools in comparison to the controls (*Student t-tests: $p \leq 0.012$*). The phenotypic characterization of VP2-specific IFN-γ producing cells is presented in Figure 5-4. On Day 42, CD8α^+ cells (upper panel) were the main VP2-specific IFN-γ producing cells whereas on Day 56 both CD8α^+ and CD8α^- cells (upper right and lower right panels) were involved in IFN-γ secretion after specific VP2 peptide pool stimulations.

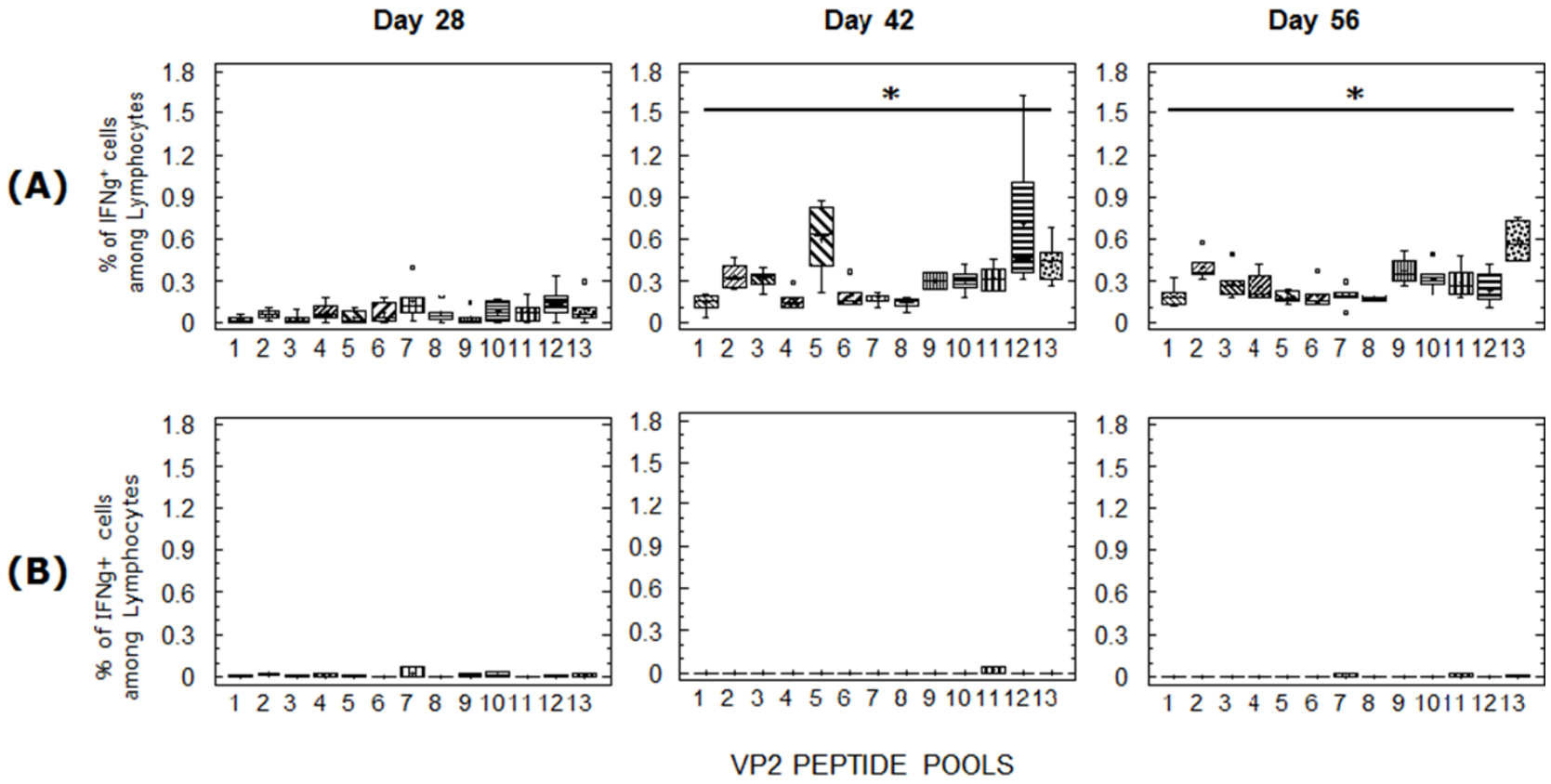

Figure 5-3 Box plots depicting VP2-specific IFN-γ^+ secreting cells from ALVAC®–AHSV4 vaccinated (A) and un-vaccinated horses (B) detected by flow cytometry on days 28, 42 and 56. Horses were vaccinated on Day 0 and Day 28. Top and bottom borders of the box represent the 90th and 10th percentiles, respectively and the solid bar indicates the median and outliers are illustrated as dots. ALVAC®–AHSV4 vaccination significantly induced IFN-γ^+ synthesis in response to all VP2 peptide pools after the second injection in comparison to control group (Student's t-test: $p \leq 0.012$).

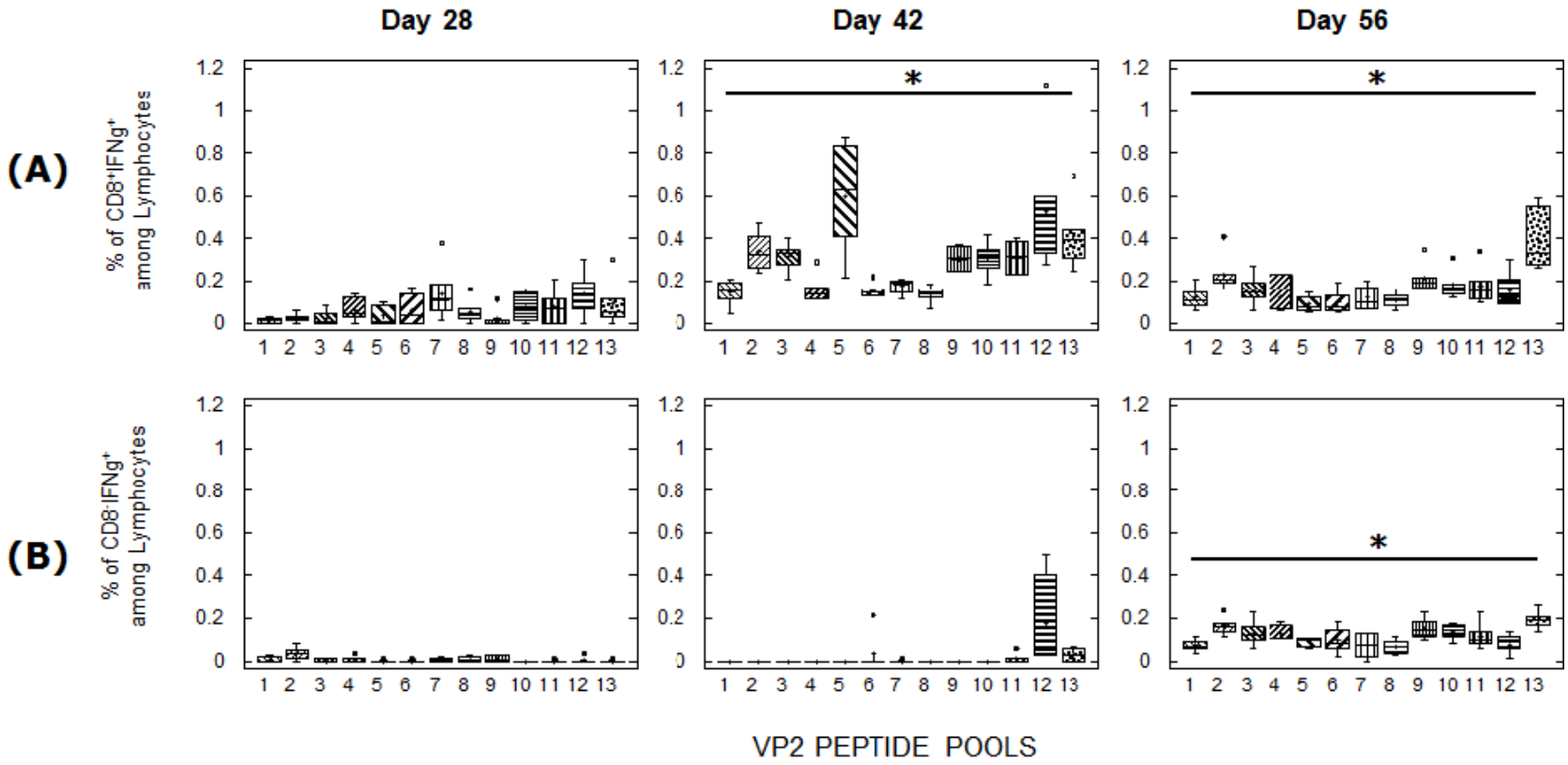

Figure 5-4 Box plots depicting VP2-specific $CD8^+$ IFN-γ^+ (A) and $CD8^-$ IFN-γ^+ (B) secreting cells among lymphocytes of six ALVAC®–AHSV4 vaccinated horses on days 28, 42 and 56. Horses were vaccinated on Day 0 and Day 28. Top and bottom borders of the box represent the 90th and 10th percentiles, respectively and the solid bar indicates the median and outliers are illustrated as dots. ALVAC®–AHSV4 vaccination significantly induced $CD8^+$ IFN-γ^+ cells on Day 42 and both $CD8^+$ IFN-γ^+ and $CD8^-$ IFN-γ^+ cells on Day 56 in response to N-Terminal region of VP5 (Student's t-test: $p \leq 0.001$)..

Detection of IFN-γ producing cells after *ex vivo* stimulation with VP5 overlapping peptide pools.

The detection and phenotype of VP5-specific IFN-γ producing cells in vaccinated and unvaccinated horses are presented in Figure 5-5 and Figure 5-6 respectively. No VP5-specific IFN-γ producing cells were detected in unvaccinated horses at any of the examined time points (Figure 5-5, lower panel). All specific reactions detected on days 28, 42 and 56 in ALVAC®-AHSV4 vaccinated group were significantly restricted to a cluster of peptide pools (peptide pools 1, 2 and 3) corresponding to the N-terminus of VP5 (Figure 5-5A, *Student t-tests: $p \leq 0.002$*). As for VP2-specific responses, VP5-specific IFN-γ producing cells were boosted after the second vaccine injection and the CD8α^+ T cells represent the main source of IFN-γ (Figure 5-6, upper panels). These responses showed a slight decrease on Day 56 although still significantly positive (*Student t-tests: $p \leq 0.002$*). Additionally and similarly to VP2, a statistical significant increase of VP5-specific IFN-γ producing CD8α^- cells was observed on Day 56 (Figure 5-6, lower right panel) (*Student t-tests: $p \leq 0.001$*).

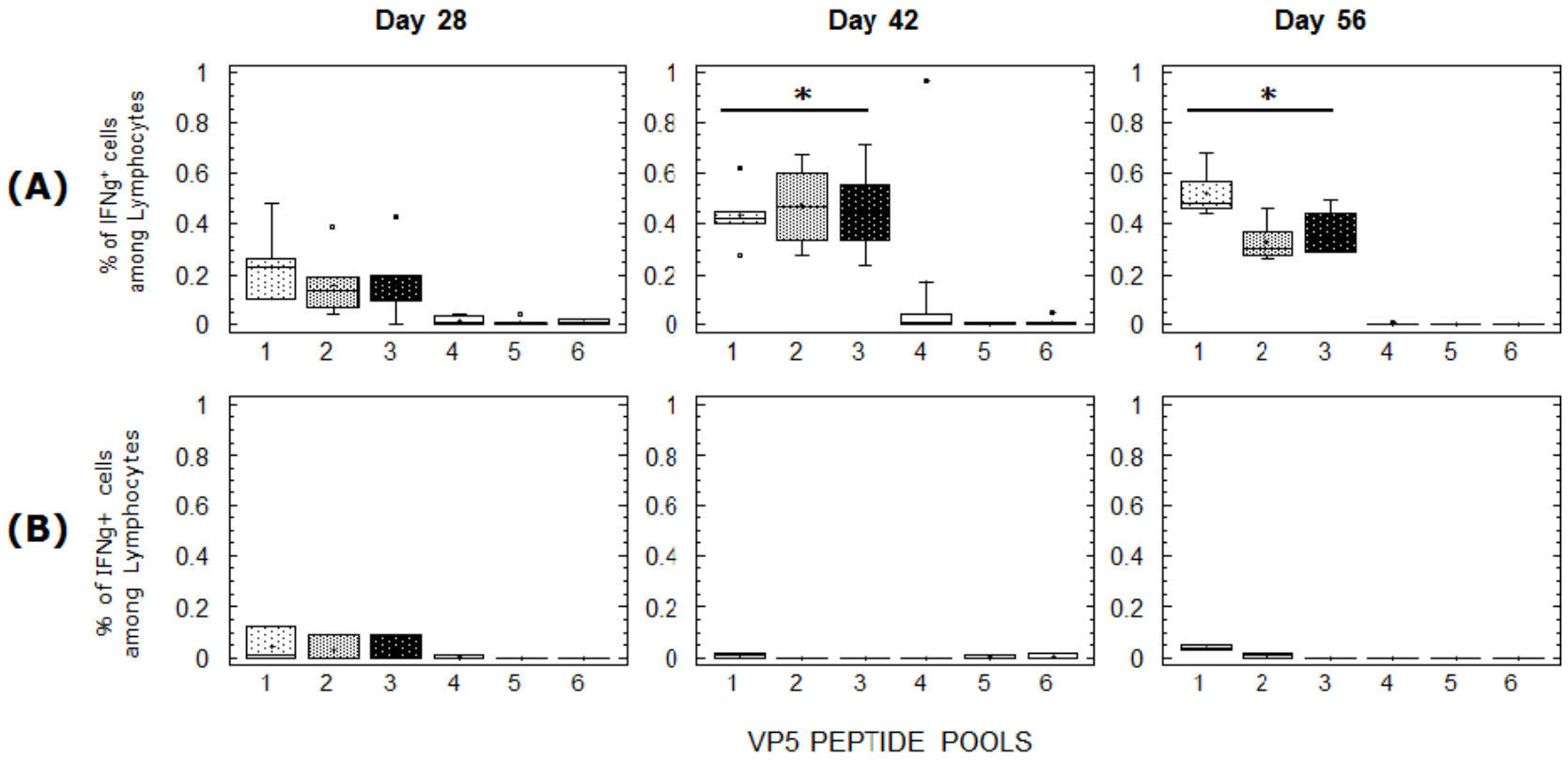

Figure 5-5 Box plots depicting VP5-specific IFN-γ^{+} secreting cells from ALVAC®–AHSV4 vaccinated (A) and un-vaccinated horses (B) detected by flow cytometry on days 28, 42 and 56. Horses were vaccinated on Day 0 and Day 28. Top and bottom borders of the box represent the 90th and 10th percentiles, respectively and the solid bar indicates the median and outliers are illustrated as dots. ALVAC®–AHSV4 vaccination significantly induced IFN-γ^{+} synthesis in response to three VP5 peptide pools after the second injection in comparison to control group (Student's t-test: $p \leq 0.002$).

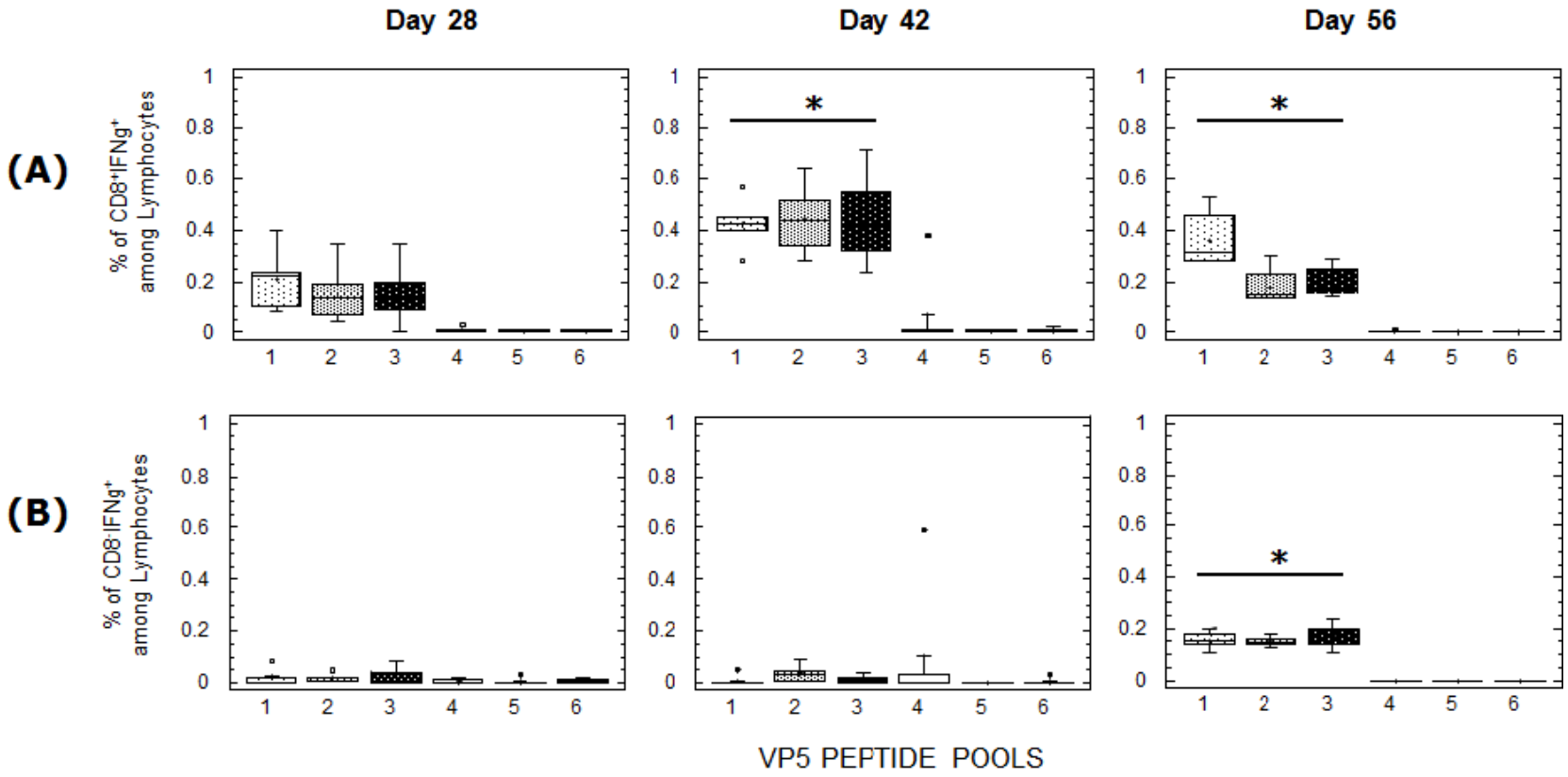

Figure 5-6 Box plots depicting VP5-specific $CD8^+$ IFN-γ^+ (A) and $CD8^-$ IFN-γ^+ (B) secreting cells among lymphocytes of six ALVAC®–AHSV4 vaccinated horses on days 28, 42 and 56. Horses were vaccinated on Day 0 and Day 28. Top and bottom borders of the box represent the 90th and 10th percentiles, respectively and the solid bar indicates the median and outliers are illustrated as dots. ALVAC®–AHSV4 vaccination significantly induced $CD8^+$ IFN-γ^+ cells on Day 42 and both $CD8^+$ IFN-γ^+ and $CD8^-$ IFN-γ^+ cells on Day 56 in response to N-Terminal region of VP5 (Student's t-test: $p \leq 0.001$).

Discussion

In the last decade, multiple experimental vaccines have been developed to prevent AHSV infection of equids, including inactivated, live attenuated virus vaccines, virus like particles (VLPs) produced from recombinant baculoviruses, recombinant vaccinia vectored vaccine, DNA vaccine and recently recombinant modified vaccinia Ankara (MVA) and canarypox vectored vaccine (Castillo-Olivares *et al*. 2011; Chiam *et al.* 2009; Guthrie *et al.* 2009; House *et al*. 1994; Martinez-Torrecuadrada *et al.* 1994; Romito *et al.* 1999; Roy *et al.* 1996; Stone-Marschat *et al.* 1996). All of these vaccines were efficient at generating neutralizing antibodies in horses which have been well-described as a surrogate marker of protection. However, one study has reported that horses vaccinated with cell extracts containing VP2 and VP7 developed significant neutralizing antibody titres after vaccination but were found to be unprotected against virulent AHSV 4 challenge (Martinez-Torrecuadrada *et al.* 1996). On the other hand, in the same study, one horse vaccinated with purified VP2 and VP7 of AHSV 4 did not develop any neutralizing antibodies but survived the challenge. Similarly to this publication and more recently, Guthrie *et al.* (2009) reported that one horse that did not seroconvert after vaccination with recombinant canarypox ALVAC®-AHSV4 was protected against virulent challenge.

Cell-mediated immune mechanisms have been suggested to play a role in the protection of these natural hosts (Martinez-Torrecuadrada *et al.* 1996). Recombinant canarypox virus based vaccines have been described for the prevention of several viral diseases in horses [influenza (Minke *et al*. 2011a; Minke *et al*. 2007; Soboll *et al*. 2010), herpes virus (Minke *et al*. 2006), West Nile virus (Minke *et al*. 2004; Minke *et al*. 2011b; Siger *et al*. 2006; Siger *et al*. 2004) and AHSV (Guthrie *et al.* 2009)] and some of them are now commercially available. Their ability to stimulate both humoral and cell-mediated immune responses has been confirmed for West Nile virus (El' Garch *et al.* 2008), influenza virus (Adams *et al*. 2011) and herpes virus (Minke *et al.* 2006; Paillot *et al.*

2005). The present paper describes both immune responses in the context of AHSV 4 vaccination.

First, we confirmed that as described by Guthrie *et al*. (2009) the recombinant canarypox virus vector that co-expresses VP2 and VP5 proteins of AHSV 4 elicits antigen-specific neutralizing antibodies. This implies that the two proteins are successfully expressed in the host cells and are excreted in a way that is sufficient to be recognized by naïve B-lymphocytes. The booster effect observed after the second vaccine injection indicated that memory B-cells have been induced after priming. Although vaccine doses were equivalent in both studies, we found higher neutralizing antibodies titres after two vaccine injections in all horses tested in the present study. This could be ascribed to the two-fold higher amount of carbopol used as adjuvant in the present study.

Cell mediated immune responses against VP2 and VP5 were characterized by the detection of TH1 cytokine, IFN-γ, secreted by a number of activated cell populations including NK-cells, alpha/beta TCR and gamma/delta TCR expressing T-cells. The ELISpot results demonstrated that ALVAC®-AHSV4 vaccine elicited specific IFN-γ responses in all vaccinated horses. Two of the six vaccinated horses responded after one injection and the remaining four after the second injection. The PBMCs in the ELISpot assay were stimulated with the ALVAC®-AHSV4 vector confirming that the appropriate proteins were successfully expressed and processed for T-cell recognition. Flow cytometry analyses also confirmed this finding and enabled us to further characterize the phenotype of VP2- and VP5-specific-IFN-γ producing cells. We observed that for both VP2 and VP5, specific IFN-γ producing cells were identified mainly as $CD8\alpha^{+}$ cells. Equine T-lymphocytes express either the homodimer $CD8\alpha\alpha$ or the heterodimer $CD8\alpha\beta$ molecules (Tschetter *et al*. 1998). Expression of these two different CD8 molecules has been associated with different cytolytic activities in humans. According to Baum *et al.* (1990) $CD8\alpha$ is expressed on both MHC restricted and non-MHC restricted lymphocytes. The MHC restricted cells expressed $CD8\alpha$ with a high density ($CD8\alpha^{bright}$). A significant population of NK cells also

expresses CD8α however its expression is at low intensity (CD8α^{dim}). Our results presented in Figure 5-2 shows that in absence of stimuli or even with an irrelevant peptide pool a weak percentage of IFN-γ was predominantly produced by CD8α^{dim} expressing cells. This population might correspond to NK cells and similar results were recently reported in horses vaccinated with a modified live AHSV 4 vaccine (Pretorius *et al*. 2012). With the antigenic stimulation with VP2 or VP5 peptide pools a significant induction of IFN-γ by both CD8α^{dim} and CD8α^{bright} cell types was observed. If these CD8α^{dim} cells represent NK cells it would be consistent with the findings of Horowitz *et al*. (2010) who described a significant effector CD4 T cell–dependent NK activation after vaccination.

Studies performed by Andrew *et al.* (1995) with BTV have demonstrated that VP2 and the non-structural protein NS1 were the major immunogens for CTLs, while VP5 and NS3 were minor immunogens in sheep. Other groups reported multiple CTL epitopes in VP2 and NS1 sequences (Janardhana *et al.* 1999; Jones *et al.* 1996). The presence of many CTL epitopes in BTV-immune sheep is in some extent also observed in the present study. Although we did not further investigate the cytotoxic ability of AHSV 4-specific IFN-γ producing CD8α^{+} cells, our results clearly highlighted multiple and broad T-cell epitopes through the entire VP2 protein sequence. Except for the CTL response described in one AHSV 3 vaccinated horse by Romito *et al.* (1999), no CTL or T-helper epitopes have been described for AHSV 4 to the best of our knowledge. Nevertheless B-epitopes and neutralising antibody epitopes were well-identified on VP2 and VP5 of AHSV 4 (Martinez-Torrecuadrada *et al.* 1999; Martinez-Torrecuadrada *et al.* 2001). Two B-cell antigenic regions in amino acid positions 83-120 and 151-200 in VP5 of AHSV 4 were also identified (Martínez-Torrecuadrada *et al.* (1999). Within each antigenic region, they have demonstrated the presence of eight B-cell epitopes with two neutralizing epitopes in position 85-92 and 179-185. Interestingly, our results also highlighted the N-terminus of this outer capsid protein as an antigenic region. The first three VP5 peptide pools stimulated efficiently specific CD8α^{+} T-cells to produced IFN-γ. Finally, a diversification of

the immune response was observed after vaccination with the appearance of $CD8\alpha^-$ cells specific to VP2 and VP5 at the latest examined time point (Day 56). As in healthy horses double positive $CD4^+CD8^+$ population in blood haven't been reported so far, one may assume that the specific $CD8\alpha^-$ cells may correspond to $CD4^+$ T-helper cells that might be helpful for the induction of memory responses. A recent paper (Rojas *et al*. 2011) reported that multiple $CD4^+$ and $CD8^+$ T-epitopes are present on VP7 of BTV 8 and can confer protection of the natural host. In this study it was found that VP2 and VP5 may also contain some epitopes that elicited both $CD8\alpha^+$ and $CD8\alpha^-$ T-cell responses.

In summary, the recombinant ALVAC®-AHSV4 experimental vaccine which has been described as fully protective against a virulent homologous challenge in horses is able to induce CMI specifically against VP2 and VP5 proteins. Further T-cell epitope mapping on VP2 and VP5 would be interesting to follow up in order to document the induced CMI that might be helpful in the process of protection against AHSV 4 infection. Additionally, as VP5 had been reported to be highly conserved among serotypes, it may be interesting to investigate how the present ALVAC®-AHSV4 vaccine would cross-react against other serotypes (Laviada *et al*. 1993).

Chapter 6

GENERAL CONCLUSIONS

The first objective of this study was to determine antibody titres to each of the 9 serotypes of AHSV in a cohort of breeding mares that were regularly vaccinated with the polyvalent cell culture-adapted MLV vaccine that is commercially available in South Africa. There was considerable variation in the antibody response of individual mares to the different AHSV serotypes suggesting that that repeated annual vaccination does not reliably induce an anamnestic response with sustained high levels of neutralising antibody to all AHSV serotypes in every horse.

Some mares developed high antibody titres to specific AHSV serotypes whereas titres to other serotypes remained low throughout the animal's life despite repeated vaccination. The transient or low antibody titres to some serotypes in individual mares could impair passive protection of their foals.

The second objective was to measure passive transfer and rate of decay of maternal antibody to the individual virus serotypes in foals. Passively-acquired maternal antibody to AHSV varied markedly among foals born to vaccinated mares, with further variation in the duration of passive immunity to individual AHSV serotypes. The mean half-live for passively-acquired maternal neutralising antibody to AHSV was 20.5 days.

To achieve sufficient protection in young foals it is important to ensure high levels of neutralising antibody in the mare before foaling. In this study the detection of maternal antibody in foals varied between to 2 to 4.5 months depending on the serotype.

The role of adjuvants could be important in this regard, as high levels of neutralising antibody (that lasted at least 10 months) was described for a inactivated adjuvanted AHS 9 vaccine (Ronchi *et al*. 2012). More data representing a larger geographical distribution and study population are needed before definitive recommendations on the optimal timing of AHSV vaccination of foals in endemic areas can be made.

The third objective was to describe the serological response to individual AHSV serotypes in horses in the field that are regularly vaccinated with the commercial MLV vaccine. Based on the assumption that neutralising antibody confers serotype specific immunity to homologous virus serotypes and the cross-neutralisation between certain virus serotypes, we expected to see a comprehensive profile of solid neutralising antibody titres to all 9 serotypes in vaccinated field horses. However, naïve foals vaccinated for the first time, more consistently seroconverted to AHSV 1 and responses to other serotypes were highly variable, and often weak or not detected.

Neutralising antibody to individual AHSV serotypes was often transient and quickly waned after the first vaccination and with a poor anamnestic response to second (booster) vaccination. This phenomenon was also evident in the neutralising antibody profile observed in mares that were repeatedly vaccinated over several years. Even foals that received selected monovalent MLV strains (representative for bottles 1 and 2 respectively, of the commercial vaccine) responded with a similar magnitude to those that received the polyvalent preparation (with exception to serotype 7). These results suggest that there is no real enhanced immune response through the administration of a monovalent vaccine as opposed to the polyvalent vaccine.

We confirmed that as described by Guthrie *et al*. (2009) the recombinant canarypox virus vector that co-expresses VP2 and VP5 proteins of AHSV 4 elicits antigen-specific neutralizing antibodies. The booster effect observed after the second vaccine injection indicated that memory B cells have been induced after priming.

The ALVAC®-AHSV4 vaccine elicited specific IFN-γ responses in all vaccinated horses confirming that the appropriate proteins were successfully expressed and processed for T-cell recognition.

We observed that for both VP2 and VP5, specific IFN-γ producing cells were identified mainly as $CD8\alpha^{+}$ cells. There was significant induction of IFN-γ by both $CD8\alpha^{dim}$ and $CD8\alpha^{bright}$ cell types. The $CD8\alpha^{dim}$ cells could represent NK cells activated by effector CD4 T-cells.

Multiple T-cell-epitopes ($CD8\alpha^{+}$) were present through all VP2 and only the N-terminus sequence of VP5.

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References

Adams, A.A., Sturgill, T.L., Breathnach, C.C., Chambers, T.M., Siger, L., Minke, J.M., & Horohov, D.W. 2011. Humoral and cell-mediated immune responses of old horses following recombinant canarypox virus vaccination and subsequent challenge infection. *Veterinary Immunology and Immunopathology*, 139, (2-4) 128-140

Alexander, R.A. 1935a. Studies on the neurotropic virus of horsesickness III The intracerebral protection test and its application to the study of immunity. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 4, (2) 349-377

Alexander, R.A. 1935b. Studies on the neurotropic virus of horsesickness IV. The pathogenesis in horses. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 4, (2) 379-388

Alexander, R.A. & Mason, J.H. 1941. Studies on the neurotropic virus of horsesicknessVII - Transmitted immunity. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 16, (1,2) 19-32

Alexander, R.A., Neitz, W.O., & Du Toit, P.J. 1936. Horsesickness: Immunization of horses and mules in the field during the season 1934-1935 with a description of the technique of preparation of polyvalent mouse neurotropic vaccine. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 7, 17-30

Allen, C.A., Payne, S.L., Harville, M., Cohen, N., & Russell, K.E. 2007. Validation of quantitative polymerase chain reaction assays for measuring cytokine expression in equine macrophages. *Journal of Immunological Methods*, 328, (1-2) 59-69

Andrew, M., Whiteley, P., Janardhana, V., Lobato, Z.I.P., Gould, A., & Coupar, B. 1995. Antigen specificity of the ovine cytotoxic T lymphocyte response to bluetongue virus. *Veterinary Immunology and Immunopathology*, 47, (3-4) 311-322

Baume, D.M., Caligiuri, M.A., Manley, T.J., Daley, J.F., & Ritz, J. 1990. Differential expression of CD8 alpha and CD8 beta associated with MHC-restricted and non-MHC-restricted cytolytic effector cells. *Cellular Immunology*, 131, (2) 352-365

Belhouchet, M., Mohd Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P.C., & Attoui, H. 2011. Detection of a Fourth Orbivirus Non-Structural Protein. *PLoS One*, 6, (10) e25697

Blackburn, N.K. & Swanepoel, R. 1988a. African horse sickness in Zimbabwe: 1972 to 1981. *Tropical Animal Health and Production*, 20, (3) 169-176

Blackburn, N.K. & Swanepoel, R. 1988b. Observations on antibody levels associated with active and passive immunity to African horse sickness. *Tropical Animal Health and Production*, 20, (4) 203-210

Boehm, U., Klamp, T., Groot, M., & Howard, J.C. 1997. Cellular responses to interferon-gamma. *Annual Review of Immunology*, 15, (1) 749-795

Boone, J.D., Balasuriya, U.B., Karaca, K., Audonnet, J.C., Yao, J., He, L., Nordgren, R., Monaco, F., Savini, G., Gardner, I.A., & MacLachlan, N.J. 2007. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine*, 25, (4) 672-678

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M., & Haller, O. 2001. Genetic Evidence for an Interferon-Antagonistic Function of Rift Valley Fever Virus Nonstructural Protein NSs. *Journal of Virology*, 75, (3) 1371-1377

Brewer, A.W. & MacLachlan, N.J. 1992. Ultrastructural Characterization of the Interaction of Bluetongue Virus with Bovine Erythrocytes in vitro. *Veterinary Pathology Online*, 29, (4) 356-359

Burrage, T.G. & Laegreid, W.W. 1994. African horsesickness: Pathogenesis and immunity. *Comparative Immunology, Microbiology and Infectious Diseases*, 17, (3-4) 275-285

Burrage, T.G., Trevejo, R., Stone-Marschat, M., & Laegreid, W.W. 1993. Neutralizing epitopes of African horsesickness virus serotype 4 are located on VP2. *Virology*, 196, (2) 799-803

Calisher, C.H. & Mertens, P.P. 1998. Taxonomy of African horse sickness viruses. *Archives of Virology, Supplementum*, 14, 3-11

Calvo-Pinilla, E., Navasa, N., Anguita, J., & Ortego, J. 2012. Multiserotype Protection Elicited by a Combinatorial Prime-Boost Vaccination Strategy against Bluetongue Virus. *PLoS One*, 7, (4) e34735

Calvo-Pinilla, E., Nieto, J.M., & Ortego, J. 2010. Experimental oral infection of bluetongue virus serotype 8 in type I interferon receptor-deficient mice. *Journal of General Virology*, 91, (11) 2821-2825

Calvo-Pinilla, E., Rodriguez-Calvo, T., Anguita, J., Sevilla, N., & Ortego, J. 2009a. Establishment of a Bluetongue Virus Infection Model in Mice that Are Deficient in the Alpha/Beta Interferon Receptor. *PLoS One*, 4, (4) e5171

Calvo-Pinilla, E., Rodriguez-Calvo, T., Sevilla, N., & Ortego, J. 2009b. Heterologous prime boost vaccination with DNA and recombinant modified vaccinia virus Ankara protects IFNAR(-/-) mice against lethal bluetongue infection. *Vaccine*, 28, (2) 437-445

Castillo-Olivares, J., Calvo-Pinilla, E., Casanova, I., Bachanek-Bankowska, K., Chiam, R., Maan, S., Nieto, J.M., Ortego, J., & Mertens, P.P. 2011. A modified vaccinia Ankara virus (MVA) vaccine expressing African horse sickness virus (AHSV) VP2 protects against AHSV challenge in an IFNAR -/- mouse model. *PLoS.One.*, 6, (1) e16503

Chiam, R., Sharp, E., Maan, S., Rao, S., Mertens, P., Blacklaws, B., vis-Poynter, N., Wood, J., & Castillo-Olivares, J. 2009. Induction of antibody responses to African horse sickness virus (AHSV) in ponies after vaccination with recombinant modified vaccinia ankara (MVA). *PLoS One*, 4, (6) 5997

Coetzer, J. A. W. & Erasmus, B. J. 1994, "African horsesickness," *In Infectious Diseases of Livestock with special reference to Southern Africa*, J. A. W. Coetzer, G. R. Thomson, & R. Tustin, eds., Cape Town: Oxford University Press, pp. 460-475.

Coetzer, J. A. W. & Guthrie, A. J. 2004, "African horse sickness," *In Infectious diseases of livestock. Volume Two*, Second ed. Oxford; UK: Oxford University Press, pp. 1231-1246.

Crafford, J.E., Lourens, C.W., Gardner, I.A., MacLachlan, N.J., & Guthrie, A.J. 2012. Passive transfer and rate of decay of maternal antibody against African horse sickness virus in South African Thoroughbred foals. *Equine Veterinary Journal*, DOI: 10.1111/evj.12015, n/a

Davies, F.G. & Lund, L.J. 1974. The application of fluorescent antibody techniques to the virus of African horse sickness. *Research in Veterinary Science*, 17, (1) 128-130

de la Poza, F., Calvo-Pinilla, E., Lopez-Gil, E., Marin-Lopez, A., Mateos, F., Castillo-Olivares, J., Lorenzo, G., & Ortego, J. 2013. Ns1 Is a Key Protein in the Vaccine Composition to Protect Ifnar(-/-) Mice against Infection with Multiple Serotypes of African Horse Sickness Virus. *PLoS One*, 8, (7) e70197

DeMaula, C.D., Jutila, M.A., Wilson, D.W., & MacLachlan, N.J. 2001. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *Journal of General Virology*, 82, (4) 787-794

DeMaula, C.D., Leutenegger, C.M., Bonneau, K.R., & MacLachlan, N.J. 2002a. The Role of Endothelial Cell-Derived Inflammatory and Vasoactive Mediators in the Pathogenesis of Bluetongue. *Virology*, 296, (2) 330-337

DeMaula, C.D., Leutenegger, C.M., Jutila, M.A., & MacLachlan, N.J. 2002b. Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. *Veterinary Immunology and Immunopathology*, 86, (3-4) 147-157

Diouf, N.D., Etter, E., Lo, M.M., Lo, M., & Akakpo, A.J. 2013. Outbreaks of African horse sickness in Senegal, and methods of control of the 2007 epidemic. *Veterinary Record*, 172, (6) 152

Drew, C.P., Gardner, I.A., Mayo, C.E., Matsuo, E., Roy, P., & MacLachlan, N.J. 2010a. Bluetongue virus infection alters the impedance of monolayers of bovine endothelial cells as a result of cell death. *Veterinary Immunology and Immunopathology*, 136, (1–2) 108-115

Drew, C.P., Heller, M.C., Mayo, C., Watson, J.L., & MacLachlan, N.J. 2010b. Bluetongue virus infection activates bovine monocyte-derived macrophages and pulmonary artery endothelial cells. *Veterinary Immunology and Immunopathology*, 136, (3–4) 292-296

El' Garch, H., Minke, J.M., Rehder, J., Richard, S., Edlund Toulemonde, C., Dinic, S., Andreoni, C., Audonnet, J.C., Nordgren, R., & Juillard, V. 2008. A West Nile virus (WNV) recombinant canarypox virus vaccine elicits WNV-specific neutralizing

antibodies and cell-mediated immune responses in the horse. *Veterinary Immunology and Immunopathology*, 123, (3-4) 230-239

Erasmus, B. J. 1973, "Pathogenesis of African horsesickness," *In Equine Infectious Diseases III*, J. T. Bryans & H. Gerber, eds., pp. 1-11.

Erasmus, B. J. 1978, "A new approach to polyvalent immunization against African horsesickness," *In Equine Infectious Diseases IV*, J. T. Bryans & H. Gerber, eds., Princeton: Veterinary Publications Incorporated, pp. 401-403.

Ghalib, H.W., Schore, C.E., & Osburn, B.I. 1985. Immune response of sheep to bluetongue virus: *In vitro* induced lymphocyte blastogenesis. *Veterinary Immunology and Immunopathology*, 10, (2-3) 177-188

Gomez-Villamandos, J.C., Sanchez, C., Carrasco, L., Laviada, M.D., Bautista, M.J., Martinez-Torrecuadrada, J., Sanchez-Vizcaino, J.M., & Sierra, M.A. 1999. Pathogenesis of African horse sickness: Ultrastructural study of the capillaries in experimental infection. *Journal of Comparative Pathology*, 121, (2) 101-116

Gould, E.A. & Higgs, S. 2009. Impact of climate change and other factors on emerging arbovirus diseases. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 103, (2) 109-121

Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Mertens, P.P.C., & Stuart, D.I. 1998. The atomic structure of the bluetongue virus core. *Nature*, 395, (6701) 470-478

Guthrie, A. J. 2007, "African horse sickness," *In Equine Infectious Diseases*, D. C. Sellon & M. T. Long, eds., St. Louis: Saunders Elsevier, pp. 164-171.

Guthrie, A. J. & Quan, M. 2009, "African horse sickness," *In Infectious diseases of the horse*, T. S. Mair & R. E. Hutchinson, eds., Fordham: Equine Veterinary Journal, pp. 72-82.

Guthrie, A.J., Quan, M., Lourens, C.W., Audonnet, J.C., Minke, J.M., Yao, J., He, L., Nordgren, R., Gardner, I.A., & MacLachlan, N.J. 2009. Protective immunization of horses with a recombinant canarypox virus vectored vaccine co-expressing genes encoding the outer capsid proteins of African horse sickness virus. *Vaccine*, 27, (33) 4434-4438

Hamblin, C., Graham, S.D., Anderson, E.C., & Crowther, J.R. 1990. A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus. *Epidemiology and Infection*, 104, (2) 303-312

Hassan, S.H., Wirblich, C., Forzan, M., & Roy, P. 2001. Expression and functional characterization of bluetongue virus VP5 protein: Role in cellular permeabilization. *Journal of Virology*, 75, (18) 8356-8367

Hassan, S.S. & Roy, P. 1999. Expression and functional characterization of bluetongue virus VP2 protein: Role in cell entry. *Journal of Virology*, 73, (12) 9832-9842

He, X.S., Rehermann, B., Boisvert, J., Mumm, J., Maecker, H.T., Roederer, M., Wright, T.L., Maino, V.C., Davis, M.M., & Greenberg, H.B. 2001. Direct functional analysis of epitope-specific CD8+ T cells in peripheral blood. *Viral Immunology*, 14, (1) 59-69

Heyman, B. 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annual Review of Immunology*, 18, 709-737

Hjelm, F., Carlsson, F., Getahun, A., & Heyman, B. 2006. Antibody-mediated regulation of the immune response. *Scandinavian Journal of Immunology*, 64, (3) 177-184

Hoffmeister, B., Kiecker, F., Tesfa, L., Volk, H.D., Picker, L.J., & Kern, F. 2003. Mapping T cell epitopes by flow cytometry. *Methods*, 29, (3) 270-281

Horowitz, A., Behrens, R.H., Okell, L., Fooks, A.R., & Riley, E.M. 2010. NK cells as effectors of acquired immune responses: effector CD4+ T cell-dependent activation of NK cells following vaccination. *Journal of Immunology*, 185, (5) 2808-2818

House, C., Mikiciuk, P.E., & Berninger, M.L. 1990. Laboratory diagnosis of African horse sickness: comparison of serological techniques and evaluation of storage methods of samples for virus isolation. *Journal of Veterinary Diagnostic Investigation*, 2, (1) 44-50

House, J.A. 1993. Recommendations for African horse sickness vaccines for use in nonendemic areas. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, 46, 77-81

House, J.A. 1998. Future international management of African horse sickness vaccines. *Archives of Virology, Supplementum*, 14, 297-304

House, J.A., Lombard, M., Dubourget, P., House, C., & Mebus, C.A. 1994. Further studies on the efficacy of an inactivated African horse sickness serotype 4 vaccine. *Vaccine*, 12, (2) 142-144

Howell, P.G. 1962. The isolation and identification of further antigenic types of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 29, 139-149

Howell, P.G. 1963. Observations on the occurence of African horsesickness amongst immunised horses. *Onderstepoort Journal of Veterinary Research*, 30, 3-10

Howell, P.G., Groenewald, D., Visage, C.W., Bosman, A.M., Coetzer, J.A.W., & Guthrie, A.J. 2002. The classification of seven serotypes of equine encephalosis virus and the

prevalence of homologous antibody in horses in South Africa. *Onderstepoort Journal of Veterinary Research*, 69, (1) 79-93

Huismans, H. & Els, H.J. 1979. Characterization of the tubules associated with the replication of three different orbiviruses. *Virology*, 92, (2) 397-406

Huismans, H., Van, S., V, Fick, W.C., van, N.M., & Meiring, T.L. 2004. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Veterinaria Italiana*, 40, (4) 417-425

Hullinger, P.J., Wilson, W.D., Rossitto, P.V., Patton, J.F., Thurmond, M.C., & MacLachlan, N.J. 1998. Passive transfer, rate of decay, and protein specificity of antibodies against equine arteritis virus in horses from a Standardbred herd with high seroprevalence. *Journal of the American Veterinary Medical Association*, 213, (6) 839-842

Hyatt, A.D., Zhao, Y., & Roy, P. 1993. Release of Bluetongue Virus-like Particles from Insect Cells is Mediated by BTV Nonstructural Protein NS3/NS3A. *Virology*, 193, (2) 592-603

Jabbar, T.K., Calvo-Pinilla, E., Mateos, F., Gubbins, S., Bin-Tarif, A., Bachanek-Bankowska, K., Alpar, O., Ortego, J., Takamatsu, H.H., Mertens, P.P.C., & Castillo-Olivares, J. 2013. Protection of IFNAR (–/–) Mice against Bluetongue Virus Serotype 8, by Heterologous (DNA/rMVA) and Homologous (rMVA/rMVA) Vaccination, Expressing Outer-Capsid Protein VP2. *PLoS One*, 8, (4) e60574

Janardhana, V., Andrew, M.E., Lobato, Z.I.P., & Coupar, B.E. 1999. The ovine cytotoxic T lymphocyte responses to bluetongue virus. *Research in Veterinary Science*, 67, (3) 213-221

Jeggo, M.H., Gumm, I.D., & Taylor, W.P. 1983. Clinical and serological response of sheep to serial challenge with different bluetongue virus types. *Research in Veterinary Science*, 34, (2) 205-211

Jeggo, M.H., Wardley, R.C., & Brownlie, J. 1984. A study of the role of cell-mediated immunity in bluetongue virus infection in sheep, using cellular adoptive transfer techniques. *Immunology*, 52, (3) 403-410

Jeggo, M.H., Wardley, R.C., & Brownlie, J. 1985. Importance of ovine cytotoxic T cells in protection against bluetongue virus infection. *Progress in Clinical Biological Research*, 178, 477-487

Jones, L.D., Chuma, T., Hails, R., Williams, T., & Roy, P. 1996. The non-structural proteins of bluetongue virus are a dominant source of cytotoxic T cell peptide determinants. *Journal of General Virology*, 77, (5) 997-1003

Jones, L.D., Williams, T., Bishop, D., & Roy, P. 1997. Baculovirus-expressed nonstructural protein NS2 of bluetongue virus induces a cytotoxic T-cell response in mice which affords partial protection. *Clinical and Diagnostic Laboratory Immunology*, 4, (3) 297-301

Kern, F., Surel, I.P., Brock, C., Freistedt, B., Radtke, H., Scheffold, A., Blasczyk, R., Reinke, P., Schneider-Mergener, J., Radbruch, A., Walden, P., & Volk, H.D. 1998. T-cell epitope mapping by flow cytometry. *Nature Medicine*, 4, (8) 975-978

Kern, F., Faulhaber, N., Frommel, C., Khatamzas, E., Prosch, S., Schonemann, C., Kretzschmar, I., Volkmer-Engert, R., Volk, H.D., & Reinke, P. 2000. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *European Journal of Immunology*, 30, (6) 1676-1682

Kiecker, F., Streitz, M., Ay, B., Cherepnev, G., Volk, H.D., Volkmer-Engert, R., & Kern, F. 2004. Analysis of antigen-specific T-cell responses with synthetic peptides--what kind of peptide for which purpose? *Human Immunology*, 65, (5) 523-536

Laviada, M.D., Arias, M., & Sanchez-Vizcaino, J.M. 1993. Characterization of African horsesickness virus seroeype 4-induced polypeptides in Vero cells and their reactivity in Western immunoblotting. *Journal of General Virology*, 74, (1) 81-87

Laviada, M.D., Roy, P., Sanchez-Vizaino, J.M., & Casal, J.I. 1995. The use of African horse sickness virus NS3 protein, expressed in bacteria, as a marker to differentiate infected from vaccinated horses. *Virus Research*, 38, (2-3) 205-218

Laviada, M. D., Roy, P., & Sánchez-Vizcaíno, J. M. 1992, "Adaptation and evaluation of an indirect ELISA and immuno-blotting test for African horse sickness antibody detection," *In Bluetongue, African horse sickness and related orbiviruses*, T. E. Walton & B. I. Osburn, eds., Boca Raton: CRC Press, pp. 646-650.

Lelli, R., Molini, U., Ronchi, G.F., Rossi, E., Franchi, P., Ulisse, S., Armillotta, G., Capista, S., Khaiseb, S., Di, V.M., & Pini, A. 2013. Inactivated and adjuvanted vaccine for the control of the African horse sickness virus serotype 9 infection: evaluation of efficacy in horses and guinea-pig model. *Veterinaria Italiana*, 49, (1) 89-98

Lobato, Z.I.P., Coupar, B.E.H., Gray, C.P., Lunt, R., & Andrew, M.E. 1997. Antibody responses and protective immunity to recombinant vaccinia virus-expressed bluetongue virus antigens. *Veterinary Immunology and Immunopathology*, 59, (3-4) 293-309

Lymperopoulos, K., Wirblich, C., Brierley, I., & Roy, P. 2003. Sequence Specificity in the Interaction of Bluetongue Virus Non-structural Protein 2 (NS2) with Viral RNA. *Journal of Biological Chemistry*, 278, (34) 31722-31730

MacLachlan, N.J., Drew, C.P., Darpel, K.E., & Worwa, G. 2009. The Pathology and Pathogenesis of Bluetongue. *Journal of Comparative Pathology*, 141, (1) 1-16

MacLachlan, N.J. & Guthrie, A.J. 2010. Re-emergence of bluetongue, African horse sickness, and other Orbivirus diseases. *Veterinary Research*, 41, (6) 35-47

MacLachlan, N.J., Balasuriya, U.B., Davis, N.L., Collier, M., Johnston, R.E., Ferraro, G.L., & Guthrie, A.J. 2007. Experiences with new generation vaccines against equine viral arteritis, West Nile disease and African horse sickness. *Vaccine*, 25, (30) 5577-5582

MacLachlan, N.J. & Mayo, C.E. 2013. Potential strategies for control of bluetongue, a globally emerging, Culicoides-transmitted viral disease of ruminant livestock and wildlife. *Antiviral Research*, 99, (2) 79-90

Maecker, H.T., Dunn, H.S., Suni, M.A., Khatamzas, E., Pitcher, C.J., Bunde, T., Persaud, N., Trigona, W., Fu, T.M., Sinclair, E., Bredt, B.M., McCune, J.M., Maino, V.C., Kern, F., & Picker, L.J. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *Journal of Immunological Methods*, 255, (1-2) 27-40

Manole, V., Laurinmaki, P., van, W.W., Potgieter, C.A., Wright, I.M., Venter, G.J., Van Dijk, A.A., Sewell, B.T., & Butcher, S.J. 2012. Structural insight into African horsesickness virus infection. *Journal of Virology*, 86, (15) 7858-7866

Maree, S. & Paweska, J.T. 2005. Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. *Journal of Virological Methods*, 125, (1) 55-65

Martinez-Torrecuadrada, J.L., Diaz-Laviada, M., Roy, P., Sanchez, C., Vela, C., Sanchez-Vizcaino, J.M., & Casal, J.I. 1996. Full protection against African horsesickness

(AHS) in horses induced by baculovirus-derived AHS virus serotype 4 VP2, VP5 and VP7. *Journal of General Virology*, 77, (6) 1211-1221

Martinez-Torrecuadrada, J.L., Iwata, H., Venteo, A., Casal, I., & Roy, P. 1994. Expression and characterization of the two outer capsid proteins of African horsesickness virus: The role of VP2 in virus neutralization. *Virology*, 202, (1) 348-359

Martinez-Torrecuadrada, J.L., Langeveld, J.P.M., Meloen, R.H., & Casal, J.I. 2001. Definition of neutralizing sites on African horse sickness virus serotype 4 VP2 at the level of peptides. *Journal of General Virology*, 82, (10) 2415-2424

Martinez-Torrecuadrada, J.L., Langeveld, J.P.M., Venteo, A., Sanz, A., Dalsgaard, K., Hamilton, W.D.O., Meloen, R.H., & Casal, J.I. 1999. Antigenic profile of African horse sickness virus serotype 4 VP5 and identification of a neutralizing epitope shared with bluetongue virus and epizootic hemorrhagic disease virus. *Virology*, 257, (2) 449-459

McIntosh, B.M. 1955. Complement fixation with horsesickness virusses. *Onderstepoort Journal of Veterinary Research*, 27, (2) 165-169

McIntosh, B.M. 1958. Immunological types of horsesickness virus and their significance in immunization. *Onderstepoort Journal of Veterinary Research*, 27, (4) 465-538

Meiswinkel, R. & Paweska, J.T. 2003. Evidence for a new field Culicoides vector of African horse sickness in South Africa. *Preventive Veterinary Medicine*, 60, (3) 243-253

Mellor, P.S. & Boorman, J. 1995. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Annals of Tropical Medicine and Parasitology*, 89, (1) 1-15

Minke, J.M., El-Hage, C.M., Tazawa, P., Homer, D., Lemaitre, L., Cozette, V., Gilkerson, J.R., & Kirkland, P.D. 2011a. Evaluation of the response to an accelerated

immunisation schedule using a canarypox-vectored equine influenza vaccine, shortened interdose intervals and vaccination of young foals. *Australian Veterinary Journal*, 89 Suppl 1, 137-139

Minke, J.M., Fischer, L., Baudu, P., Guigal, P.M., Sindle, T., Mumford, J.A., & Audonnet, J.C. 2006. Use of DNA and recombinant canarypox viral (ALVAC) vectors for equine herpes virus vaccination. *Veterinary Immunology and Immunopathology*, 111, (1-2) 47-57

Minke, J.M., Siger, L., Cupillard, L., Powers, B., Bakonyi, T., Boyum, S., Nowotny, N., & Bowen, R. 2011b. Protection provided by a recombinant ALVAC(R)-WNV vaccine expressing the prM/E genes of a lineage 1 strain of WNV against a virulent challenge with a lineage 2 strain. *Vaccine*, 29, (28) 4608-4612

Minke, J.M., Siger, L., Karaca, K., Austgen, L., Gordy, P., Bowen, R., Renshaw, R.W., Loosmore, S., Audonnet, J.C., & Nordgren, B. 2004. Recombinant canarypoxvirus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. *Archives of Virology*, Suppl. 18, (Suppl. 18) 221-230

Minke, J.M., Toulemonde, C.E., Coupier, H., Guigal, P.M., Dinic, S., Sindle, T., Jessett, D., Black, L., Bublot, M., Pardo, M.C., & Audonnet, J.C. 2007. Efficacy of a canarypox-vectored recombinant vaccine expressing the hemagglutinin gene of equine influenza H3N8 virus in the protection of ponies from viral challenge. *American Journal of Veterinary Research*, 68, (2) 213-219

Morris, C.R., Gardner, I.A., Hietala, S.K., Carpenter, T.E., Anderson, R.J., & Parker, K.M. 1994. Persistence of Passively Acquired Antibodies to Mycoplasma-Hyopneumoniae in A Swine Herd. *Preventive Veterinary Medicine*, 21, (1) 29-41

Mosmann, T.R. & Coffman, R.L. 1989. TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. *Annual Review of Immunology*, 7, (1) 145-173

Nielsen, M. & Lund, O. 2009. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics*, 10, 296

Nielsen, M., Lundegaard, C., Worning, P., Lauemoller, S.L., Lamberth, K., Buus, S., Brunak, S., & Lund, O. 2003. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. *Protein Science*, 12, (5) 1007-1017

O'Neill, T., Kydd, J.H., Allen, G.P., Wattrang, E., Mumford, J.A., & Hannant, D. 1999. Determination of equid herpesvirus 1-specific, CD8+, cytotoxic T lymphocyte precursor frequencies in ponies. *Veterinary Immunology and Immunopathology*, 70, (1-2) 43-54

Odeon, A.C., Schore, C.E., & Osburn, B.I. 1997. The role of cell-mediated immunity in the pathogenesis of bluetongue virus serotype 11 in the experimental infection of vaccine/sensitized calves. *Comparative Immunology, Microbiology and Infectious Diseases*, 20, (3) 219-231

Ohka, S., Igarashi, H., Nagata, N., Sakai, M., Koike, S., Nochi, T., Kiyono, H., & Nomoto, A. 2007. Establishment of a Poliovirus Oral Infection System in Human Poliovirus Receptor-Expressing Transgenic Mice That Are Deficient in Alpha/Beta Interferon Receptor. *Journal of Virology*, 81, (15) 7902-7912

Ohno, S., Ono, N., Seki, F., Takeda, M., Kura, S., Tsuzuki, T., & Yanagi, Y. 2007. Measles Virus Infection of SLAM (CD150) Knockin Mice Reproduces Tropism and Immunosuppression in Human Infection. *Journal of Virology*, 81, (4) 1650-1659

OIE Code Commission 2010. *Terrestrial Animal Health Code*, 19 ed. Paris, OIE - World Organisation for Animal Health.

OIE Code Commission 2012a, "African horse sickness," *In Terrestrial Animal Health Code*, 21 ed. OIE Code Commission, ed., Paris: OIE - World Organisation for Animal Health.

OIE Code Commission 2012b. *Terrestrial Animal Health Code*, 21 ed. Paris, OIE - World Organisation for Animal Health.

Osburn, B. I. 1992, "Immune responses to orbiviruses," *In Second international Symposium on Bluetongue, African Horse Sickness and Related Orbiviruses*, T. E. Walton & B. I. Osburn, eds., Boca Raton: CRC Press, pp. 511-524.

Owens, R.J., Limn, C., & Roy, P. 2004. Role of an Arbovirus Nonstructural Protein in Cellular Pathogenesis and Virus Release. *Journal of Virology*, 78, (12) 6649-6656

Paillot, R., Daly, J.M., Juillard, V., Minke, J.M., Hannant, D., & Kydd, J.H. 2005. Equine interferon gamma synthesis in lymphocytes after *in vivo* infection and *in vitro* stimulation with EHV-1. *Vaccine*, 23, (36) 4541-4551

Paillot, R., Ellis, S.A., Daly, J.M., Audonnet, J.C., Minke, J.M., vis-Poynter, N., Hannant, D., & Kydd, J.H. 2006a. Characterisation of CTL and IFN-[gamma] synthesis in ponies following vaccination with a NYVAC-based construct coding for EHV-1 immediate early gene, followed by challenge infection. *Vaccine*, 24, (10) 1490-1500

Paillot, R., Kydd, J.H., MacRae, S., Minke, J.M., Hannant, D., & Daly, J.M. 2007. New assays to measure equine influenza virus-specific Type 1 immunity in horses. *Vaccine*, 25, (42) 7385-7398

Paillot, R., Kydd, J.H., Sindle, T., Hannant, D., Edlund Toulemonde, C., Audonnet, J.C., Minke, J.M., & Daly, J.M. 2006b. Antibody and IFN-[gamma] responses induced

by a recombinant canarypox vaccine and challenge infection with equine influenza virus. *Veterinary Immunology and Immunopathology*, 112, (3-4) 225-233

Poulet, H., Minke, J., Pardo, M.C., Juillard, V., Nordgren, B., & Audonnet, J.C. 2007. Development and registration of recombinant veterinary vaccines: The example of the canarypox vector platform. *Vaccine*, 25, (30) 5606-5612

Pretorius, A., Van, K.M., van, W.W., & Heath, J. 2012. Virus-specific CD8(+) T-cells detected in PBMC from horses vaccinated against African horse sickness virus. *Veterinary Immunology and Immunopathology*, 146, (1) 81-86

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P.C., & Baylis, M. 2005. Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology*, 3, (2) 171-181

Quinlivan, M., Nelly, M., Prendergast, M., Breathnach, C., Horohov, D., Arkins, S., Chiang, Y.W., Chu, H.J., Ng, T., & Cullinane, A. 2007. Pro-inflammatory and antiviral cytokine expression in vaccinated and unvaccinated horses exposed to equine influenza virus. *Vaccine*, 25, (41) 7056-7064

Quist, C.F., Howerth, E.W., Bounous, D.I., & Stallknecht, D.E. 1997. Cell-mediated immune response and IL-2 production in white-tailed deer experimentally infected with hemorrhagic disease viruses. *Veterinary Immunology and Immunopathology*, 56, (3-4) 283-297

Rojas, J.M., Rodriguez-Calvo, T., Pena, L., & Sevilla, N. 2011. T cell responses to bluetongue virus are directed against multiple and identical CD4+ and CD8+ T cell epitopes from the VP7 core protein in mouse and sheep. *Vaccine*, 29, (40) 6848-6857

Romito, M., Du Plessis, D.H., & Viljoen, G.J. 1999. Immune responses in a horse inoculated with the VP2 gene of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 66, (2) 139-144

Ronchi, G.F., Ulisse, S., Rossi, E., Franchi, P., Armillotta, G., Capista, S., Peccio, A., Di, V.M., & Pini, A. 2012. Immunogenicity of two adjuvant formulations of an inactivated African horse sickness vaccine in guinea-pigs and target animals. *Veterinaria Italiana*, 48, (1) 55-76

Roy, P., Bishop, D.H.L., Howard, S., Aitchison, H., & Erasmus, B. 1996. Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge. *Journal of General Virology*, 77, (9) 2053-2057

Roy, P., Mertens, P.P.C., & Casal, I. 1994. African horse sickness virus structure. *Comparative Immunology, Microbiology and Infectious Diseases*, 17, (3-4) 243-273

Sanchez-Matamoros, A., Kukielka, D., De las Heras, A.I., & Sanchez-Vizcaino, J.M. 2013. Development and evaluation of a SYBR Green real-time RT-PCR assay for evaluation of cytokine gene expression in horse. *Cytokine*, 61, (1) 50-53

Scanlen, M., Paweska, J.T., Verschoor, J.A., & Van Dijk, A.A. 2002. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine*, 20, (7-8) 1079-1088

Scheffold, A. & Kern, F. 2000. Recent developments in flow cytometry. *Journal of Clinical Immunology*, 20, (6) 400-407

Shrestha, B. & Diamond, M.S. 2004. Role of CD8+ T cells in control of West Nile virus infection. *Journal of Virology*, 78, (15) 8312-8321

Shrestha, B., Samuel, M.A., & Diamond, M.S. 2006. CD8+ T cells require perforin to clear West Nile virus from infected neurons. *Journal of Virology*, 80, (1) 119-129

Siger, L., Bowen, R., Karaca, K., Murray, M., Jagannatha, S., Echols, B., Nordgren, R., & Minke, J.M. 2006. Evaluation of the efficacy provided by a recombinant canarypox-vectored Equine West Nile Virus vaccine against an experimental West Nile Virus intrathecal challenge in horses. *Veterinary Therapeutics*, 7, (3) 249-256

Siger, L., Bowen, R.A., Karaca, K., Murray, M.J., Gordy, P.W., Loosmore, S.M., Audonnet, J.C., Robert, M., & Minke, J.M. 2004. Assessment of the efficacy of a single dose of a recombinant vaccine against West Nile virus in response to natural challenge with West Nile virus-infected mosquitoes in horses. *American Journal of Veterinary Research*, 65, (11) 1459-1462

Soboll, G., Hussey, S.B., Minke, J.M., Landolt, G.A., Hunter, J.S., Jagannatha, S., & Lunn, D.P. 2010. Onset and duration of immunity to equine influenza virus resulting from canarypox-vectored (ALVAC) vaccination. *Veterinary Immunology and Immunopathology*, 135, (1-2) 100-107

Stauber, N., McCullough, K. C., & Kihm, U. 1992, "Interaction of African horse sickness viruses with leukocytes and the capacity to induce an immune response *in vitro*," *In Bluetongue, African horse sickness, and related orbiviruses*, T. E. Walton & B. I. Osburn, eds., Boca Raton: CRC Press, pp. 815-824.

Stone-Marschat, M.A., Moss, S.R., Burrage, T.G., Barber, M.L., Roy, P., & Laegreid, W.W. 1996. Immunization of VP2 is sufficient for protection against lethal challenge with African horsesickness virus type 4. *Virology*, 220, (1) 219-222

Theiler, A. 1921. African horse sickness (pestis equorum). *Science Bulletin*, 19, 1-29

Tizard, I.R. 2013. *Veterinary Immunology*, 9th ed. St Louis, Missouri, Elsevier.

Tschetter, J.R., Davis, W.C., Perryman, L.E., & McGuire, T.C. 1998. CD8 dimer usage on alpha beta and gama delta T lymphocytes from equine lymphoid tissues. *Immunobiology*, 198, (4) 424-438

Umeshappa, C.S., Singh, K.P., Pandey, A.B., Singh, R.P., & Nanjundappa, R.H. 2010. Cell-mediated immune response and cross-protective efficacy of binary ethylenimine-inactivated bluetongue virus serotype-1 vaccine in sheep. *Vaccine*, 28, (13) 2522-2531

van den Boom, R. & Sloet van Oldruitenborgh-Oosterbaan, M.M. 2013. Can Europe learn lessons from African horse sickness in Senegal? *Veterinary Record*, 172, (6) 150-151

Verwoerd, D. W., Huismans, H., & Erasmus, B. J. 1979, "Orbiviruses," *In Comprehensive virology*, H. Fraenkel-Conrat & R. R. Wagner, eds., London: Plenum Press, pp. 285-345.

von Teichman, B.F., Dungu, B., & Smit, T.K. 2010. *In vivo* cross-protection to African horse sickness Serotypes 5 and 9 after vaccination with Serotypes 8 and 6. *Vaccine*, 28, (39) 6505-6517

von Teichman, B.F. & Smit, T.K. 2008. Evaluation of the pathogenicity of African Horsesickness (AHS) isolates in vaccinated animals. *Vaccine*, 26, (39) 5014-5021

Wade-Evans, A.M., Romero, C.H., Mellor, P., Takamatsu, H., Anderson, J., Thevasagayam, J., Fleming, M.J., Mertens, P.P.C., & Black, D.N. 1996. Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. *Virology*, 220, (1) 227-231

Wade-Evans, A.M., Woolhouse, T., O'Hara, R., & Hamblin, C. 1993. The use of African horse sickness virus VP7 antigen, synthesised in bacteria, and anti-VP7

monoclonal antibodies in a competitive ELISA. *Journal of Virological Methods*, 45, (2) 179-188

Williams, R. 1987. A single dilution enzyme-linked immunosorbent assay for the quantitative detection of antibodies to African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 54, 67-70

Wilson, W.D., Mihalyi, J.E., Hussey, S., & Lunn, D.P. 2001. Passive transfer of maternal immunoglobulin isotype antibodies against tetanus and influenza and their effect on the response of foals to vaccination. *Equine Veterinary Journal*, 33, (7) 644-650

Appendices

Appendix 1: Serum-virus microneutralisation test

All procedures must be carried out under sterile conditions.

1. A 1:5 dilution of the test serum is made in phosphate buffered saline [with Ca^{2+} and Mg^{2+} (PBS^{+})] and inactivated in a water bath for 30 min at 56 °C.
2. A series of six, two-fold dilutions in Minimum Essential Medium[1] (MEM) with 2 g/l $NaHCO_3$[2], gentamycin sulphate (Genta 50)[3] 0.05 mg/ml and 5% foetal calf serum[4] (FCS)(MEM complete medium) is made in 96-well microtitre plates from the 1:5 dilution – volumes of 100µl are used.
3. The stock virus is diluted in MEM complete medium to obtain $100TCID_{50}$.
4. A series of four, ten-fold dilutions is made from the $100TCID_{50}$ antigen, to be used as the virus control.
5. 100µl of the $100TCID_{50}$ antigen is added to all the wells containing the diluted test sera.
6. A virus control is set up over three rows and six columns as follows:
    - 100µl MEM complete medium is added to all the wells.
    - 100µl of the $100TCID_{50}$ virus is added to the first two columns.
    - 100µl of the four dilutions is added accordingly to the remaining four columns, starting with the highest virus dilution.
7. The plates are incubated for one hour at 37 °C (± 2°C) in a humid atmosphere of 5% CO_2 in air.

[1] Highveld Biological, Modderfontein, Gauteng, South Africa
[2] Merck, Wadeville, Gauteng, South Africa
[3] Virbac Animal Health, Centurion, Gauteng, South Africa
[4] Sigma-Aldrich, Johannesburg, Gauteng, South Africa

8. 80 µl of Vero (African green monkey kidney) ATCC CCL81 cell suspension (1 680 cells/µl) are added to all the wells to an estimated 480,000 cells/ml.
9. The cell control is set up in duplicate rows as follows:
    - 200 µl MEM complete medium.
    - 80 µl of the cell suspension.
10. The plates are incubated at 37 °C (± 2 °C) in a humid atmosphere of 5% CO_2 in air for 4-5 days, until the back titration indicates that the stock virus shows 50% cytopathic effect. The presence of specific antibodies in the test serum inhibits the production of cytopathic effect. The end point is taken as the dilution at which 50% of the cells are infected.
11. A positive and negative control is included on each plate. A virus control as well as a cell control is also included.

Appendix 2: Isolation of Equine PBMC

1. Collect blood in 3 × 8 ml heparin tubes.
2. Spin for 10 min at 400 × g (with brake).
3. The following procedures must be performed under sterile conditions.
4. Aspirate and discard the plasma to about 1 ml above the buffy-coat without disturbing the cells. Place the pipette at the level of the buffy-coat and with a circular movement aspirate the buffy-coat including the remaining plasma and some of the red blood cells (RBCs). Collect buffy-coat from 3 tubes (from the same animals) into one 15 ml sterile centrifuge tube. Add PBS without Ca^{2+} and Mg^{2+} (PBS^{-}) up to a volume of 15 ml.
5. Overlay 7.5 ml of the diluted buffy-coat on to 5 ml ficol[5] (2 tubes) by slowly adding the blood suspension while holding the tube at an angle, making sure that the blood and ficol does not mix.
6. Spin for 10 min at 1 100 × g (without brake).
7. Remove and discard the supernatant to about 1 ml above the ring of PBMCs and collect the ring of PBMCs with most of the ficol avoiding any of the RBCs (with horses some cells remain in the lower part of the ficol fraction). Pool the cells from two tubes into one and top up to 15 ml with PBS^{-}. This is the first wash.
8. Spin for 10 min at 400 × g (with brake).
9. Discard the supernatant and re-suspend the cells by dragging the tube over the tube rack. Top up to 15 ml with PBS^{-}. This is the second wash.
10. Spin for 10 min at 400 × g (with brake).
11. Discard the supernatant and re-suspend the cells. The re-suspended cells will have a volume of about 200 µl. To count the cells add 1.8 ml PBS^{-} to make a

[5] Sigma-Aldrich, Johannesburg, Gauteng, South Africa

final volume of 2 ml and mix well (invert 5 times). Collect 200 µl for counting (see counting of cells). Top up the remainder of cells to 15 ml with PBS^-.

12. Spin for 10 min at 400 × g (with brake). Discard the supernatant and re-suspend the cells. This is the third wash.
13. To count the cells in an automated cell counter add 800 µl PBS^- to the 200 µl fraction that was collected for counting in step 10 to make a 1:5 dilution and count with cell counter. Cells per ml are calculated taking the 1:5 dilution into account and then multiplied by two to get the total number of cells.
14. After counting, the cells are adjusted to 10 million cells/ml in RPMI 1640 medium[6] containing 10% FCS, 1% Penicillin[6] Streptomycin[6], 0.01% 2β mercaptoethanol[6] (RPMI complete).

[6] Sigma-Aldrich, Johannesburg, Gauteng, South Africa

Appendix 3: Stimulation of cells for flow cytometry analysis

1. A final volume of 200 µl/well is used throughout the procedure.
2. Mark the plate layout for the different stimulators.
3. Add 500 000 cells to each well (50 µl of the 10 million cells/ml suspension). Use 1 200 µl tips and electronic single channel pipette. Change tips for every animal.
4. Add 50 µl of RPMI complete medium per well (total volume = 100 µl/well). Use 1 200 µl tips and electronic 8 channel pipette. Use same tips for all plates.
5. Add 100 µl of the various activators to a final volume of 200 µl/well.
    - Peptide pools are added at a final concentration of 5 µg/ml per well. (E.g. prepare a 10 µg/ml pre-dilution in RPMI complete medium – it will be diluted 1:2 in the well). Note - as negative control a well with only medium and cells must be added for each animal.
    - Canarypox virus is diluted in RPMI 1640 without FCS and usually added at 250 000 PFU per 500 000 cells (e.g. prepare 250 000 PFU/50 µl and add to the all the wells followed by 50 µl RPMI medium without FCS to represent the 100 µl of activator). Note - for the virus a separate control of cells and virus medium must be added for each animal.
    - PMA-Ionomycin[6] as activator is used as positive control. Add in 100 µl volume. Prepare by adding 5 µl PMA (0.1 mg/ml in PBS) plus 25 µl Ionomycin (1 mM) plus 2.5 ml RPMI complete medium.(PMA final concentration in the well = 25 ng/well/200 µl or 100 ng/ml) (Ionomycin final concentration = 12.5 µM
6. Negative control well = cells plus RPMI complete medium.
7. Positive control well = PM-Ionomycin. For the ELISpot this is done by preparing a 20 times concentrated solution (1µl PMA (0.1 mg/ml in PBS) plus 5µl Ionomycin (1 mM) plus 244 µl RPMI complete medium). Add 10 µl per well containing 200 µl cells.

8. Mix all the wells with a multi-channel pipet changing tips.
9. Incubate for 2 hours at 37 ˚C and 5% CO_2 (Incubation for virus is usually for 5 hours).
10. Add brefeldin A (BFA) (BD GolgiPlug Cat: 555029) as a protein transport inhibitor. Recommended dose for BFA = 1µl/1ml tissue culture (1:1 000 final dilution in well). Prepare a 1:50 pre dilution of BFA in RPMI complete medium – when adding 10 µl of the 1:50 pre-dilution to 200µl of cells this is a further 1:20 dilution to give a final dilution of 1:1 000. Add 10 µl to each well with single channel multi-step pipette and mix afterwards with multi-channel changing tips.
11. Incubate overnight at 37 ˚C and 5% CO_2.
12. Cells are ready to be labelled with various fluorescent antibodies.

Appendix 4: Fluorescein isothiocyanate labelling of CD8 cells for flow cytometry analysis

1. Don't have to work under sterile conditions. All reagents must be cold and incubation steps must be on ice.
2. Plates are centrifuged at 400 – 450 × g at 4 ˚C for 5 min to pellet the cells. Discard the supernatant in the wells. Use a gentle flick motion and then blot keeping the plate upside-down and avoiding any bubbles. This is used for all spinning steps.
3. Re-suspend cells by brief shaking on vortex. Wash by adding 200 µl FACS buffer (Tpon FACS buffer - 2 L PBS plus 10 g bovine serum albumin (BSA) plus 4 ml Sodium azide 0.05 mg/ml) This is to remove the BFA and RPMI complete medium.
4. Spin plates and discard supernatant. Re-suspend cells by brief shake on vortex.
5. Add 50 µl per well of mouse anti-CD8 antibody (VMRD Cat: 73/6.9.1 with 0.09% NaN_3).at 1:100 dilution (0.5 µl anti-CD8 plus 49.5 µl FACS buffer).
6. Mix all the wells 2 twice by multi-channel pipette changing the tips.
7. Incubate 30 min on ice in the dark.
8. Cells must be washed twice to remove unbound anti-CD8 Ab. For the first wash add 150 µl FACS buffer.
9. Spin plates and discard supernatant.
10. Add 200 µl FACS buffer (second wash).
11. Spin plates and discard supernatant.
12. Add 50 µl per well of goat anti-mouse IgG3 FITC conjugate (Southern Biotech Cat: 1100-02) at 1:100 dilution (0.5 µl anti-mouse plus 49.5 µl FACS buffer).
13. Mix all the wells twice by multi-channel pipette changing the tips.
14. Incubate 30 min on ice in the dark.
15. Cells must be washed twice to remove unbound anti-mouse Ab. For the first wash add 150 µl FACS buffer.

16. Spin plates and discard supernatant.
17. Add 200 µl FACS buffer (second wash)
18. Spin plates and discard supernatant. Re-suspend cells by brief shake on vortex.

Appendix 5: Intracellular staining of cells for gamma interferon

1. Open pores in cell membrane with BD Cytofix/Cytoperm kit (Cat: 554714).
2. Add 100 µl/well of the Cytofix/Cytoperm solution.
3. Mix 2 – 3 times with multi-channel changing tips.
4. Incubate for 20 min on ice in the dark.
5. For the first wash add 100 µl of the Perm wash solution.
6. Spin at 400 – 450 × g at 4 ˚C for 6 min to pellet the cells. Discard the supernatant.
7. For the second wash add 200 µl of the Perm wash solution and spin as before. Discard the supernatant. Re-suspend cells by brief shake on vortex.
8. Add 50 µl per well of mouse anti-bovine INF-γ conjugated to biotin (AbD Serotech Cat: MCA1783B) at 1:100 dilution (0.5 µl anti-mouse plus 49.5 µl Perm wash solution).
9. Mix 2 – 3 times with multi-channel changing tips.
10. Incubate for 30 min on ice in the dark.
11. For the first wash add 150 µl of the Perm wash solution.
12. Spin as before and discard the supernatant.
13. For the second wash add 200 µl of the Perm wash solution.
14. Spin as before and discard the supernatant. Re-suspend cells by brief shake on vortex.
15. Add 50 µl per well of streptavidin-PerCP conjugate (BD Cat: 554064) at 1:100 dilution (0.5 µl streptavidin-PerCP plus 49.5 µl Perm wash solution)
16. Mix 2 – 3 times with multi-channel changing tips.
17. Incubate for 30 min on ice in the dark.
18. For the first wash add 150 µl of the Perm wash solution.
19. Spin as before and discard the supernatant.
20. For the second wash add 200 µl of the Perm wash solution.
21. Spin as before and discard the supernatant.

22. Add 150 µl/well of FACS buffer containing 1% formaldehyde to fix and preserve the cells.
23. Mix 2 – 3 times with multi-channel changing tips.
24. Cells are ready to be counted and can be stored at 4 ˚C wrapped in tin foil for 1 to 2 weeks. Before reading add 80 µl PBS plus 0.05% EDTA.

Appendix 6: ELISpot assay for gamma interferon

Coating of plates

1. Work in sterile conditions.
2. Use IP ELISpot plates (Millipore Cat: MSIPS4W10)
3. Be very careful never to touch the bottom of the plates with the tips throughout the entire procedure as this will damage the membrane.
4. Activate plates with 50 µl/well 70% Ethanol for 1 ½ min.
5. Wash plates 5 times with 200 µl/well PBS. Contents of plates are discarded on sterile absorptive cotton pads in order to prevent splatter in the biosafety cabinet.
6. Use antibodies from INF-γ ELISpot kit for bovine/ovine/equine (Mabtech, Cat: 3115-2A).
7. Coat with 100 µl/well of anti-INF-γ antibody at 1:66 in PBS and incubate overnight at 4 ˚C (can also incubate over the weekend).
8. Wash 5 times with PBS as above.
9. Block plate with 200 µl/well RPMI 10% FCS medium (leave the plate at room temp till needed for next step).

Assay setup

1. Work in sterile conditions.
2. Use the same plate layout as for ELISA and flow cytometry.
3. Usually use 500 000 cells/well but must use less cells if high response is expected (evaluate different cell concentrations e.g. 500 000, 200 000 or 125 000 cells/well). To use 125 000 cells/well start with 10 million cells/ml and pre-dilute 1:4 (2.5 million cells/ml) add 50 µl/well. Note: always use the same number of cells when comparing time line responses.
4. Add 100 µl of the various activators (see stimulation of cells for flow cytometry analysis).

5. Incubate for 24 h at 37 ˚C 5% CO_2.

Staining of spots

1. The rest of the procedure can be carried out in non-sterile conditions.
2. Discard all the cells and add 200 µl of cold distilled water to haemolyse any RBCs.
3. Plates are incubated for 5 min at room temp. During this incubation time the plates are washed vigorously by 5 times aspiration and dispensing of the same content with a multi-channel pipette. Can use the same tips for all the plates. (Don't touch the membrane!) Plates must never be dry. Can keep for short periods with PBS-T.
4. Wash plates 5 times by adding 200 µl PBS with 0.05% Tween 20 (PBS-T) and then discarding. Blot gently on absorptive pad.
5. Prepare detection antibody from kit (anti-bovine INF γ monoclonal conjugated to biotin) use at 1:200 diluted in PBS-T with 0.5% FCS. (Prepare enough dilution buffer for both anti-bov-INF-γ and streptavidin - alkaline phosphatise) (Don't let the plates stand dry).
6. Add 100 µl/well of the anti-bov-INF-γ and incubate overnight at 4 ˚C.
7. Wash 5 times as above with 200 µl PBS-T.
8. Prepare streptavidin – alkaline phosphatise at 1:1 000 in PBS-T 0.5% FCS.
9. Add 100 µl/well and incubate for 1 hour at room temp in the dark.
10. Wash 5 times with PBS-T as above.
11. Add 100 µl/well substrate (BCIP/NBT substrate for ELISpot cat: 895866 R&D Systems)
12. Incubate for 30 to 40 min at room temp in the dark (cover with tin foil) on an orbital shaker. Start with 30 min and check for the development of spots. Add 5 min extra time until spots are clear.
13. Discard the substrate and wash 5 times under running tap water.

14. Carefully remove the protective layer at the back of the plate and rinse under tap.
15. Dry the plates upside down in an incubator for 1 hour at 37 ˚C
16. Plates are ready to be read and can be stored for many weeks wrapped in tin foil at 4 ˚C.