Investigation of transmission of vaccine strains of African horse sickness virus in weanling foals kept under field conditions, following the use of a commercial live attenuated vaccine

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ABBREVIATIONS

AGID	Agar gel immunodiffusion
AHS	African horse sickness
AHSV-LAV Comb 1	AHS vaccine combination 1 (AHS type 1, 3 & 4)
AHSV-LAV Comb 2	AHS vaccine combination 2 (AHS type 2, 6, 7 & 8)
AHSV	African horse sickness virus
BHK	Baby hamster kidney
BT	Bluetongue
BTV	Bluetongue virus
CFT	Complement fixation test
CPE	Cytopathic effects
Cq	Quantification cycle
DAFF	Department of Agriculture, Forestry and Fisheries
DISA	Disabled infectious single animal vaccine
DISC	Disabled infectious single cycle vaccine
DIVA	Differentiation of infected from vaccinated animals
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EEV	Equine encephalosis virus
ELISA	Enzyme-linked immunosorbent assay
ERC	Equine Research Centre
GS RT-qPCR	Group-specific RT-qPCR
I-ELISA	Indirect enzyme-linked immunosorbent assay
IFA	Immunofluorescent assay
KC	Insect (*Culicoides sonorensis*) cells
LAV	Live attenuated vaccine
NS	Non-structural protein
OBP	Onderstepoort Biological Products
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction

RT-qPCR	Reverse transcription quantitative PCR
SNT	Serum neutralisation test
TS RT-qPCR	Type-specific RT-qPCR
Vero	African green monkey kidney
VP	Viral protein

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Investigation of transmission of vaccine strains of African horse sickness virus in weanling foals kept under field conditions, following the use of a commercial live attenuated vaccine

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SUMMARY

African horse sickness (AHS) is a World Organisation for Animal Health (OIE) listed disease and is a controlled disease in terms of the Animal Diseases Act (Act No 35 of 1984) in South Africa. It affects equids with horses being the most susceptible and often suffering high mortalities. This makes the

disease very important to the South African equine industry and international equine trade. In Southern Africa, control of the disease relies heavily on annual vaccination with the Onderstepoort Biological Products (OBP) polyvalent AHSV live attenuated vaccine (LAV). It is divided into two combinations: AHSV-LAV Combination (Comb) 1 contains a trivalent vaccine including AHSV types 1, 3 and 4 and AHSV-LAV Comb 2 contains a quadrivalent vaccine including AHSV types 2, 6, 7 and 8. The causative agent of AHS is the AHS virus (AHSV). AHSV, bluetongue virus (BTV) and equine encephalosis virus (EEV) belong to the genus *Orbivirus* in the family *Reoviridae*. The virus is transmitted between equids by haematophagous midges (*Culicoides* spp.). According to some field studies, BTV-LAV strains can be transmitted by *Culicoides* midges. No studies have yet been carried out to investigate whether AHSV-LAV strains can be transmitted in the same manner.

The aim of this study was to determine if AHSV can be detected using RT-qPCR in *Culicoides* midges and unvaccinated weanlings following first vaccination of roughly half the weanlings in a group using a commercial polyvalent AHSV-LAV.

This study started in March and ran until June 2014. The AHS controlled area in the Western Cape Province, has historically been free from AHS with only isolated outbreaks occurring periodically. These outbreaks typically occur from February to May (Sinclair, Buhrmann & Gummow 2006, Venter, Koekemoer & Paweska 2006, Grewar et al. 2013). Approximately 130 Thoroughbred weanlings were kept in open camps on two Thoroughbred stud farms in the AHS protection zone of the AHS controlled area where surveillance has shown that field AHSV has not circulated since at least 1996. Approximately half of the foals on each farm were vaccinated with a commercial polyvalent AHSV-LAV and the other half were unvaccinated for the duration of the study. Weekly blood samples were collected and subjected to group-specific and type-specific RT-qPCR assays to assess the presence of AHSV nucleic acid for the duration of the study. One Onderstepoort (220V ultraviolet down-draught suction) light trap was set close to where the horses were kept on each farm overnight once a week starting on the day of first vaccination. Insect catches were cleaned so that they contained only *Culicoides*. The largest catch from each month on each farm was selected for species composition analysis under a stereomicroscope. *Culicoides* were tested for the presence of AHSV nucleic acid

using RT-qPCR assays. Seven recently blood-fed *Culicoides* were tested to determine the host species on which they had fed using a multiplex PCR

This study showed that RNAaemia can be detected with RT-qPCR in weanlings after vaccination with the commercial LAV under field conditions. The age of weanlings at first vaccination seems to affect the detection of RNAaemia due to interference by maternal antibody. The order in which the vaccine combinations are administered affects the AHSV types detected using type-specific RT-qPCR assays. Multiple AHSV types were detected in individual weanlings which increases the chances of reassortment occurring. AHSV type 1 and 3 were detected in weanlings following vaccination with AHSV-LAV Comb 2 only. Possible reasons for this occurrence include mechanical transmission, naturally circulating wild virus or the presence of these AHSV types in the vaccine bottle. The lack of AHSV detection in the midges supports the theory that large numbers of *Culicoides* need to be present to compensate for the low infection prevalence and possibly transmit vaccine virus from the small proportion of horses with a high enough viraemia (Venter, Koekemoer & Paweska 2006).

Although the study did not show the transmission of vaccine virus, it reaffirms the fact that the behaviour of the LAV is not fully understood and needs further investigation and possibly replacement. This is especially important in the light of the spread of BTV into areas where it previously did not occur and the fact that vaccine transmission was suspected to be the cause of the AHSV outbreak in the Western Cape Province in 2014.

CHAPTER 1

1. General Introduction

African horse sickness (AHS) is an infectious, noncontagious, arthropod-borne disease of equids caused by the African horse sickness virus (AHSV). The virus causes vascular injury and clinical signs usually appear in one of four forms of the disease: horse sickness fever form; the “pulmonary” or “dunkop” form (peracute); the “cardiac” or “dikkop” form (subacute) or “mixed” form (acute) (Theiler 1921). AHS is a World Organisation for Animal Health (OIE) listed disease and is a controlled disease in terms of the Animal Diseases Act (Act No 35 of 1984) in South Africa. The high mortality rate and disruption of overseas horse movement make it very important to the South African equine industry. It is endemic in the tropical and sub-tropical parts of sub-Saharan Africa (Howell 1963, Mellor & Boorman 1995). The first account of a disease in horses resembling AHS was made by Father Monclaro in 1569 while travelling to East Africa (Theiler 1921). Official documents of the Cape of Good Hope dated 1719 contain the term “perreziekte” or “pardenziekte”, this is the earliest record of an outbreak in South Africa. Particularly severe outbreaks seem to occur periodically, approximately 20 years apart (Bayley 1856, Theiler 1921, Guthrie 1996). The outbreak of 1854/55 was most severe with a mortality of over 40% (Bayley 1856). These outbreaks are associated with above average rainfall and increased numbers of *Culicoides* biting midges (Bayley 1856, Meiswinkel 1998).

The AHS outbreak that occurred in 1960 in the Middle East and South-West Asia resulted in the suspension of horse exports from Africa, the “Horse Sickness Ban” (Howell 1960, Guthrie 1996). An outbreak in Spain occurred in 1966 (Hazrati 1967) and 1987 and in Portugal in 1989 (Rodriguez, Hooghuis & Castano 1992). All these outbreaks outside Africa were caused by AHSV type 9 (AHSV 9) except for the outbreak in Spain and Portugal in 1987-1989 which was due to AHSV 4 (Lubroth 1988). The 1966 Spanish outbreak was thought to be the result of infected midge vectors reaching the area by wind or boat from Morocco (Rodriguez, Hooghuis & Castano 1992). The 1989 outbreak was initiated by zebra imported from Namibia to Spain (Lubroth 1988, Rodriguez, Hooghuis &

Castano 1992). An outbreak of AHSV 9 also occurred in Saudi Arabia in 1989 (Anderson, Mellor & Hamblin 1989). AHS was reported in Saudi Arabia and Yemen in 1997 and on the Cape Verde Islands in 1999 (MacLachlan & Guthrie 2010).

The area around the Cape Peninsula in the Western Cape Province has historically been free of AHS except for outbreaks reported in 1967 and 1990 that were thought to be due to the transport of infected horses from the northern parts of the country (Du Plessis et al. 1991, Bosman, Bruckner & Faul 1995, Coetzer & Guthrie 2004). Therefore, in 1997 South Africa was divided into an AHS controlled area in the Western Cape Province and an infected area covering the rest of the country to facilitate the export of horses from South Africa. The controlled area is divided into a free, surveillance and protection zone. The boundaries of these zones correspond to geographical barriers that hamper the spread of the virus. A horse moving into the controlled area from the infected area of the country must have been vaccinated against AHS using a registered vaccine at least 60 days before movement. The horse must be certified healthy by a veterinarian within 48 hours of movement and originate from an area that has not had a case of AHS, as confirmed by the relevant state veterinarian, within 30km in the last 40 days.

In 1999 and 2004 outbreaks of AHS were confirmed in the Stellenbosch district. AHSV 7 was isolated in 1999 and AHSV 1 in 2004 (Bell 1999, Sinclair, Buhrmann & Gummow 2006, Venter, Koekemoer & Paweska 2006, Grewar et al. 2013). An outbreak also occurred in 2011 in the Malmesbury Magisterial District due to AHSV 1 (Grewar et al. 2013). An outbreak of AHSV 1 occurred in the Porterville, Wellington, Tulbagh, Piketberg area of the Western Cape from March to May 2014. These areas fall into the AHS protection and surveillance zones of the Western Cape Province. In this outbreak, morbidity, mortality and case fatality rates were significantly lower and clinical signs were mild in comparison to previous AHS outbreaks. Large scale vaccination was not carried out because the area is, by law, supposed to have a fully vaccinated horse population and the testing methods used at the time did not differentiate between vaccine and field strain virus. Additionally the outbreak occurred at a time of year when the vector was present and transmission of vaccine by the vector could not be ruled out at the time (Grewar 2014a, Grewar 2014b).

Insecticides applied to *Culicoides* breeding sites and insect repellents on animals reduce the risk of infection. Horses can be stabled from before sunset until after sunrise to reduce exposure to the midges. Movement control of horses also hinders spread of the virus. However, the mainstay of control is annual vaccination, especially in endemic areas, with a polyvalent AHSV-LAV being the only registered, commercially available option in South Africa.

Concerns have been raised regarding the use of AHSV-LAVs. Bluetongue-LAV type 2 (BTV 2) (produced by OBP) has been shown to circulate in cattle and *Culicoides* midges (Ferrari et al. 2005). AHSV and BTV are very similar on a morphological and molecular level (Long & Guthrie 2014) and it is therefore possible that AHS demonstrates the same ability for circulation of vaccine virus (Batten et al. 2008, Maan et al. 2010). If circulation of the virus by midges from one mammalian host to another, with subsequent replication of the virus, is possible then the risk for reassortment of vaccine virus with wild-type virus in the vertebrate host or insect vector exists (Paweska, Prinsloo & Venter 2003). The latter is a real risk when vaccination is carried out in the face of an AHS outbreak. Additionally, it is possible for the progeny reassortant viruses to have enhanced ability for multiplication and pathogenicity in comparison to the parental viruses.

Transmission of vaccine virus to unvaccinated animals also complicates testing as the RT-qPCR does not differentiate between the vaccine and wild-type virus. According to the OIE Terrestrial Animal Health Code, if “viral antigen or viral ribonucleic acid specific to AHSV has been identified in samples from an equid showing clinical signs consistent with AHS, or epidemiologically linked to a suspected or confirmed case” this constitutes an “infection with AHSV” (World Organisation for Animal Health (OIE) 2015). This does not differentiate between the LAV and field virus. Thus, the transmission of vaccine virus would be treated in the same manner as a field virus outbreak and result in the same limitations to movement and trade of equids. This has important implications for export of equines from South Africa. This study was undertaken to address these concerns.

CHAPTER 2

2. Literature Review

2.1 Virus

AHS is an infectious, noncontagious, arthropod-borne disease of equids caused by the AHSV. The virus belongs to the genus *Orbivirus* in the family *Reoviridae*. Nine AHSV types have been recognized (McIntosh 1958, Howell 1962). The AHSV is unenveloped and about 70 nm in diameter. The double-stranded ribonucleic acid (dsRNA) genome comprises ten segments which code for seven structural (VP1-7) and four non-structural proteins (NS1-3 and NS3a). It is surrounded by a double layered icosahedral capsid made up of the seven structural proteins. The inner capsid or core consists of two major proteins, viral protein (VP) 3 and VP7, and three minor proteins, VP1, VP4 and VP6. The outer capsid contains VP2 and VP5. VP2 is the main type-specific antigen on the surface of the virus and plays an important role in cell attachment and penetration (Burrage et al. 1993, Roy, Mertens & Casal 1994). It is also likely to be the main determinant of virulence for the virus in mammalian hosts (O'Hara et al. 1998). VP3 and VP7 both play a role in group-specific antigenicity and are highly conserved between the nine AHSV types (Oellermann, Els & Erasmus 1970, Huismans & Erasmus 1981, Roy 1992, Roy, Mertens & Casal 1994, Mellor & Hamblin 2004). The virus is able to maintain virulence for at least six months when stored at 4°C and seems to be stable between a pH of 6 and 10.

2.2 Disease

Horses are most susceptible and the disease is usually acute to peracute with the possibility of more than 90% mortality in immunologically naïve populations (Long & Guthrie 2014). There is no known

therapy for AHS and treatment is thus supportive (Lubroth 1988). Rest for at least four weeks after recovery is advised (Coetzer & Guthrie 2004). Donkeys, mules and zebra usually suffer less severe clinical signs (Mellor & Hamblin 2004) and may act as reservoirs for the virus (Bosman, Bruckner & Faul 1995, Hamblin et al. 1998, Barnard 1998). The mortality rate in donkeys and mules is usually less than 50% (Sailleau et al. 1997). Zebra are regarded as a natural maintenance host for AHSV and play a significant role in the epidemiology of the virus in Africa (Lord, Woolhouse & Barnard 1997, Barnard 1998). Continuous circulation of the virus is thought to occur in the herd for as long as herd and vector numbers are high enough as is the case in the Kruger National Park (Barnard 1993). Horses are thought to be incidental hosts due to the high mortality caused by the virus in this species. Dogs may also be infected when they consume the meat of a horse infected with AHSV (Lubroth 1988). They usually develop the pulmonary form of the disease (Long & Guthrie 2014).

2.3 Clinical signs

The incubation period in experimental cases is usually between five and seven days (Long & Guthrie 2014). Most cases involve dysfunction of the cardiac or pulmonary systems (Lubroth 1988). Detectable clinical signs are often non-specific and may include depression, pyrexia, anorexia, congested mucous membranes, frothy nasal discharge, dyspnoea, recumbency and death (Skowronek et al. 1995). Peracute cases can result in death before the detection of any clinical signs and post-mortem lesions in these cases are often non-specific (Lubroth 1988).

The disease usually presents as one of four forms: horse sickness fever form; the “pulmonary” or “dunkop” form (peracute); the “cardiac” or “dikkop” form (subacute) or “mixed” form (acute). Although such rigid classification may not always be appropriate due to the range in combinations of different clinical signs in individual cases (Newsholme 1983).

In the horse sickness fever form obvious clinical signs may be absent or there may be mild fever and the animal recovers (Lubroth 1988). Diagnosis frequently does not occur. The incubation period is between five and nine days. Fever may reach 40°C. Depression, mild anorexia, conjunctival

hyperaemia, mild dyspnoea and increased heart rate may occur. This form usually occurs in donkeys, zebra and immune horses (Long & Guthrie 2014).

The pulmonary form is often fatal and usually affects fully susceptible horses and foals that have lost their passive immunity (Long & Guthrie 2014). Mortality can reach 95% (Long & Guthrie 2014). The incubation period is usually three to four days when the temperature can rise to 40-41°C. Depression, sweating and recumbency, resembling colic, may occur (Lubroth 1988, Long & Guthrie 2014). Tachypnoea may exceed 50 breaths per minute (Long & Guthrie 2014). Dyspnoea may be visible as a wide foreleg stance, head extended, nares dilated, abdominal heave lines and a paroxysmal cough of increasing severity. Large amounts of white or blood tinged foam may be seen escaping from the nostrils shortly before death which usually occurs three to four days after depression develops. Anorexia is not typical and horses often die with food still present in the oral cavity (Lubroth 1988).

The incubation period in the cardiac form may be as long as 21 days (Lubroth 1988) but is usually between five and seven days (Long & Guthrie 2014). This is followed by a fever of 39-41°C that starts to decline after three to four days when other clinical signs start to develop (Long & Guthrie 2014). Generalized subcutaneous and intermuscular oedema, that is not gravity dependent, develops in the head and neck, especially the supraorbital fossae, conjunctiva, lips, cheeks, tongue, intermandibular space, laryngeal region, and some distance down the neck which may include the jugular groove or ligamentum nuchae (Lubroth 1988, Skowronek et al. 1995, Long & Guthrie 2014). Dyspnea and cyanosis may then develop (Long & Guthrie 2014). Hyperaemia of the oral and conjunctival mucous membranes occurs. Petechiation may be evident on the conjunctivae, gums, lips and tongue shortly before death (Lubroth 1988, Long & Guthrie 2014). Colic symptoms may develop due to anoxia in the gut because of cardiovascular compromise. Oedema of the legs may also be present. Hydrothorax and hydropericardium may be evident on thoracic auscultation. Oesophageal paralysis may result in the inability to swallow and food being present in the mouth but not the stomach at the time of death (Lubroth 1988). This form is usually milder than the pulmonary form but mortality can exceed 50% with death usually occurring four to eight days after the start of fever (Long & Guthrie 2014).

In the mixed form any combination of these clinical signs may occur together (Gomez-Villamandos et al. 1999). It is rarely diagnosed but is the most common form seen on post mortem of horses and mules. Death usually occurs three to six days after the onset of fever (Long & Guthrie 2014).

2.3.1 Pathogenesis

Vertebrate hosts are infected by haematophagous midges. Viral replication then occurs in the regional lymph nodes and is followed by a primary viraemia (Erasmus 1973, Long & Guthrie 2014). The virus is associated with the erythrocytes during this viraemia (Henning 1956, Ozawa, Salama & Dardiri 1973, Burrage & Laegreid 1994, Sailleau et al. 1997) and fever develops at this point (Laegreid et al. 1993, Sailleau et al. 1997). Virus disseminates to target organs, such as the lungs, spleen and other lymphoid tissues, where multiplication then takes place.

Infection of endothelial cells in the target organs has been shown to occur (Laegreid et al. 1992b, Brown, Meyer & Grubman 1994, Skowronek et al. 1995). This leads to protein and fluid leakage from vasculature resulting in oedema, effusions and visceral and serosal haemorrhages (Long & Guthrie 2014). It has been suggested that viral isolates of differing virulence may cause endothelial damage by different mechanisms, for example, with or without endothelial infection (Laegreid et al. 1992a). This was thought to lead to the variety in clinical signs and different forms of the disease. The extent of viral infection of the endothelial cells may vary according to target organ, being most common in myocardial tissue, less in the lung and least in the spleen and liver (Gomez-Villamandos et al. 1999). AHSV has also been shown to infect large mononuclear cells in the spleen (Brown, Meyer & Grubman 1994). It has been suggested that myocardial damage is not a consistent finding (Skowronek et al. 1995) but may occur due to vascular damage (Brown, Meyer & Grubman 1994) or excessive endogenous catecholamine release possibly due to severe stress caused by the disease (Newsholme 1983). There is evidence to support consumption coagulopathy after activation of the coagulation pathways probably resulting from endothelial cell damage (Skowronek et al. 1995). Disseminated intravascular coagulation seems to be an inconsistent finding (Skowronek et al. 1995) as may be evidenced by microthrombi in capillaries of the lung (Laegreid et al. 1992a).

After target organ infection a secondary viraemia develops which usually lasts four to eight days in the horse but can rarely be as long as 21 days (Mellor 1993, Mellor 1994). The time between infection and secondary viraemia marks the incubation period and is usually no longer than nine days (Mellor & Hamblin 2004). In donkeys and zebra the secondary viraemia is usually at a lower level and longer lasting (Barnard et al. 1994, Hamblin et al. 1998). Viraemia may last up to 40 days in zebra (Barnard et al. 1994).

2.4 Reassortment

Genetic reassortment is an accepted evolutionary mechanism of RNA viruses possessing segmented genomes. The genome structure allows for the exchange of RNA segments when more than one strain of virus is present simultaneously in a single vertebrate or insect cell. This has been shown to occur in mammalian reoviruses *in vivo* under laboratory conditions (Wenske et al. 1985). BTV, the prototype virus of the genus *Orbivirus,* is responsible for causing BT in sheep, goats, cattle and wild ruminants. As with AHSV, the dsRNA BTV genome consists of ten segments. Reassortment of BTV has been demonstrated in the vertebrate host (sheep) (Samal et al. 1987b) and insect vector (*Culicoides variipennis*) (Samal et al. 1987a) under experimental conditions. A BTV 16 strain was detected in Italy in 2002 that appeared to be a reassortant between BTV 2 and BTV 16 vaccine strains (Batten et al. 2008). Another study indicated that BTV 6 vaccine virus had reassorted with BTV 2 vaccine and BTV 8 field strain virus in the Netherlands (Maan et al. 2010). An *in vitro* study involving BTV showed that some wild-type/LAV “reassortants replicated to high titres” (Coetzee et al. 2014). The study also highlighted the unpredictable nature of reassortant strains and the need for further investigation into the possibility of these strains resulting in a higher viraemia for a longer period of time and thus more effective transmission. Reassortent progeny viruses have been produced from two parental AHSV types, virulent AHSV 3 (attenuated)^{att} and an avirulent AHSV 8 (wild type)^{wt} in a mouse model (O'Hara et al. 1998). Recently, three of the seven AHSV types incorporated in the OBP AHS vaccine, i.e. AHSV 3, 7 and 8, have also been shown to be reassortants of two or more progenitor viruses (Guthrie et al. 2015a, Guthrie et al. 2015b).

2.5 Vectors

Culicoides (biting midges) is reportedly the most important vector for AHSV in the field but mosquitoes (*Aedes*, *Anopheles* and *Culex* genera) and the brown dog tick (*Rhipicephalus sanguineus*) have also been shown to transmit the virus to susceptible animals (Ozawa & Nakata 1965, Ozawa et al. 1966, Dardiri & Brown 1989, Mellor 1994). It has been suggested that mosquitoes, *Tabanus pluto* and *Stomoxys calcitrans* flies may be able to transmit AHS mechanically (Schuberg & Kuhn 1912, du Toit 1944, Ozawa & Nakata 1965, Mellor 1994).

The virus infects the adult of certain species of *Culicoides* (biting midges) when they consume a blood meal from animals in the febrile or viraemic stage of the disease (Roy, Mertens & Casal 1994). An incubation period of approximately eight days in the midge is then followed by concentration in the salivary glands. From here the virus can infect a new vertebrate host during the midge's next blood meal. The adult midge will remain infected for the rest of its life (Lubroth 1988).

The *Culicoides* life cycle has four phases: egg, larva, pupa and adult. The adult midge life span averages 21-22 days. Eggs are laid and the duration of the larval stage that follows can vary according to the suitability of weather conditions. This allows the midge to overwinter. Transovarial transmission and overwintering of AHSV in the larvae has not been documented. A maintenance and/or amplifying vertebrate host may serve as a source of infection when adult midge activity resumes under favourable weather conditions (Lubroth 1988). However, more recent findings suggest that BTV infected adult *C. sonorensis* midges have a prolonged survival and thus play a role in the maintenance of BTV during the interseasonal period until seasonal transmission resumes (Mayo et al. 2014). This has global relevance due to the similarities between BTV and AHSV. *Culicoides* abundance is seasonal in South Africa with an increase in the number of parous females in late summer in summer rainfall areas. This results in a higher vector potential for AHSV at this time of year. Numbers diminish drastically after the first frost. In frost-free areas of the country, such as the Western Cape and parts of Limpopo Province, *Culicoides* can be collected throughout the year (Venter, Nevill & Van Der Linde 1997). More recently, it has been shown that adult *Culicoices* remain present and breeding and blood-feeding continues throughout the year in the Onderstepoort area of

Gauteng Province where temperatures drop below 0°C in winter. This implies that virus can be present in the midges for the duration of winter and transmission can resume as soon as midge population numbers are large enough after winter (Venter et al. 2014). *Culicoides* are most active between sunset and sunrise and so animals kept on open land are most likely to be infected at this time (Coetzer & Guthrie 2004). However, *Culicoides* have been collected from horses before sunset and diurnal *Culicoides* may play a role in transmission of AHS (Scheffer et al. 2012, Venter et al. 2014). It was observed that losses due to AHS could be minimized by stabling of horses from before sunset until after sunrise (Theiler 1921), however, there is evidence indicating that *Culicoides* do enter stables (Barnard 1997).

Viral infection of, and replication in, the midge vector is affected by insect genotype and ambient temperature. At higher temperatures infection rate is higher and viral replication and transmission is quicker. Conversely, *Culicoides* survival decreases with increasing temperature (Mellor & Hamblin 2004).

Culicoides imicola and *C. bolitinos* seem to be the most important vectors of AHSV in Africa (Meiswinkel & Paweska 2003, Scheffer et al. 2012, Long & Guthrie 2014). In a survey of 17 light traps throughout South Africa conducted by Venter and co-workers (1987), *C. imicola* was found to be the most abundant of the ten predominant *Culicoides* species caught (Venter, Nevill & Meiswinkel 1987, Nevill et al. 1988). *Culicoides imicola* was also the second most commonly found *Culicoides* midge in the Stellenbosch area of the AHS control zone of the Western Cape Province (Nevill et al. 1988). *Culicoides imicola* breeds in soil and is dependent on moisture and soil quality. *Culicoides bolitinos* prefers the cool, wet high lying and sandy, coastal regions of South Africa and can occur where the dung of cattle, African buffalo and blue wildebeest is available for breeding. This allows *C. bolitinos* to thrive in the mountainous and cooler parts of the country where *C. imicola* does not occur and maximizes the AHS vector distribution area (Meiswinkel & Paweska 2003). *Culicoides* have a broad global distribution so the potential exists for AHSV to spread from endemic areas to areas historically free of the disease (Scheffer et al. 2012).

There is evidence to support the oral susceptibility of *C. bolitinos* and *C. imicola* to infection with the majority of the AHSV vaccine strains in use currently in South Africa (Paweska, Prinsloo & Venter 2003). In this study, *C. imicola* was susceptible to all seven vaccine AHSV types and *C. bolitinos* was susceptible to all AHSV types except AHSV 3. It is also reported that the level of viraemia required for midge infection is unknown and midges with differing geographical distribution may differ in susceptibility to different isolates/strains within an AHSV type. Another study showed a higher viral recovery rate of AHSV 7 vaccine strain compared to wild-type virus of the same AHSV type in *C. bolitinos* and *C. imicola*, indicating a difference in viral replication ability (Venter & Paweska 2007).

2.6 Midge trapping

Various models of suction light traps are the primary method used for the monitoring of *Culicoides* midges. The Onderstepoort trap is the most commonly used biting midge trap (Probst et al. 2015). It is manufactured in South Africa and can be left in place for a few months at a time due to its robust nature. It is a 220V down-draught trap with an ultraviolet (black light) tube and polyester netting to exclude larger insects. The Rieb trap is a 12V down-draught trap made of durable plastic and is fairly light and compact. It contains an ultraviolet (light blue) tube and plastic mesh to exclude larger insets. The Miniature Center for Disease Control light trap is a lightweight down-draught trap powered by a rechargeable 6V battery. The light source is an ultraviolet (black light) tube and larger insects are excluded by a filter between the light source and the fan. The Pirbright trap is a light down-draught trap. The light source is a white incandescent bulb and larger insects are excluded by a metal grid between the fan and light source. This light trap has been replaced by the Onderstepoort trap. The BG-sentinel (Biogents) mosquito trap is designed for the collection of mosquitoes but has been used for *Culicoides* collections. It is light weight with an ultraviolet (black light) tube and plastic netting to exclude larger insects (Venter et al. 2009b).

Data collected by the various light traps is difficult to compare due to structural differences and effectiveness of collection of *Culicoides* (Venter et al. 2009b, del Río et al. 2013, Probst et al. 2015). In one study comparing these five light traps, the UV light was found to be superior to the white incandescent light for *Culicoides* collections (Venter et al. 2009b). The Onderstepoort light trap

collects high numbers of *Culicoides* compared to other light traps (Venter et al. 2009b, del Río et al. 2013, Probst et al. 2015). This means that it should yield the highest numbers of parous individuals for viral testing and is the most sensitive trap when infection prevalence and vector abundance is low. It was also found to be the most effective and sensitive light trap for catching less abundant species of midges (Venter et al. 2009b). However, the composition of the catch may vary according to the time of year (Scheffer et al. 2012). It has been observed that the largest midge catches in light traps are obtained at least two hours after sunset (Page et al. 2009, Scheffer et al. 2012). Height at which the trap is set also results in differences in catch sizes. Venter et al. (2009a) found an optimal height of 2.8m while Probst et al. (2015) found an optimal height of 1m, although other external factors may have resulted in this difference (Venter et al. 2009a). Baiting traps with carbon dioxide can increase efficiency (Venter et al. 2015). Odours, such as animal hair extracts, may also attract *Culicoides* midges (Mands, Kline & Blackwell 2004).

Mechanical aspiration directly from horses with a modified hand-held vacuum cleaner has also been used for *Culicoides* collections at the time around sunset, the time that *Culicoides* midges appear to most actively feed on horses. This seems to be the more effective method during cooler weather as the body heat of the horses may be more evident to the midges than the ultraviolet light of the light trap. It also seems to provide more accurate information on biting rates of the midges. However, it can only be performed on a limited number of horses at a time and is labour intensive. Individual horses also seem to differ in attractiveness to midges (Scheffer et al. 2012).

2.7 Diagnosis

The clinical diagnosis of AHS is very difficult in the early stages of infection and laboratory testing is essential. Clinical signs are not specific and can be similar to other diseases such as equine encephalosis virus (EEV) which may also result in oedema of the supraorbital fossae, eyelids or lips. The epidemiology of EEV is similar to that of AHS but the mortality rate is lower with EEV infection (Long & Guthrie 2014). Widespread petechiation also occurs in purpura haemorrhagica and equine viral arteritis. Piroplasmosis can also complicate or be confused with AHS (Lubroth 1988, Long &

Guthrie 2014). Primary colic also needs to be ruled out (Lubroth 1988). A combination of history and clinical signs can lead to a presumptive diagnosis in some cases. However this should always be confirmed by some form of agent identification in order for a case to be confirmed. In 2012 the OIE Terrestrial Animal Health Code and Terrestrial Manual was amended to recognize the reliability of polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) to detect virus nucleic acid and antigen, respectively (World Organisation for Animal Health (OIE) 2012, Long & Guthrie 2014).

2.7.1 Laboratory diagnosis

2.7.1.1 Viral isolation and identification

Virus isolation is historically the gold standard to confirm the presence of AHSV. Blood samples should be collected in heparin in the febrile stage or samples of lung, spleen and lymph nodes, where viral concentration is high, collected on post mortem and kept at 4°C (Erasmus 1973). Inoculation can be intravenous injection of embryonated eggs or intracerebral in suckling mice (World Organisation for Animal Health (OIE) 2012). Isolation is also performed by inoculation of cell cultures such as baby hamster kidney-21 (BHK-21), monkey stable (MS), African green monkey kidney (Vero) or insect (*Culicoides sonorensis*) cell lines (KC). Cell cultures are then observed for cytopathic effects (CPE) (World Organisation for Animal Health (OIE) 2012). This can be evident three to seven days after inoculation (Long & Guthrie 2014) but could extend from two to ten days after inoculation (World Organisation for Animal Health (OIE) 2012). This confirms the presence of a virus which must then be identified as AHSV by an indirect sandwich enzyme-linked immunosorbent assay (I-ELISA) (Hamblin et al. 1991, Crafford 2001, Weyer 2013). Only when no CPE are observed after three passages should the sample be classified as negative for AHSV (Quan et al. 2008, World Organisation for Animal Health (OIE) 2012). This test can also be used to detect the presence of AHSV in *Culicoides* midges and may be useful in the investigation of overwintering of the virus (Hamblin et al. 1991).

2.7.1.2 Virus serotyping

Virus serotyping is achieved by a virus neutralization test using type-specific antisera in mice or on a variety of cell cultures. The test can also be used to identify AHSV but takes at least five days (World Organisation for Animal Health (OIE) 2012). Vero cell monolayers are inoculated with dilutions of the virus sample. After adsorption the inoculum is removed and replaced with an agarose overlay. Electrical insulating fish-spine beads are immersed in type-specific antisera and placed in strict sequence on top of the overlay followed by incubation. The absence of plaques around a bead after five to six days incubation indicates a zone of viral neutralisation by the homologous antibody (Porterfield 1960, Quan et al. 2008, Weyer et al. 2015). Positive controls exhibit viral plaque formation distributed over the Vero cell monolayer and the negative control exhibits no plaques as no virus is inoculated into this monolayer.

2.7.1.3 Group-specific serology

Serological testing includes the detection of group-specific antibodies by complement fixation test (CFT) (mainly IgM) (Crafford 2001), agar gel immunodiffusion tests (AGID) (House, Mikiciuk & Beminger 1990), indirect immunofluorescent antibody tests (IFA) (House, Mikiciuk & Beminger 1990) and ELISA tests.

The CFT test has been replaced in many instances by other screening tests such as ELISA because of the higher sensitivity and level of standardization and the fact that some sera have an anticomplementary effect. However, it may be useful primarily in establishing the presence and titre of group-specific IgM antibodies against AHSV especially after infection or vaccination (McIntosh 1956, Crafford et al. 2003, Blood & Studdert 2005, World Organisation for Animal Health (OIE) 2012).

In AGID, antibody and antigen diffuse from separate wells and result in the formation of precipitation lines in the agar between the wells when complexes exceed the gel's capacity and precipitate out (Blood & Studdert 2005). The AGID uses a crude antigen and lines can be nonspecific and interfere with reading of results. It also lacks sensitivity (House, Mikiciuk & Beminger 1990).

IFA is based on a standardised antigen that reacts with a specific antibody. A labelled antibody to the specific antibody is then added to the slide. This labelled antibody will bind to the specific antibody that is already bound to the antigen and fluoresce (Blood & Studdert 2005). Nonspecific staining can occur in recently vaccinated cases. However, the test has been used successfully for screening purposes (House, Mikiciuk & Beminger 1990).

Indirect ELISA (I-ELISA) tests are available for detection of group-specific IgG antibodies to soluble AHSV antigen or recombinant VP7 (Maree & Paweska 2005). The I-ELISA employing the recombinant VP7 is more sensitive than traditional serological tests in detecting early immunological response after infection and declining maternal immunity in foals (Maree & Paweska 2005). The antigen is also stable and lacks infectivity (World Organisation for Animal Health (OIE) 2012). The cut-off value is calculated from the value of the negative control. Test samples with an adsorbance value lower than the cut-off are reported as negative and those greater than the cut-off are reported as positive. Test samples that result in intermediate adsorbance values should be subjected to a second technique to confirm the result (Maree & Paweska 2005, World Organisation for Animal Health (OIE) 2012, Weyer 2013).

Competitive ELISA tests are also available for detection of group-specific antibody to AHSV (Hamblin et al. 1990, Wade-Evans et al. 1993, Kweon et al. 2003) and are useful in all species as species-specific anti-globulin is not required with this method (World Organisation for Animal Health (OIE) 2012). A monoclonal antibody directed against VP7 is used which permits high levels of sensitivity and specificity (World Organisation for Animal Health (OIE) 2012). The test relies on the specific reaction between the recombinant VP7 (adsorbed to the ELISA plate) and a conjugated monoclonal antibody against VP7. The control antibody must be from a different species to the test samples otherwise the conjugate will react with both (Crafford 2001, World Organisation for Animal Health (OIE) 2012).

The antigen (BHK-21 infected with AHSV) is allowed to adsorb to the ELISA plate. The test serum and the specific (control) antibody (guinea-pig anti-AHS serum) are added to the plate at the same

time. The amount of competing antibody present in the test serum will determine how much of the control antibody binds to the antigen. An enzyme-conjugated antiserum (rabbit anti-guinea-pig horse radish peroxidase) directed against the control antibody is then added. Chromogen and substrate is then added and the colour reaction develops. The reaction is stopped and the result is measured spectrophotometrically. Washing of the plate to remove unbound reagents and incubation at recommended temperatures occurs between each step of the procedure (Hamblin et al. 1990, Crafford 2001, World Organisation for Animal Health (OIE) 2012).

2.7.1.4 Serotype-specific serology

Serotype-specific antibody to AHSV and serum antibody titres are tested using the serum neutralization test (SNT) with appropriate reference antigens in microtitre plates (House, Mikiciuk & Beminger 1990). Serial dilutions of test serum are prepared in a microtitre system. Stock viral antigen, at a predetermined concentration, is added and time for neutralization is allowed. Vero cells are then added. Daily observation follows and cytopathic changes are noted. The presence of specific antibodies in the test serum will inhibit these cytopathic effects. Antibody titres are recorded as the reciprocal of the highest final dilution of serum that provides at least 50% protection of the BHK-21 cell monolayer. A titre greater than ten is considered positive and a four-fold increase in titre of paired samples is considered seroconversion (Guthrie et al. 2009).

2.7.2 Molecular laboratory techniques

2.7.2.1 Group-specific PCR

Various PCR assays exist that detect AHSV. Viral genetic material is detected in blood from viraemic animals or in infected organs (Stone-Marschat et al. 1994, Zientara et al. 1994, Zientara et al. 1995, Sailleau et al. 1997). These tests are sensitive, specific and rapid (24 hours) in comparison with other available tests such as virus isolation which may take a week or longer. They are more sensitive than

virus isolation in detecting AHSV in situations of low viral concentration or infection with attenuated strains, as, for example, in the case of vaccinated animals (Stone-Marschat et al. 1994, Sailleau et al. 1997, Quan et al. 2010, Guthrie et al. 2013). Since the test detects nucleic acid, a positive result does not necessarily equate to the presence of viable infectious virus (World Organisation for Animal Health (OIE) 2012). They are useful when cases of AHS result in death before an antibody response can be demonstrated by serological methods (Sailleau et al. 1997) or for samples that do not contain live virus such as formalin-fixed specimens. PCR also seems to be the better choice when samples contain partially or totally inactivated virus due to unfavourable storage conditions. However, intrinsic factors such as the porphyrin ring of haem may have an inhibitory effect on gene amplification and virus isolation may provide a more reliable result (Sailleau et al. 1997). The inhibitory effects of anticoagulants, such as heparin, and chelating agents, such as EDTA, on PCR amplification have been described (Barker et al. 1992). However, it has been reported that there is no significant difference in viral genome detection between samples collected in heparin or EDTA (Sailleau et al. 1997). There is a risk of contamination occurring in PCR testing (Zientara et al. 1995). PCR tests currently in use for AHSV cannot differentiate between natural infection and vaccination with the LAV (Quan et al. 2010).

Conventional reverse transcription PCR (RT-PCR) has been developed for AHSV where virus isolates are propagated on cell culture before extraction and PCR and the product is visualized by agarose gel electrophoresis (Mizukoshi et al. 1994, Stone-Marschat et al. 1994, Aradaib et al. 2006). This technique is susceptible to cross contamination between tubes and false positive results in assays performed in the same laboratory space (Aradaib et al. 2006, Bachanek-Bankowska et al. 2014). Single tube RT-PCR assays followed by agarose gel electrophoresis (Bremer & Viljoen 1998) and restriction enzyme digestion for detection of AHSV in clinical samples are described (Zientara et al. 1994). The combination of reverse transcription and PCR in one tube is designed to reduce contamination. An RT-nested PCR has been developed but is also susceptible to contamination (Zientara et al. 1995). Nested PCR is designed to increase the specificity of the test.

Real-time RT-PCR assays allow for the test to be completed in a closed tube format which reduces contamination and false positive results. These assays also provide for high sensitivity and specificity

and the ability to quantify viral RNA. The use of short amplicons minimizes the problems caused by the partial degradation of the RNA target. Automation of the process increases throughput and reproducibility which is useful for surveillance and in outbreak situations (Aguero et al. 2008, Fernandez-Pinero et al. 2009, Bachanek-Bankowska et al. 2014). The inclusion of sequence specific primers increases the specificity of the assay (Aguero et al. 2008, Fernandez-Pinero et al. 2009). However, the use of random hexamers during RNA denaturation and instead of gene-specific primers during reverse transcription increases the sensitivity of the assay especially in cases of low RNA concentration (Quan et al. 2010). The equipment and reagents needed for real-time PCR are, however, usually more expensive than that required for conventional PCR (Rodriguez-Sanchez et al. 2008).

Depending on the purpose of the test, the PCR may target different genome segments of the virus. Assays that have been developed target genome segment 3 which encodes VP3 (Aradaib et al. 2006), segment 5 which encodes NS1 (Mizukoshi et al. 1994), segment 7 which encodes VP7 (Zientara et al. 1993, Zientara et al. 1995), segment 8 which encodes NS2 (Stone-Marschat et al. 1994), segment 10 which encodes NS3 (Zientara et al. 1995). Some real-time RT-PCR assays for AHSV that have been developed target the VP1 and VP3 (Bachanek-Bankowska et al. 2014), VP7 (Aguero et al. 2008, Fernandez-Pinero et al. 2009, Quan et al. 2010), NS1 (Rodriguez-Sanchez et al. 2008) or NS2 encoding genes (Quan et al. 2010, Monaco et al. 2011).

A real-time quantitative reverse transcription PCR (RT-qPCR) assay has recently been developed in the ERC that is more sensitive, faster and has lower potential for contamination than conventional PCR assays using agarose gel electrophoresis. It is significantly more sensitive than virus isolation and specific as it does not detect other orbiviruses (Quan et al. 2010, Guthrie et al. 2013). RNA viruses have a high mutation rate and so sequence variation in the AHSV genome can occur (Drake 2001, Belshaw et al. 2008). Thus the test is in a duplex format which reduces the number of false negative results that could otherwise have occurred due to this nucleotide variation in the target areas (Quan et al. 2010). The test has been optimized for AHSV detection in blood samples of naturally infected horses (Quan et al. 2010, Guthrie et al. 2013). It has also been refined by automation of the extraction and denaturing steps making the process faster, more suitable for high throughput

situations and less susceptible to contamination (Guthrie et al. 2013). XenoRNA and Xeno primers and a probe are included as an internal control to minimize false negative results (Monaco et al. 2011). A positive result for the control indicates that RNA extraction was successful and that PCR inhibitors are not present (Hoffmann et al. 2006). The test targets the segment 7 gene encoding VP7 and is quantitative. RT-qPCR involves transcription of RNA into complementary DNA (cDNA) by reverse transcriptase. The cDNA then functions as a template for amplification in the qPCR reaction. In this reaction the probes are released from the DNA which results in the generation of fluorescence. The results, fluorescence exceeding a specific threshold in relation to the number of cycles, are then viewed in real time after each PCR cycle as opposed to at the end point.

2.7.2.2 Type-specific PCR

Samples that test positive on the group-specific RT-qPCR may undergo testing with type-specific PCR assays. Restriction enzymes have previously been used and the restriction fragment length polymorphisms allow for differentiation of groups of the AHSV types (Zientara et al. 1993). A conventional type-specific RT-PCR targeting genome segment 2 encoding VP2 has been developed (Sailleau et al. 2000). A real-time type-specific PCR using probe hybridization and melting curve analysis targeting segment 2 also exists (Koekemoer 2008). Another real-time type-specific RT-PCR also targets segment 2 (Bachanek-Bankowska et al. 2014). Type-specific RT-qPCR assays in a triplex format have been developed in the ERC and are designed for blood samples collected in the field (Weyer et al. 2015).

2.7.2.3 AHSV detection in *Culicoides*

It is also useful to test *Culicoides* midges for surveillance purposes to determine whether they are infected with AHSV and, in studies such as this, to investigate the transmission mechanisms of the disease. This may be done with an indirect sandwich ELISA (Hamblin, Mellor & Boned 1991, Hamblin et al. 1991). RT-PCR assays designed for the detection of BTV in blood samples have been used for detection of BTV in *Culicoides* midges (Toussaint et al. 2007, Shaw et al. 2007, Mayo et al. 2012,

Schulz et al. 2015). An RT-PCR assay using infrared-dye-labelled primers for detecting BTV in *Culicoides* midges has also been developed (Kato & Mayer 2007). An adaptation of the RT-qPCR designed for the detection of AHSV in blood samples was used in this study for the detection of AHSV in the *Culicoides* midges (Quan et al. 2010, Scheffer et al. 2011, Scheffer et al. 2012).

2.7.2.4 *Culicoides* blood meal testing

Many *Culicoides* are opportunistic feeders and so blood meal analysis is useful to determine actual feeding patterns (Scheffer et al. 2012). Blood meal analysis to identify the species fed upon by haematophagous arthropods has traditionally been done by immunological methods (Blackwell, Mordue & Mordue 1994). These tests might not distinguish between blood meals from closely related host species (Mukabana, Takken & Knols 2002). DNA sequencing is useful when the host range of an arthropod is very wide or unknown. The technique is, however, expensive and thus not suitable for large numbers of samples. Molecular genetic techniques have been developed based on variations in nuclear and mitochondrial DNA sequences providing greater specificity. Nuclear genes provide a small amount of starting material which may make their use challenging. Mitochondria occur in high numbers in cells and each contains an independent genome making them suitable for analysis in tiny arthropod blood meals. Mitochondrial genes that may be utilized include *cytochrome b* and *cytochrome c oxidase 1* (Kent 2009). Group-specific PCR primers can be used when there are only a few possible hosts or only broad classification of hosts is necessary (Kent & Norris 2005). PCR-restriction fragment length polymorphism can be useful when sequencing and group-specific primers are not suitable (Oshaghi, Chavshin & Vatandoost 2006). Real-time PCR provides increased sensitivity and the ability to quantify the amount of template in the original sample (van den Hurk, Smith & Smith 2007). Another technique, the reverse line blot has been useful for tick blood meal identification (Pichon et al. 2003).

A multiplex PCR to test for the host species on which the midges have been feeding is available in the Veterinary Genetics Laboratory, University of Pretoria. The PCR employs universal and species-specific primers based on the mitochondrial *cytochrome b* gene. The universal primers react with all

mammalian species and the species-specific primers react only with the species for which they were designed (Tobe & Linacre 2008, Garros et al. 2011).

2.8 Control of AHS

A few strategies can be employed to manage the disease but control relies heavily on annual vaccination with the OBP polyvalent AHSV-LAV, especially in endemic areas. Control of insect vectors can be attempted by insecticide spraying of areas where insect breeding occurs and horses are kept. Horses can be sprayed with insect repellents. Standing water should be drained to eliminate possible insect breeding sites (Lubroth 1988). Stabling of horses from before sunset until after sunrise can reduce potential exposure because many species of *Culicoides* are nocturnal and do not readily enter buildings (Blackburn & Swanepoel 1988).

2.8.1 Vaccination

Vaccination is still the mainstay of controlling the disease. Implementation of a ring-vaccination campaign of 25 km diameter from the affected area in the 1966 outbreak in Spain helped prevent northerly spread of the disease (Lubroth 1988). If the AHSV type involved in an outbreak can be established then a monovalent vaccine can be produced which may be less expensive and provide more efficient protection in endemic areas (Lubroth 1988, World Organisation for Animal Health (OIE) 2012). In South Africa, it is recommended that the polyvalent AHSV-LAV (OBP) is administered on an annual basis in endemic areas. However, this is controversial due to the potential circulation and reassortment of vaccine virus as has been shown to occur with BTV (Lubroth 1988, Batten et al. 2008, De Clercq et al. 2009, Maan et al. 2010, Eschbaumer et al. 2010). Additionally, laboratory testing often cannot distinguish between the LAV and wild-type virus infection (House, House & Mebus 1992, House et al. 1992, Stone-Marschat et al. 1996, Guthrie et al. 2009).

The serum-virus method of immunization was developed in 1905 (McIntosh 1958). Blood was transfused from a sick animal infected with AHSV into an immune animal and the serum was collected

from this animal about 14 days later. Virus strains were attenuated in a horse or mule and then used to inoculate horses in combination with serum injections at various time intervals (Theiler 1915).

Serial passage of AHSV in mice and guinea pigs was used to produce an AHSV live attenuated vaccine in the 1930s (Alexander & Du Toit 1934). Subsequently, a cell culture LAV has been developed. The current OBP vaccine was introduced in 1994 and is the only AHS vaccine registered for use in South Africa. It is divided into two combinations: AHSV-LAV Comb 1 contains a trivalent vaccine including AHSV 1, 3 and 4 and AHSV-LAV Comb 2 contains a quadrivalent vaccine including AHSV 2, 6, 7 and 8 (von Teichman, Dungu & Smit 2010). Von Teichman et al. (2010) cited Erasmus (1994) reporting that AHSV 4 and 5 caused severe vaccine reactions and deaths in weanlings in 1990 (Erasmus 1994). This may have been due to a virulent reassortant strain produced through reassortment of vaccine AHSV 4 and 5 but apparently this could not be investigated at the time (von Teichman & Smit 2008). AHSV 5 was subsequently excluded from the vaccine as AHSV 8 provides cross protection and AHSV 4 underwent additional attenuation for re-inclusion in the vaccine. AHSV 9 has not been prevalent in South Africa and thus has never been included in the vaccine. Subsequent to a number of deaths due to AHSV 9 in the Western Cape Province in 2006, a study was undertaken to confirm that cross protection is obtained by AHSV 6 (von Teichman, Dungu & Smit 2010).

The attenuation of virus for inclusion into vaccines involves passage in BHK cells. Selection of large plaques in Vero cells ensures that avirulent clones are used for further passage in Vero cells (Erasmus 1978, Venter & Paweska 2007). However, House et al. (1992) suggests that “vaccination with a large plaque variant in Spain resulted in a variant population of AHSV-4” although no molecular studies were carried out to investigate whether there was reassortment between vaccine and circulating field virus (House, House & Mebus 1992). In South Africa attenuated viruses destined for use in vaccines may not produce a viraemia exceeding 10^3PFU/mL in blood of susceptible horses. However, this testing is performed in a finite number of animals and it is therefore difficult to predict the individual effect on the many equines that are vaccinated in South Africa each year (Paweska, Prinsloo & Venter 2003). These authors also stated that viraemia cannot be detected in most vaccinated horses following vaccination with attenuated AHSV strains. However, the diagnostic tests referred to in this report were not specified and currently used tests i.e. real-time RT-qPCR assays,

are much more sensitive in detecting AHSV in cases of low viral concentration or infection with attenuated strains (Stone-Marschat et al. 1994, Sailleau et al. 1997, Quan et al. 2010, Guthrie et al. 2013).

One injection of each combination is administered subcutaneously at least three weeks apart. According to the manufacturer (OBP), this should be done in late winter to early summer (September to November) so that there is an adequate immune response before challenge during the AHS peak season (von Teichman, Dungu & Smit 2010).

Annual vaccination with an LAV is an efficient and relatively cheap method of minimizing serious losses although it does not guarantee freedom from infection or disease. According to the manufacturer (OBP), it takes two or three vaccinations for horses to become immune to all AHSV types in the vaccine. However, individual horses have been shown to have differing responses indicating that the AHSV types contained in the vaccine may differ in their immunogenicity (Blackburn & Swanepoel 1988, Crafford et al. 2013). Although, according to von Teichman & Smit (2008) personal communication with Dr Baltus Erasmus, the AHSV type combinations were chosen at the time of vaccine development “based on cross-neutralization results and minimal interference between AHSV types upon vaccination allowing the production of antibodies to each strain”. Marked differences have been found in the immunogenicity of individual vaccine virus AHSV types in foals vaccinated between seven and ten months of age (Crafford et al. 2014). These foals more consistently seroconverted to AHSV 1 while responses to the other AHSV types were often weak and transient. Annual vaccination is thus advised by the manufacturer. Natural infection is reported to result in broader cross-immunity to the other AHSV types than vaccination (Blackburn & Swanepoel 1988, Coetzer & Guthrie 2004).

Another study found considerable variation in the antibody response of individual mares to the different AHSV types after vaccination (Crafford et al. 2013). AHSV 1, 4, 6 and 9 produced the strongest response and AHSV 5 and 8 a weaker response. They also found marked variation in the level and duration of passively acquired maternal antibody of foals born to these vaccinated mares. The antibody level of the serum of the dam is related to the antibody level in the colostrum and this

determines the duration of passive immunity in the foal. Foals born to immune mares should not be vaccinated before reaching six months of age due to the interference of this passive immunity with the response to vaccination (Long & Guthrie 2014). However, in some cases antibody levels to individual AHSV types may decline to levels that may result in susceptibility to infection well before the age of six months. It was also found that vaccination at three to four months of age seemed to elicit only a weak immune response but did not seem to affect existing antibody levels (Blackburn & Swanepoel 1988). The Animal Diseases Act 1984 (Act 35 of 84) stipulates that vaccination should occur between the age of six and 12 months, then again between the age of 12 and 18 months and then again once every year thereafter.

Detectable viraemia due to low level virus replication following vaccination is unusual but if present seems to occur from day 10 to 14 after vaccination and lasts for three to five days (Paweska, Prinsloo & Venter 2003, von Teichman, Dungu & Smit 2010). Clinical signs resulting from vaccination with the OBP vaccine are unusual but a mild fever five to 13 days post-vaccination may occur. Horses should not be excessively stressed for about three weeks following the first immunization (Coetzer & Guthrie 2004). More severe clinical signs of AHS occur infrequently in animals receiving the vaccine for the first time (Long & Guthrie 2014). Some local horse breeds may have inherent adaptation to AHS making them less susceptible to reaction to the vaccine (von Teichman & Smit 2008). Vaccinating too frequently has been reported to result in unresponsiveness or hypersensitivity (Erasmus 1978).

Concerns exist regarding the reassortment and mutation of the LAV and, as a result, a change in the phenotypic characteristics of the viruses e.g. reversion to virulence (Stone-Marschat et al. 1996). The potential for reassortment may be greater in polyvalent vaccines due to the simultaneous presence of genomic segments from multiple strains in the host (von Teichman & Smit 2008). According to von Teichman & Smit (2008), a virulent vaccine AHSV 3 strain has been isolated in vaccine trials and some field cases of foals that died after the first vaccination. Unpublished data (OBP and OVI) suggests that this virulent AHSV type has mainly been isolated after the first vaccination with AHSV-LAV Comb 1 of young or immune-compromised horses and often after AHSV-LAV Comb 2 but never after booster administration (von Teichman & Smit 2008). This is thought to be related to the high replication rate and immunogenicity of AHSV 3. The Department of Agriculture, Forestry and Fisheries

(DAFF) has recently introduced regulations that vaccination be restricted from 1 June to 31 October each year in the controlled area due to recent evidence that has emerged indicating that certain outbreaks in the area may be due to vaccine transmission by midges (World Organisation for Animal Health (OIE) 2014, Grewar 2014a, Grewar 2014b). This is a period of generally low midge activity and it is strongly recommended that the same be done in the rest of the country.

Killed (inactivated) vaccines may be viewed as a safer alternative because they do not result in viraemia, reversion to virulence or reassortment with wild-type viruses (House et al. 1992, Dubourget et al. 1992). However, the level and duration of immune protection is inferior to that of the LAV and are also apparently expensive to produce (Paweska, Prinsloo & Venter 2003, Guthrie et al. 2009).

Production of subunit vaccines has also been investigated. A recombinant vaccinia virus expressing VP2 of AHSV 4 was constructed and produced a protective immune response with no clinical signs or viraemia after homologous challenge (Stone-Marschat et al. 1996). A study was also conducted using a baculovirus-expressed VP2 protein of AHSV 5 but further investigation is necessary (Scanlen et al. 2002).

Some work has been done with virus like particles (Roy & Sutton 1998). They have demonstrated safety and efficacy in laboratory trials but they are not available commercially because of difficulties in production, high cost and long-term stability (Guthrie et al. 2009).

Advances have been made in the development of recombinant vaccines. A recombinant modified vaccinia Ankara virus (MVA) expressing major capsid protein VP2 of AHSV 9 provided complete clinical protection against challenge (Alberca et al. 2014). A canarypox virus vectored vaccine has been developed that co-expresses outer capsid proteins, VP2 and VP5, of AHSV 4. It appears to be safe and provides effective immunity against AHSV 4. Virus replication does not seem to occur after challenge. This type of vaccine would allow differentiation of infected from vaccinated animals (DIVA). However, none are currently registered for use (Guthrie et al. 2009, El Garch et al. 2012).

Developments have also been made in disabled infectious single animal (DISA) vaccines for AHSV. One study involves reverse genetics to exchange segment 2 encoding the AHSV type-determining VP2 and deletion of segment 10 encoding NS3/NS3a. NS3/NS3a is not essential for viral replication but its deletion inhibits release of virus from cells which blocks further transmission by the insect vector. DISA vaccine candidates for the nine AHSV types would share nine segments and only differ in segment 2. Thus, reassortment between the AHSV types in the vaccine would not occur. However, there is not yet any evidence ruling out reassotment with wild virus (Coetzee et al. 2014). The development of a disabled infectious single cycle (DISC) vaccine using reverse genetics that stimulates an immune response but does not replicate may be safer with regards to reversion to virulence and reassortment (Matsuo et al. 2011, Celma et al. 2013). DIVA would also be possible with this type of vaccine (van de Water et al. 2015).

An ideal vaccine should be effective, produce no viraemia after administration and only allow viraemia following infection below the level capable of infecting the vector (House, House & Mebus 1992). House and co-workers (1992) suggests that the vaccine should be tested in the particular population in which it is to be used due to the variation in susceptibility of horses in different areas to AHS.

2.8.2 Movement Control

South Africa is divided into an AHS controlled area in the Western Cape Province and an infected area covering the rest of the country. This is based on the fact that the disease prevalence is much lower in the south western parts of the country, partly due to the distance from the Kruger National Park where substantial numbers of zebra naturally occur. The controlled area is divided into a free, surveillance and protection zone. Control of the disease relies heavily on annual vaccination, especially in endemic areas, using the polyvalent AHSV-LAV produced by OBP. Vaccination during late winter or early summer provides the greatest chance of immunity by the time of high season but does not necessarily prevent infection or disease. According to regulations promulgated under the Animal Diseases Act (Act No. 35 of 1984) (South African Government 1984), vaccination is compulsory in all areas of South Africa except the AHS free and surveillance zones, where written permission from the Director of Veterinary Services (Western Cape Province) is required before

vaccination may occur. However, it has been estimated that the South African horse population has increased from 230 000 in 1993 to 270 000 in 2005 but that less than 150 000 doses of vaccine have been sold by OBP annually indicating that there are many horses not receiving vaccination (von Teichman & Smit 2008).

AHS affects equids with horses being the most susceptible and often suffering high mortalities. This makes the disease very important to the South African equine industry and international equine trade. In southern Africa, control of the disease relies heavily on annual vaccination with the OBP polyvalent AHSV-LAV. The use of the AHSV-LAV is sometimes questioned due to the potential for reversion to virulence as well as circulation of vaccine virus and reassortment as has been demonstrated for BTV, the prototype virus of the genus *Orbivirus* (Batten et al. 2008, Maan et al. 2010, Coetzee et al. 2014, McVey & MacLachlan 2015). Transmission of vaccine virus from vaccinated horses to *Culicoides* midges with further transmission to unvaccinated horses in the field, with subsequent replication of the virus, increases the risk of reassortment of vaccine virus with wild-type virus in the vertebrate host or insect vector (Paweska, Prinsloo & Venter 2003). The latter is a real risk when vaccination is carried out in the face of an AHS outbreak. Additionally, it is possible for the progeny reassortant viruses to have enhanced ability for multiplication and pathogenicity in comparison to the parental viruses. Transmission of vaccine virus to unvaccinated animals also complicates testing as the RT-qPCR does not differentiate between the vaccine and wild-type virus. The emergence of BTV in Europe is linked to the expansion of the natural range of *C. imicola* due to climate change and transmission by other *Culicoides* spp. outside of this range. This implies that if AHSV were to appear in Europe, the risk of widespread transmission would be great as the two diseases rely on the same vector species (Purse et al. 2005). This has important implications for export of equines from South Africa. No studies have yet been carried out to investigate whether the AHSV-LAV strains can be transmitted in the same manner as BTV. It is due to these concerns that this study was undertaken.

OBJECTIVES

1. To determine the incidence and duration of viral nucleic acid detection (RNAaemia) by RT-qPCR in weanlings (vaccinated and unvaccinated) following first vaccination of roughly half the weanlings using a commercial polyvalent AHSV-LAV vaccine.
2. To determine if AHSV nucleic acid can be detected by RT-qPCR in *Culicoides* midges caught in light traps following vaccination of weanlings on farms in the AHS controlled area of South Africa.
3. To determine if the AHSV-LAV can be transmitted from *Culicoides* midges to unvaccinated weanlings on farms in the AHS controlled area of South Africa where other weanlings are vaccinated.

CHAPTER 3

3. Investigation of transmission of vaccine strains of African horse sickness virus in weanling foals kept under field conditions, following the use of a commercial live attenuated vaccine

3.1 Abstract

African horse sickness (AHS), caused by African horse sickness virus (AHSV), is a disease of equids that can result in high mortality in horse populations. Control of the disease in South Africa relies heavily on annual vaccination with a live attenuated vaccine (LAV). The aim of this study was to investigate whether the AHSV-LAV can be transmitted by *Culicoides* midges to unvaccinated weanlings following first vaccination of roughly half the weanlings in a group using a commercial polyvalent AHSV-LAV. Age of weanlings at first vaccination seems to affect the detection of RNAaemia due to interference by maternal antibody. The order in which the vaccine combinations are administered affects the AHSV types detected using group-specific RT-qPCR. Multiple AHSV types were detected in individual weanlings which increases the chances of reassortment occurring. AHSV type 1 and 3 were detected in weanlings following vaccination with AHSV-LAV Comb 2 only. Possible reasons for this occurrence include mechanical transmission, naturally circulating wild virus or the presence of these AHSV types in the vaccine bottle. The lack of AHSV detection in the midges supports the theory that large numbers of *Culicoides* need to be present to compensate for the low infection prevalence and possibly transmit vaccine virus from the small proportion of horses with a high enough viraemia (Venter, Koekemoer & Paweska 2006). Although the study did not show the transmission of vaccine virus, it reaffirms the fact that the behaviour of the AHSV-LAV is not fully understood and needs further investigation.

3.2 Introduction

African horse sickness (AHS) is an OIE listed disease and is a controlled disease in terms of the Animal Diseases Act (Act No 35 of 1984) in South Africa. It affects equids with horses being the most susceptible and often suffering high mortalities (Long & Guthrie 2014). This makes the disease very important to the South African equine industry and international equine trade. The causative agent of AHS is AHS virus (AHSV) and *Culicoides* (haematophagous midges) are the biological vector of the disease (Lubroth 1988). Chemical control of *Culicoides* does not seem viable as they occur in such vast numbers (Meiswinkel 1998). Thus, control of the disease relies heavily on annual vaccination with the polyvalent AHSV live attenuated vaccine (LAV) manufactured by Onderstepoort Biological Products (OBP), especially in endemic areas. This is the only vaccine registered for use in South Africa. Susceptible horse populations have suffered very high mortalities where vaccination is absent (Guthrie 1996, Grewar et al. 2013).

Concerns have been raised regarding the use of the AHSV-LAV. AHSV and BTV are very similar on a morphological and molecular level (Long & Guthrie 2014) and it is therefore possible that AHSV demonstrates the same ability for circulation of vaccine virus and reassortment that has been demonstrated for BTV (Batten et al. 2008, Maan et al. 2010). There is evidence to support the susceptibility of *C. bolitinos* and *C. imicola* to oral infection with the majority of the AHSV vaccine strains currently in use in South Africa (Paweska, Prinsloo & Venter 2003). Another study showed higher viral recovery rates of AHSV 7 vaccine strain compared to wild-type virus in *C. bolitinos* and *C. imicola*, indicating significant viral replication ability (Venter & Paweska 2007). If circulation of the vaccine by midges from one mammalian host to another, with subsequent replication of the virus, is possible then the risk for reassortment of vaccine virus with wild-type virus in the vertebrate host or insect vector exists (Paweska, Prinsloo & Venter 2003). This is a real risk when vaccination is carried out in the face of an AHS outbreak. Additionally, it is possible for the progeny reassortant viruses to have changed their phenotype and have an enhanced ability for multiplication and pathogenicity in comparison to the parental viruses. Transmission of vaccine virus to unvaccinated animals also complicates testing as the RT-qPCR does not differentiate between the LAV and natural infection with wild-type virus (Quan et al. 2010).

Transmission of a commercial AHSV-LAV from vaccinated foals to *Culicoides* midges with further transmission to unvaccinated foals in the field is therefore possible. The possibility of this phenomenon confuses diagnostic testing and increases the risk of reassortment, reversion to virulence of vaccine strains and outbreaks of the disease in susceptible populations.

In this study a group of weanlings were monitored longitudinally while half the group received the AHSV-LAV and half did not. The aim of this study was to establish whether transmission of the AHSV-LAV could be documented from one horse to another via the insect vector under field conditions where field AHSV does not regularly occur.

3.3 Materials and Methods

3.3.1 Study design

A group of registered Thoroughbred weanlings (n=130) from two Thoroughbred stud farms, Wilgerbosdrift (n=57) and Moutonshoek (n=73), were selected for data collection. The OBP polyvalent AHSV-LAV was used for vaccinations according to the manufacturer's instructions (except for the reversed order of administration of the vaccine combinations in Group 2). Foals were divided into four groups, each group containing a minimum of 25 foals.

- Group 1 (n=29) (Wilgerbosdrift) received AHSV-LAV Comb 1 (Batch #108) at week zero and AHSV-LAV Comb 2 (Batch #240) four weeks later.
- Group 2 (n=38) (Moutonshoek) received AHSV-LAV Comb 2 (Batch #240) at week zero and AHSV-LAV Comb 1 (Batch #108) four weeks later.
- Group 3 (n=28) (Wilgerbosdrift) and 4 (n=35) (Moutonshoek) received AHSV-LAV Comb 2 at week 12 and AHSV-LAV Comb 1 four weeks later.

The timeline of events for each Group are summarized in Figure 1. Ethical approval for the sampling strategy and use of these animals was granted by the Animal Use and Care Committee of the University of Pretoria (V012-14).

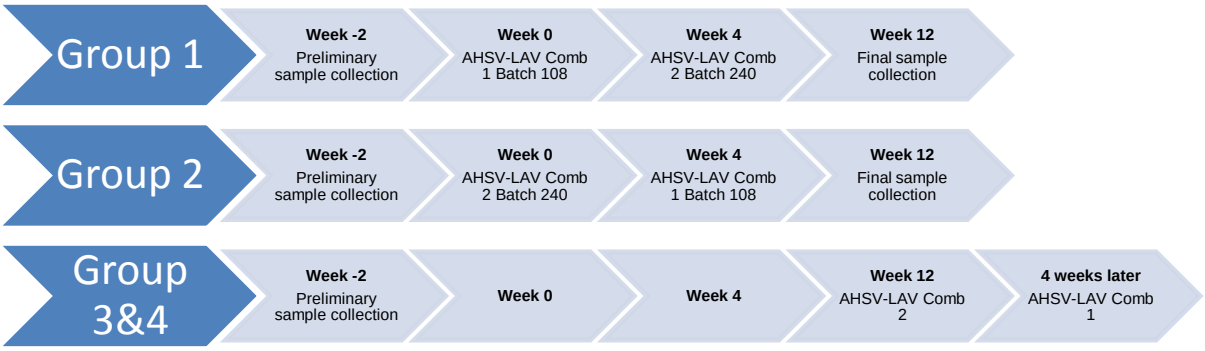

Figure 1: Summary of timeline of events for foals in each treatment Group

3.3.2 Sampling area

The two farms, Wilgerbosdrift (-32.65; 18.69) and Moutonshoek (-32.71; 18.71), included in the study were situated in the Piketberg area which falls into the AHS Protection Zone of the AHS Controlled Area of the Western Cape Province (Figure 2) where wild virus is unlikely to occur. The two farms are approximately 7km apart.

Figure 2: Location of farms used in this study in the AHS Protection Zone

An outbreak of AHS occurred in the Porterville, Wellington, Tulbagh, Piketberg area of the Western Cape Province from March to May 2014. A single case of AHS was confirmed at Wolfkloof in the Pikerberg area on 29 April 2014 which corresponded to week 3 of the study. Wolfkloof was situated approximately 17.5 km from Moutonshoek and there is a mountain range between the two farms (Figure 3).

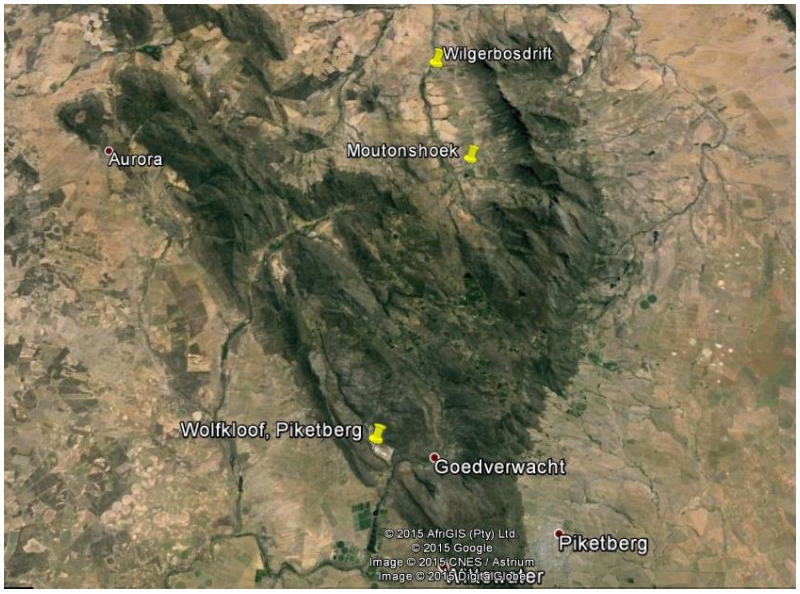

Figure 3: Location of farms in relation to outbreak case (Wolfkloof)

3.3.3 Sampling

Sampling began in March and continued until the end of June 2014. This allowed all foals in the program to be vaccinated between the age of six and 12 months according to Animal Diseases Act (Act No 35 of 1984) in South Africa and the rules of the National Horse Racing Authority (National Horseracing Authority 2014). Blood samples were collected by jugular venepuncture in EDTA and serum blood collection tubes from foals two weeks before the first day of vaccination (week -2), on the day of first vaccination (week 0) and every week thereafter until Groups 3 and 4 received their first vaccination.

3.3.4 Vector trapping

One Onderstepoort (220V ultraviolet down-draught suction) light trap was set on each farm close to the areas where the horses were kept from before sunset until after sunrise once every week starting

on the first day of vaccination (week 0) for the duration of the study. Catches were made into a mixture of approximately 250 ml tap water and 15 ml “Savlon” (Johnson & Johnson, South Africa) to break the surface tension. The contents was then filtered through gauze cloth and the insects stored in 75% ethanol until *Culicoides* numbers were determined under a stereomicroscope. *Culicoides* were sorted into pools of maximum 200 midges according to date of catch. Species sorting was performed on the largest catches from each location from each month under a stereomicroscope. The entire catch if, small enough, or a sub-sample of at least 500 midges from each larger catch was sorted into species using the method described previously (Van Ark & Meiswinkel 1992). Catches were small enough to allow all *Culicoides* to be tested using an RT-qPCR assay for the presence of AHSV nucleic acid (Scheffer et al. 2011). Seven *Culicoides* that had recently had a blood meal were tested individually. These seven *Culicoides* were tested to determine the presence of AHSV nucleic acid using RT-qPCR and the host species on which they fed using a multiplex PCR that employs universal and species-specific primers based on the mitochondrial *cytochrome b* gene (Tobe & Linacre 2008). Samples with a quantification cycle (Cq) of 33 or less on the group-specific RT-qPCR were tested with type-specific RT-qPCR assays (Weyer et al. 2015).

3.3.5 Laboratory tests

To assess the presence or loss of maternal antibody before vaccination commenced, preliminary serum samples (week -2) were subjected to a validated in-house I-ELISA (ARC-OVI) to test for group-specific IgG antibodies to AHSV (Maree & Paweska 2005). All further serum samples were stored at -20°C. ELISA plates were coated with a recombinant AHSV 4 VP7 diluted in carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were then blocked with PBS and skimmed milk. Diluted test sera and positive and negative controls were added to the plate in duplicates and incubated. Horseradish peroxidase conjugated anti-horse gamma-globulin diluted 1:20 000 was added and the plate was incubated. Substrate solution was then added. 3N H_2SO_4 was added after five to ten minutes to stop the colour development and the plates were read at 450 nm. Plates were washed between each step of the procedure. Test samples with a percentage positive (pp) value less than five were reported as negative. Those greater than or equal to five and less than ten were reported as

suspect for AHSV antibody and those greater than ten were classified as positive (Maree & Paweska 2005, World Organisation for Animal Health (OIE) 2012, Weyer 2013).

EDTA samples were tested with an AHS group-specific RT-qPCR assay (Quan et al. 2010, Guthrie et al. 2013). Preliminary EDTA samples (week -2) were tested to ensure that all foals were negative for AHSV nucleic acid before the start of vaccination. Samples were pooled into groups of ten or less for this testing. EDTA samples were tested individually for the remainder of the study. Samples with a Cq of 33 or less on the group-specific RT-qPCR were tested with type-specific RT-qPCR assays (Weyer et al. 2015).

A real-time RT-qPCR assay developed in the ERC was used for AHSV nucleic acid detection (Quan et al. 2010, Guthrie et al. 2013). Briefly, 100 µl sample blood was added to 20 µl of magnetic bead mixture in each well of a deep well plate. This sample plate was then sealed with foil and vortexed at 1000 rpm for 1 min. 400 µl of lysis/binding solution (containing 200 µl Lysis Binding Solution, 200 µl Isopropanol and 2 µl Xeno RNA) was then added to each well. The plate was again sealed and vortexed. The sample plate was then loaded into the Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) along with two ethanol based wash plates, 2 isopropanol based wash plates and an elution plate containing 90 µl elution buffer in each well. RNA extraction was done using the MagMAX™ pathogen RNA/DNA kit in the magnetic particle processor. The elution plate was then removed from the machine, sealed and cooled at -20°C for 5 min. 20 µl VetMax™-Plus one-step RT-PCR mastermix (Thermo Fisher Scientific) (containing ABI AHS/Xeno Assay primers and probes) was added to each well of the PCR plate. A volume of 5 µl of extracted sample from each well of the elution plate was then transferred to the corresponding well on the PCR plate. The elution plates were then stored after RNA extraction for possible use in the AHSV type-specific RT-qPCR assays. Three wells on the PCR plate each contained an AHSV-negative template and a low and high AHSV-positive control. The plate was then covered and processed through the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) according to the Standard Operating Procedure of the ERC (Quan et al. 2010, Guthrie et al. 2013). Samples were classified as positive if the fluorescence exceeded the threshold of 0.1 within a maximum of 37 PCR cycles. The Cq was defined as the cycle number during which the threshold was reached.

Samples with a Cq of 33 or less on the group-specific RT-qPCR were tested with the type-specific RT-qPCR assays that have recently been developed in the ERC (Weyer et al. 2015). Elution plates described above were thawed and a volume of 5 µl of eluate was transferred to each of three separate wells on a PCR plate. A volume of 5 µl of each of three triplex primer probe mixes were added to each of the three wells. The plate was sealed and heated at 95°C for 1 min to denature the dsRNA. The plate was then frozen at -20°C for 5 min. A volume of 15 µl of VetMax™-Plus One-Step RT-PCR mastermix (Applied Biosystems) was added to each well on a 96-well PCR plate. The plate was sealed and the AHSV type-specific RT-qPCR assays were performed according to the manufacturer's recommendations on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Samples were classified as positive if the fluorescence exceeded a 0.1 threshold within 40 PCR cycles. The Cq was defined as the cycle number during which the threshold was reached (Weyer et al. 2015).

The *Culicoides* were tested to determine the presence of AHSV nucleic acid using RT-qPCR (Quan et al. 2010, Scheffer et al. 2012). Briefly, 400 µl of lysis/binding solution (containing 200 µl Lysis Binding Solution, 200 µl Isopropanol and 2 µl Xeno RNA) was added to each midge pool or individual midge and this was homogenized in a MagnaLyser (Roche Products). Each sample was then centrifuged and the supernatant used for RNA extraction in the Kingfisher 96 Magnetic Particle Processor (Thermo Scientific). RT-qPCR was then performed as described above for blood samples (Quan et al. 2010, Scheffer et al. 2011, Scheffer et al. 2012).

The seven *Culicoides* were also tested to determine the host species on which they fed using a multiplex PCR. The PCR tests for cow, pig, sheep, horse, donkey, goat, cat, dog and human. The test employs universal and species-specific primers based on the mitochondrial cytochrome *b* gene. The universal primers were designed to react with all mammalian species and the species-specific primers only with the species for which they were designed (Tobe & Linacre 2008, Garros et al. 2011). The RNA extracted from the *Culicoides* used in the AHSV RT-qPCR described above, was mixed with buffer, the universal and species-specific primers and sterile water. The assay used in this study included two different species-specific primers for cow, horse, sheep, goat, donkey, pig, dog, cat and

human. The PCR was then performed and the products were subjected to fragment analysis on a genetic analyser (3500XL, ThermoFisher Scientific). The data were visualized and analysed using STRand (Board of Regents, University of California).

Three horses in Group 4 (horse no 37, 50, 65) that were meant to remain unvaccinated for the duration of the study were inadvertently vaccinated on the second day of vaccination (week 4). They were thus excluded from any further analysis.

The mares on Moutonshoek were vaccinated on 6 (AHSV-LAV Comb 2) and 30 (AHSV-LAV Comb 1) June 2014 and the mares on Wilgerbosdrift were vaccinated on 9 (AHSV-LAV Comb 1) and 30 (AHSV-LAV Comb 2) June 2014.

3.3.6 Data presentation

The distribution of the Cq values of the group-specific RT-qPCR positive weanlings in Groups 1 and 2 was presented in a box and whisker plot (Figure 4 below). The upper and lower “hinges” correspond to the first and third quartiles (the 25^{th} and 75^{th} percentiles). The upper whisker extends from the hinge to the highest value that is within 1.5*IQR of the hinge, where IQR is the inter-quartile range, or distance between the first and third quartiles. The lower whisker extends from the hinge to the lowest value within 1.5*IQR of the hinge. Data beyond the end of the whiskers are outliers and are plotted as points.

The number of weanlings positive on group-specific RT-qPCR over time in each of the weanling groups was shown in a bar plot (Figure 5 below). The percentage of weanlings positive on group-specific RT-qPCR in Groups 1 and 2 was shown in a line graph as a cumulative positive percentage over time (Figure 6 below). This was worked out as the percentage of weanlings that tested positive in each group each week added, cumulatively, to that of the following week.

The number of weanlings that tested positive for each of the 9 AHSV types in the first 4 weeks, between weeks 4 and 8 and weeks 8 and 12 was depicted in stacked bar plots (Figure 7 below).

The number of *Culicoides* midges caught in the light traps on each farm each week were shown in a bar plot (Figure 8 below).

3.4 Results

3.4.1 Preliminary sampling

All weanlings were negative for AHSV nucleic acid using RT-qPCR at the beginning of the study, two weeks before the first vaccination was administered (week -2). Serum samples collected at the same time were subjected to the I-ELISA. The total number of weanlings that tested positive using the I-ELISA was 24 (18%) (Table 1). All of the weanlings that were going to be vaccinated (Groups 1 and 2) were negative except for three on Wilgerbosdrift (Group 1) and three on Moutonshoek (Group 2) (Table 1). Of the weanlings that were going to remain unvaccinated, eleven tested positive on Wilgerbosdrift (Group 3) and seven on Moutonshoek (Group 4) (Table 1).

Table 1: Number of weanlings that tested positive and negative before the onset of the study using the I-ELISA on the respective farms

	Weanlings to be vaccinated		Weanlings to remain unvaccinated		Total
	I-ELISA +ve	I-ELISA -ve	I-ELISA +ve	I-ELISA -ve	
Moutonshoek	3	35	7	28	73
Wilgerbosdrift	3	26	11	17	57

3.4.2 Group-specific RT-qPCR results

In both vaccinated groups, positive AHSV RT-qPCR results were obtained from two weeks after first vaccination until the last samples were collected eight weeks after the second vaccination (Tables 2 & 3). None of the weanlings in any of the groups showed obvious signs of illness at any stage during the study.

Table 2: A summary of the group-specific (GS) RT-qPCR and type-specific (TS) RT-qPCR results over time for each of the weanlings in Group 1. The Cq value for each of the group-specific results is given, and the AHS type obtained is given below each corresponding positive group-specific result. N indicates that the Cq value for the group-specific PCR was > 37. The column highlighted in red indicates the week when AHSV-LAV Comb 1 was given and the column highlighted in green indicates the week AHSV-LAV Comb 2 was given. The age of each weanling at the time of initial vaccination (week 0) is given in days (d).

			Weeks post initial vaccination												
Horse	Age (d)	Test	0	1	2	3	4	5	6	7	8	9	10	11	12
102	215	GS	N	N	29.7	32.8	33.1	33.2	34.3	35.2	N	N	31.9	34.2	N
		TS			1	1							1		
103	215	GS	N	N	32.4	32.6	33.4	33.0	34.6	N	N	N	N	36.5	N
		TS			1	1		1							
104	244	GS	N	N	34.3	35.2	34.2	N	N	N	N	N	N	N	N
		TS													
105	207	GS	N	N	29.7	30.0	32.6	N	36.6	35.1	N	N	N	N	N
		TS			1	1	1								
107	204	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
110	214	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
111	232	GS	N	N	37.0	N	35.7	34.2	34.2	N	N	N	N	N	N
		TS													
113	184	GS	N	N	N	N	N	N	36.5	N	N	N	N	N	N
		TS													
114	211	GS	N	N	N	N	36.7	N	N	N	N	36.9	N	N	N
		TS													
119	238	GS	N	N	35.6	N	N	34.6	34.6	N	N	36.7	N	N	N
		TS													
122	203	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
125	216	GS	N	N	N	N	N	N	36.6	N	N	N	N	N	N
		TS													
126	222	GS	N	N	N	N	N	35.4	33.2	36.9	N	N	N	N	N
		TS													
127	196	GS	N	N	N	33.7	31.6	33.0	34.7	N	N	N	N	N	N
		TS					1	1							
128	203	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
129	197	GS	N	N	N	33.4	32.9	N	34.9	35.2	N	N	N	N	N
		TS					1								
131	199	GS	N	N	N	30.4	30.2	34.0	28.1	29.7	32.5	31.5	33.9	34.4	36.3
		TS				1	1		1, 3	1, 3	3	3			
136	183	GS	N	N	N	N	N	N	N	N	N	N	35.1	36.7	N
		TS													
137	222	GS	N	N	N	31.6	32.7	N	36.4	N	N	N	N	N	N
		TS				1	1								
138	206	GS	N	N	35.6	33.2	30.4	34.9	32.0	32.9	32.5	32.0	35.0	35.8	N
		TS					1		3	1,3,4	1, 3	1, 3			
143	209	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
144	224	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
146	242	GS	N	N	N	N	N	N	N	N	N	36.3	N	N	N
		TS													
148	217	GS	N	N	N	N	N	36.1	N	N	N	N	N	N	N
		TS													
149	221	GS	N	N	33.4	29.4	31.6	35.6	35.8	N	N	N	N	N	N
		TS				1	1								
150	199	GS	N	N	N	33.9	35.2	N	32.9	N	N	N	N	N	N
		TS							3						
151	184	GS	N	N	N	N	N	N	33.2	35.8	N	N	N	N	36.4
		TS													
157	212	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
158	196	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
Min. Age	183	Min Cq	N	N	29.7	29.4	31.6	33.0	28.1	29.7	32.5	31.5	31.9	34.2	36.3
Max. Age	244														
Ave. Age	211	Total Pos	0	0	8	11	13	10	16	7	2	5	4	5	2

Table 3: A summary of the group-specific (GS) RT-qPCR and type-specific (TS) RT-qPCR results over time for each of the weanlings in Group 2. The Cq value for each of the group-specific results is given, and the AHS type obtained is given below each corresponding positive group-specific result. N indicates that the Cq value for the group-specific PCR was > 37. The column highlighted in red indicates the week when AHSV-LAV Comb 1 was given and the column highlighted in green indicates the week AHSV-LAV Comb 2 was given. The age of each weanling at the time of initial vaccination (week 0) is given in days (d).

			Weeks post initial vaccination												
Horse	Age (d)	Test	0	1	2	3	4	5	6	7	8	9	10	11	12
2	200	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
4	199	GS	N	N	N	N	N	N	35.5	N	N	N	35.1	N	N
		TS													
5	224	GS	N	N	N	N	N	N	N	N	32.8	34.0	N	32.3	31.3
		TS									1			1	1
10	249	GS	N	N	31.8	33.3	35.2	N	N	N	N	N	35.4	N	N
		TS			8										
11	211	GS	N	N	N	N	N	N	N	N	35.3	34.1	N	N	N
		TS													
13	190	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
16	209	GS	N	N	N	34.5	36.5	N	N	N	N	N	N	N	N
		TS													
18	240	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
21	210	GS	N	N	N	N	N	N	33.1	33.0	31.7	33.2	N	N	N
		TS								1	1				
22	228	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
23	218	GS	N	N	35.2	32.5	33.0	34.7	35.7	N	N	N	N	N	N
		TS				8									
29	218	GS	N	N	N	N	N	N	31.0	N	N	32.1	N	N	N
		TS							1						
30	225	GS	N	N	N	N	N	N	N	N	N	N	35.3	N	N
		TS													
31	206	GS	N	N	N	N	N	36.0	35.8	N	35.2	35.5	N	N	N
		TS													
34	200	GS	N	N	N	N	N	N	36.2	N	N	32.1	32.9	N	N
		TS										8	8		
36	205	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
38	205	GS	N	N	N	N	N	N	N	N	N	36.8	36.0	N	33.8
		TS													
41	218	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
42	213	GS	N	N	N	N	N	34.1	34.1	N	N	N	N	N	N
		TS													
45	220	GS	N	N	N	N	35.8	32.6	N	N	N	N	N	N	N
		TS						8							
46	188	GS	N	N	N	N	N	N	N	N	N	36.1	34.2	N	N
		TS													
47	194	GS	N	N	N	N	N	N	31.9	36.0	N	35.4	N	N	N
		TS							3						
51	225	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
54	238	GS	N	N	N	N	N	N	N	N	N	34.8	35.5	N	N
		TS													
56	208	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
58	213	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
59	223	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
60	221	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
62	191	GS	N	N	N	N	N	N	36.7	N	N	N	N	N	N
		TS													
63	224	GS	N	N	N	N	35.3	N	N	N	N	N	N	N	N
		TS													
66	207	GS	N	N	N	N	N	N	N	N	N	35.3	N	N	N

		TS													
69	235	GS	N	N	N	N	N	N	N	N	N	35.7	N	N	N
		TS													
70	204	GS	N	N	N	33.4	35.2	36.1	N	N	36.3	35.5	N	N	N
		TS													
73	200	GS	N	N	N	N	N	N	N	N	N	N	N	N	N
		TS													
Min. Age	**188**	**Min Cq**	**N**	**N**	**31.8**	**32.5**	**33.0**	**32.6**	**31.0**	**33.0**	**31.7**	**32.1**	**32.9**	**32.3**	**31.3**
Max. Age	**249**														
Ave. Age	**213.5**	**Total Pos**	**0**	**0**	**2**	**4**	**6**	**5**	**10**	**2**	**5**	**13**	**7**	**1**	**2**

3.4.2.1 Treatment Group 1

In this group (n=29), where the weanlings received AHSV-LAV Comb 1 and then AHSV-LAV Comb 2, a minimum Cq of 28.1 was obtained at week 6, two weeks after vaccination with AHSV-LAV Comb 2 (Table 2 & Figure 4). Twenty-one (72%) of the vaccinated weanlings tested positive for AHSV nucleic acid using RT-qPCR (Figures 5 & 6). Eight vaccinated weanlings and all the unvaccinated weanlings on the farm (Group 3) remained negative for the duration of the study. Peak numbers of positive weanlings (n=16) occurred at week 6, two weeks after vaccination with AHSV-LAV Comb 2 (Table 2 & Figure 5). A small second peak (n=5) occurred at week 9, five weeks after AHSV-LAV Comb 2 vaccination. The duration of consecutive positive results ranged from one to ten weeks.

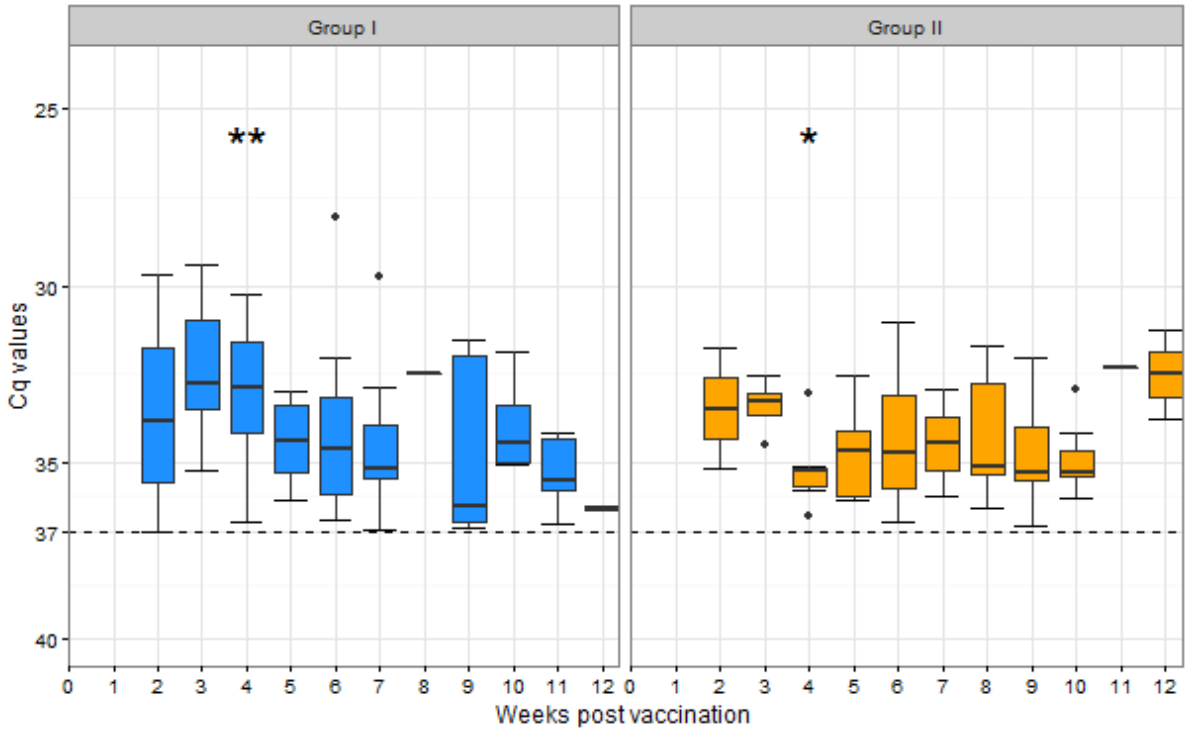

Figure 4: A box and whisker plot describing the Cq value distributions of the group-specific RT-qPCR positive weanlings in each of the AHS vaccination groups (Groups 1 and 2). The upper and lower "hinges" correspond to the first and third quartiles (the 25th and 75th percentiles). The solid line within the boxes represents the median Cq value. The outlier results are represented by the dots. Weanling group 1 (a) received AHSV-LAV Comb 1 at week 0, and AHSV-LAV Comb 2 4 weeks later (indicated by the **). Weanling group 2 (b) received AHSV-LAV Comb 2 at week 0 and AHSV-LAV Comb 1 4 weeks later (indicated by the *).

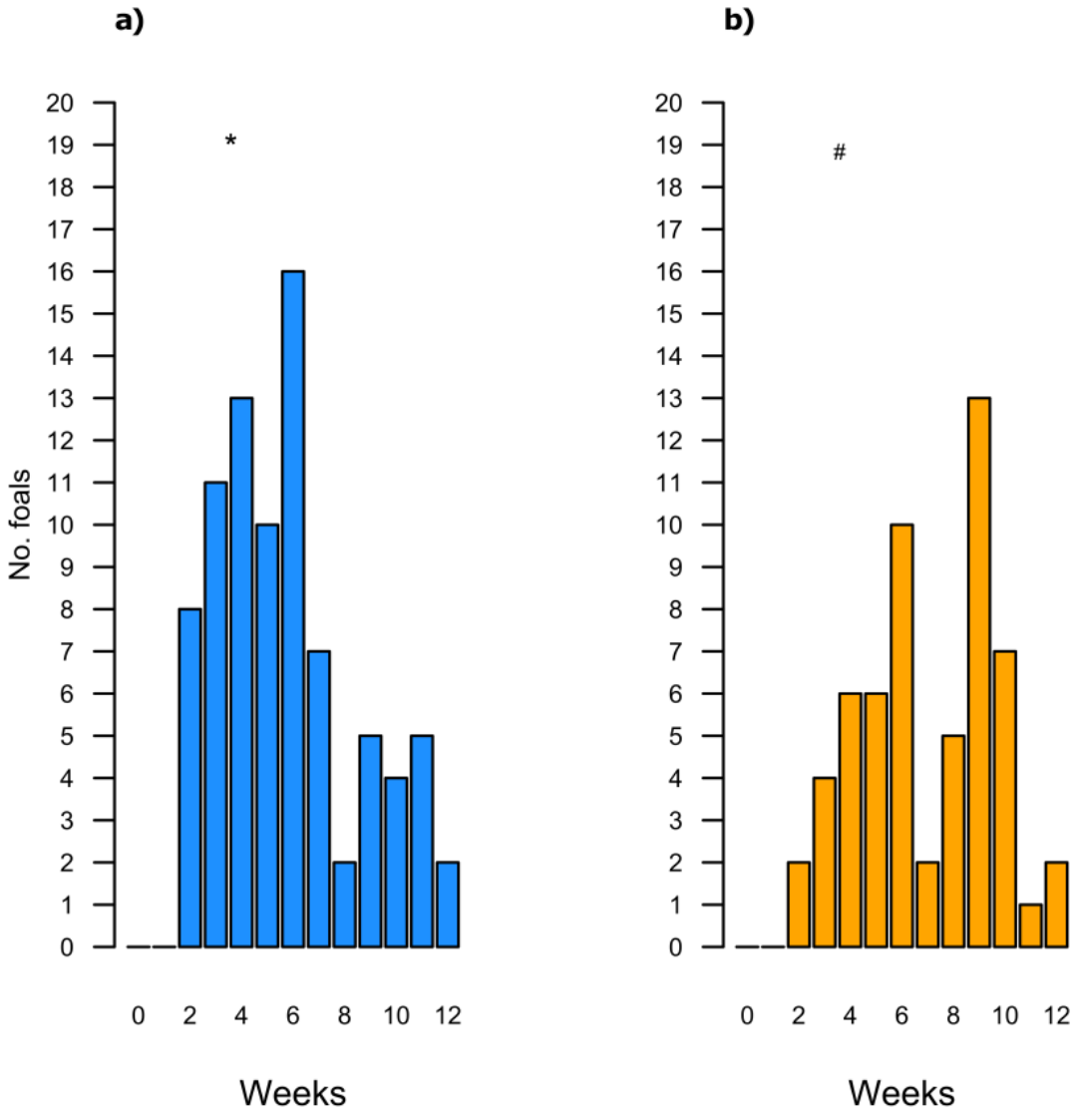

Figure 5: Bar plots showing the number of weanlings positive on group-specific RT-qPCR over time in each of the weanling groups. Weanling group 1 (a) received AHSV-LAV Comb 1 at week 0, and AHSV-LAV Comb 2 4 weeks later (indicated by the *). Weanling group 2 (b) received AHSV-LAV Comb 2 at week 0 and AHSV-LAV Comb 1 4 weeks later (indicated by the #).

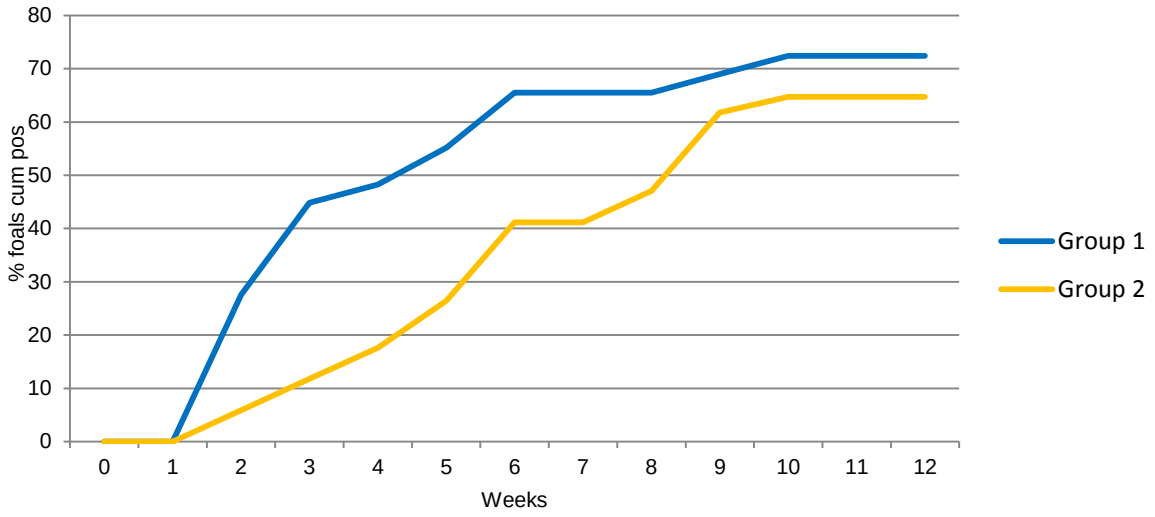

Figure 6: Line graph comparing the percentage of weanlings positive on group-specific RT-qPCR in Groups 1 and 2 as a cumulative positive percentage over time. Weanling group 1 (blue line) received AHSV-LAV Comb 1 at week 0, and AHSV-LAV Comb 2 4 weeks later. Weanling group 2 (orange line) received AHSV-LAV Comb 2 at week 0 and AHSV-LAV Comb 1 4 weeks later.

3.4.2.2 Treatment Group 2

In this group (n=34), where the weanlings received AHSV-LAV Comb 2 and then AHSV-LAV Comb 1, a minimum Cq of 31.0 was obtained at week 6, two weeks after vaccination with AHSV-LAV Comb 1 (Table 3 & Figure 4). Twenty-two (65%) of the vaccinated weanlings tested positive for AHSV nucleic acid using the RT-qPCR (Figures 5 & 6). Twelve vaccinated weanlings and all the unvaccinated weanlings on the farm (Group 4) remained negative for the duration of the study. Peak numbers of positive weanlings (n=10) occurred at week 6, two weeks after vaccination with AHSV-LAV Comb 1 (Table 3 & Figure 5). A second peak (n=13) occurred at week 9, five weeks after AHSV-LAV Comb 1 vaccination. The duration of consecutive positive results ranged from one to five weeks. In this group, results obtained for four weanlings using type-specific RT-qPCR assays (see Section 3.4.3.2) were not consistent with having been vaccinated with AHSV-LAV Comb 2 initially and therefore their results were excluded from this analysis.

3.4.2.3 Comparison of I-ELISA and RT-qPCR results

In the vaccinated groups of weanlings (Groups 1 and 2), the majority of the weanlings that had a positive I-ELISA result also tested positive using RT-qPCR. In the unvaccinated groups of weanlings (Groups 3 and 4), none of the weanlings had positive results on both the I-ELISA and RT-qPCR (Table 4).

Table 4: Comparison of I-ELISA and RT-qPCR results for vaccinated and unvaccinated weanlings

	Vaccinated weanlings		Unvaccinated weanlings	
	I-ELISA +ve	**I-ELISA -ve**	**I-ELISA +ve**	**I-ELISA -ve**
RT-qPCR +ve	5	38	0	0
RT-qPCR -ve	1	19	18	42

3.4.3 Type-specific RT-qPCR results

3.4.3.1 Treatment Group 1

In Group 1, ten vaccinated weanlings tested positive on the type-specific RT-qPCR assays (Figure 7). Seven horses tested positive for AHSV 1, one for AHSV 3, one for AHSV 1 and 3 and one for AHSV 1, 3 and 4. It is interesting to note that one horse (horse 131) tested positive for AHSV 1 after vaccination with AHSV-LAV Comb 1 and then for AHSV 3 only after vaccination with AHSV-LAV Comb 2. Another horse (horse 138) tested positive for AHSV 3 after vaccination with AHSV-LAV Comb 1 and then for AHSV 1 and 4 only after vaccination with AHSV-LAV Comb 2. A third horse (horse 150) only developed a positive reaction to AHSV 3 after vaccination with AHSV-LAV Comb 2. No AHSV types were detected from AHSV-LAV Comb 2 after the second vaccination in this group.

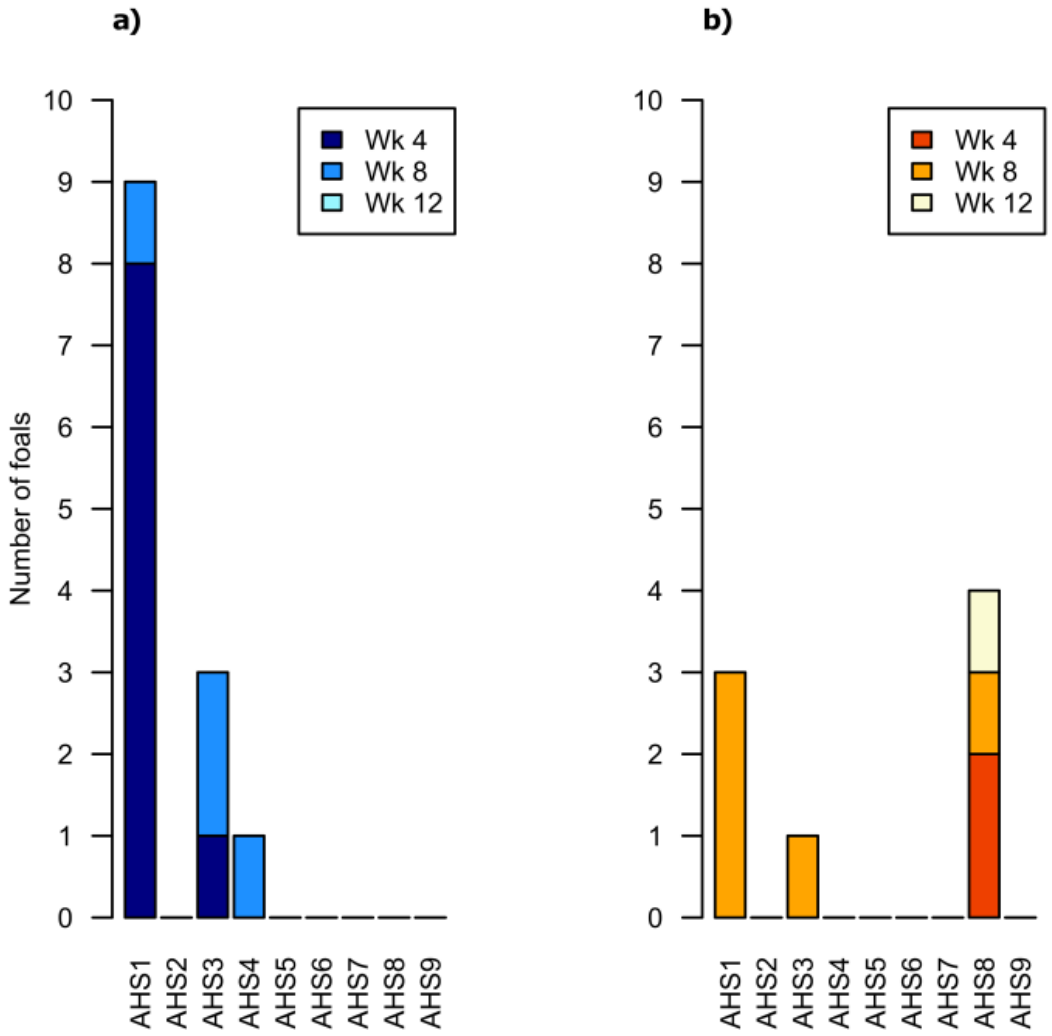

Figure 7: Stacked bar plots depicting the number of weanlings that tested positive for each of the 9 AHSV types on type-specific RT-qPCR. Plot a) depicts Group 1, b) Group 2. Plot a) received AHSV-LAV Comb 1 at week 0 and AHSV-LAV Comb 2 at week 4. Plot b) received AHSV-LAV Comb 2 at week 0 and AHSV-LAV Comb 1 at week 4. The stacks indicate the number of weanlings that tested positive on type-specific RT-qPCR per AHSV type in the first 4 weeks, between weeks 4 and 8, weeks 8 and 12.

3.4.3.2 Treatment Group 2

In Group 2, eight vaccinated weanlings tested positive on the type-specific RT-qPCR (Figure 7). Of these vaccinated weanlings, three tested positive for AHSV 1, one for AHSV 3, four for AHSV 8. Interestingly, two horses tested positive for AHSV 8 only after the second vaccination (AHSV-LAV Comb 1).

Some inconsistent results were obtained in Group 2 after vaccination with AHSV-LAV Comb 2 (first vaccination). One horse tested positive for AHSV 1, one for AHSV 3 and two for AHSV 3 and 8 in the four weeks prior to being vaccinated with AHSV-LAV Comb 1, which contains AHSV 1, 3 and 4. As these results were inconsistent with having only been vaccinated with AHSV-LAV Comb 2, the original stored duplicate sample from each of these four horses for weeks 2, 3 and 4 was retested with the group-specific and type-specific RT-qPCR assays to verify repeatability of the results. Repeat testing provided the same results as the initial testing and therefore these inconsistent results are presented separately (Table 5).

Table 5: A summary of the group-specific (GS) RT-qPCR and type-specific (TS) RT-qPCR results over time for each of the weanlings in Group 2 that had results inconsistent with having been vaccinated with AHSV-LAV Comb 2 in the first 4 weeks. The Cq value for each of the group-specific results is given, and the AHS type obtained is given below each corresponding positive group-specific result. N indicates that the Cq value for the group-specific PCR was > 37. The column highlighted in red indicates the week when AHSV-LAV Comb 1 was given and the column highlighted in green indicates the week AHSV-LAV Comb 2 was given. The age of each weanling at the time of initial vaccination (week 0) is given in days (d).

			Weeks post initial vaccination												
Horse	Age (d)	Test	0	1	2	3	4	5	6	7	8	9	10	11	12
14	211	GS	N	N	33.0	31.2	30.3	33.8	35.5	N	N	N	N	N	N
		TS			8	3	3								
43	216	GS	N	N	32.4	29.1	30.8	36.8	35.0	N	N	N	37.0	N	N
		TS			3, 8	3, 8	3								
52	191	GS	N	N	N	32.6	31.9	34.2	36.9	N	N	N	N	N	N
		TS				1	1								
72	217	GS	N	N	31.3	30.8	31.4	35.8	N	N	N	N	N	N	N
		TS			3	3	3								
Min. Age	183	Min Cq	N	N	31.3	29.1	30.3	33.8	35.0	N	N	N	37.0	N	N
Max. Age	244														
Ave. Age	211	Total Pos	0	0	3	4	4	4	3	0	0	0	1	0	0

3.4.4 *Culicoides* sampling

The number of *Culicoides* midges caught per light trap per night ranged from 0 to 765 on Moutonshoek (average = 226) and 1095 on Wilgerbosdrift (average = 362) (Figure 8). The dominant species based on the analysed catches on Moutonshoek was *C. leucostictus* and on Wilgerbosdrift was *C. subschultzei* (Table 6). *Culicoides imicola* was present in all analysed catches and represented 23% of the *Culicoides* caught on Moutonshoek and 8% on Wilgerbosdrift. *Culicoides bolitinos* was caught in five of the analysed catches and represented 4% of the *Culicoides* caught on Moutonshoek and 1% on Wilgerbosdrift. A further 18 species of *Culicoides* were also identified (Table 6).

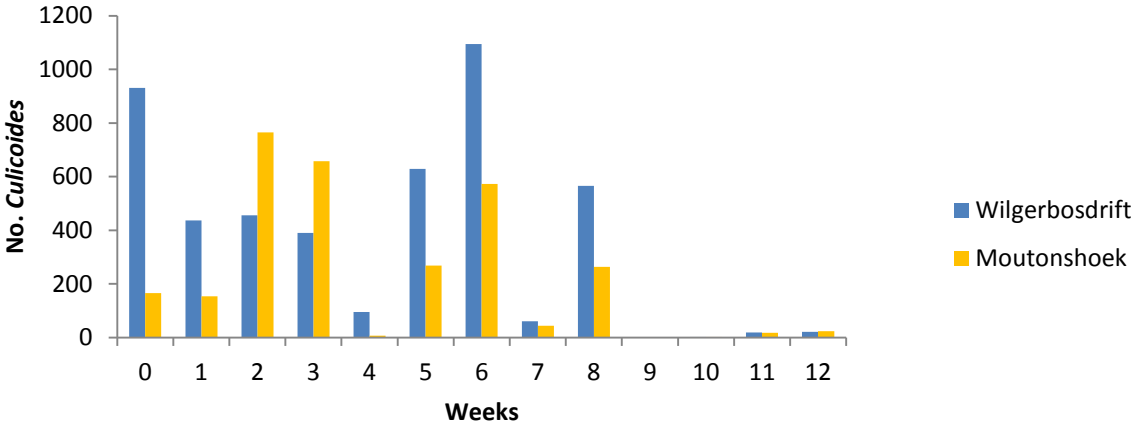

Figure 8: Bar plot depicting the number of *Culicoides* midges caught in the light traps on each farm over time. Blue bars show numbers caught on Wilgerbosdrift and orange bars show numbers caught on Moutonshoek.

Table 6: The number of each of the *Culicoides* species caught in light traps on each of the farms in selected catches from each month.

Species	Moutonshoek					Wilgerbosdrift				
	Weeks post initial vaccination									
	0	6	8	12	Average	0	6	8	12	Average
C. bedfordi	6	2	0	0	4	54	16	6	0	25
C. bolitinos	0	12	21	1	11	0	6	21	0	14
C. brucei	2	9	2	0	4	6	6	5	0	6
C. engubandei	6	31	0	2	13	0	0	0	0	0
C. herero	1	1	0	0	1	33	0	3	0	18
C. imicola	32	107	69	6	54	39	56	40	2	34
C. leucostictus	36	130	80	8	64	87	61	74	0	74
C. magnus	3	9	12	3	7	5	9	21	10	11
C. neavei	2	8	9	0	6	18	12	18	0	16
C. nivosus	2	23	13	0	13	11	27	33	0	24
C. nr. angolensis	1	7	1	0	3	14	4	5	0	8
C. onderstepoortensis	0	1	0	0	1	3	3	13	0	6
C. pycnostictus	21	75	29	1	32	27	45	33	5	28
C. ravus	15	29	1	0	15	7	3	0	0	5
C. schultzei	0	0	0	0	0	2	0	2	0	2
C. similis	2	1	0	0	2	7	0	1	0	4
C. subschultzei	3	28	14	0	15	149	246	205	0	200
C. tropicalis	31	86	3	0	40	70	21	6	0	32
C. zuluensis	0	3	4	2	3	1	1	7	3	3
C. Sp. #90	0	7	5	0	6	32	51	71	1	39
C. Sp. #94	2	4	0	0	3	0	0	0	1	1
C. Sp. #119	0	0	0	0	0	0	0	1	0	1
Sample size	**165**	**573**	**263**	**23**		**565**	**567**	**566**	**21**	
Catch size	**165**	**573**	**263**	**23**		**931**	**1095**	**566**	**21**	

None of the pools of *Culicoides* or recently blood-fed *Culicoides* tested positive for AHSV nucleic acid using the RT-qPCR.

Of the seven *Culicoides* tested to determine the host species on which they fed using the multiplex PCR, two tested positive for horse and one for cow. None of the other species tested by the assay were detected in the midges.

3.5 Discussion

Since the group-specific RT-qPCR assay used in this study is quantitative, the number of horses that test positive for AHSV each week together with the Cq values give an indication of the viral RNA present in the environment available for transmission by *Culicoides* midges. The highest proportion of positive results in vaccinated weanlings occurred in Group 1 (AHSV-LAV Comb 1 followed by AHSV-LAV Comb 2). The lowest Cq value was obtained in Group 1 (28.1) and the maximum duration of consecutive positive results obtained in the same weanling was longest in Group 1. However, it is difficult to comment on the duration of RNAaemia detected by the RT-qPCR in this study due to weeks where a negative result was obtained in between the weeks of positive results. The fact that an AHSV type could not always be obtained made it impossible to know whether this phenomenon was due to the same or two different AHSV types. There was a clear difference in the occurrence of different AHSV types in the two groups. When AHSV-LAV Comb 1 was given first, AHSV 1 was the predominant AHSV type. When AHSV-LAV Comb 2 was administered first, AHSV 8 occurred most often. This is consistent with other work that has recently been done characterising the dynamics of the AHSV following vaccination (Dr Camilla Weyer, personal communication, 2015). However, the Cq values obtained in the present study were consistently higher than those obtained by Weyer (2015). The average age of vaccinated weanlings in the present study was lower than that in the study by Weyer (2015) and all the foals were born to regularly vaccinated mares. It is therefore possible that a proportion of the negative RT-qPCR results in weanlings in the present study may have been due to interference of response to vaccination by maternal antibody although this was not supported by the results of the group-specific I-ELISA performed on serum samples collected prior to first vaccination (Alexander & Mason 1941, Crafford et al. 2013).

The results for AHSV 1 and 3 in Group 2 after vaccination with AHSV-LAV Comb 2 only were unexpected. Detectable viraemia seems to occur from days 10 to 14 after vaccination and lasts for

three to five days (Paweska, Prinsloo & Venter 2003, von Teichman, Dungu & Smit 2010). Incubation in the midge lasts approximately eight days before infection of the next vertebrate host may occur (Lubroth 1988). This indicates that it should take approximately 28 days for detectable viraemia to develop in a vertebrate host following transmission of vaccine virus (ten days from vaccination to viraemia, eight days in the midge and ten days from infection to viraemia in the next vertebrate host). The unexpected results in Group 2 occurred 14 to 21 days after the first vaccination (AHSV-LAV Comb 2). This is too soon for vaccine transmission to have occurred from Wilgerbosdrift, where weanlings had received only AHSV-LAV Comb 1. Accidental vaccination is very unlikely as the study protocol and checklists were strictly adhered to and this would have been noticed as it was for the three horses that were inadvertently vaccinated in week 4. Additionally, it would not explain the cases where AHSV 3 and 8 occurred in the same weanlings. Incorrect vaccine bottle labelling at the time of manufacture is also possible but would also not explain these two cases. Mechanical transmission of vaccine virus by mosquito, biting fly (such as *Tabanus* and *Stomoxys*) or tick (Schuberg & Kuhn 1912, du Toit 1944, Ozawa & Nakata 1965, Mellor 1994) from Wilgerbosdrift is a remote possibility. A false positive RT-qPCR result is also very unlikely because all these RT-qPCR positive results were recognised as irregular and the original stored duplicate samples gave similar results when they were independently extracted and analysed. Transmission of circulating field virus also needs to be considered. An outbreak of AHS occurred in the Porterville, Wellington, Tulbagh, Piketberg area of the Western Cape Province from March to May 2014. A distance of approximately 17.5km and a mountain range separate Moutonshoek from Wolfkloof, the farm where the case closest to the study area occurred. The outbreak case on Wolfkloof was not subjected to type-specific RT-qPCR assays but was presumed to be AHSV 1 as this was the AHSV type detected in this outbreak. Its source was suspected to be transmission of vaccine virus (World Organisation for Animal Health (OIE) 2014, Grewar 2014a, Grewar 2014b). It was detected on 29 April 2014 which corresponds to week 3 of this study. The unusual positive results in Group 2 were first detected in weeks 2 and 3. A large proportion of the outbreak cases were characterised by a lack of clinical signs. Therefore the presence of naturally circulating virus cannot, at this stage, be ruled out. Another possibility is that these two AHSV types were present together in the vaccine bottle.

The presence of multiple AHSV types in the same weanling, as seen in both groups of vaccinated weanlings, may allow for virus reassortment to occur in the mammalian host or insect vector. A BTV 16 strain detected in Italy in 2002 was found to be a reassortant between BTV 2 and BTV 16 vaccine strains (Batten et al. 2008). A study by Maan et al. (2010) indicated that BTV 6 vaccine virus had reassorted with BTV 2 vaccine and BTV 8 field strain virus in the Netherlands (Maan et al. 2010). AHSV and BTV are very similar on a morphological and molecular level and it is therefore of grave concern that if similar events were to occur with AHSV they could have devastating consequences including high mortality in naïve populations (Long & Guthrie 2014). The Porterville outbreak was characterised by very mild clinical signs which is unusual for AHS. However, this outbreak occurred in a population of horses that were mostly vaccinated. If it had spread into a population of naïve horses the consequences could have been dramatic.

The number of *Culicoides* midges caught per light trap per night ranged from 0 to 765 on Moutonshoek (average = 226) and 1095 on Wilgerbosdrift (average = 362). Numbers of *Culicoides* caught in this study seem to be significantly lower than those collected in the same area from January to December 2006 (Venter 2008). In that study the average collection size was 4066 (range: 2 - 15 855) for Moutonshoek and 2663 (range: 0 – 20 202) for Wilgerbosdrift. The Onderstepoort light trap was used as it has been shown to collect high numbers of *Culicoides* midges (Venter et al. 2009b, del Río et al. 2013, Probst et al. 2015). Additionally, it has been observed that the largest midge catches in light traps are obtained at least two hours after sunset and light traps were thus set overnight close to plenty of water for midge breeding (Page et al. 2009, Scheffer et al. 2012). However the composition of the catch may vary according to the time of year (Scheffer et al. 2012). *Culicoides* abundance in South Africa is seasonal with an increase in numbers after summer rainfall but in frost-free areas of the country, such as the Western Cape, *Culicoides* can be collected throughout the year (Venter, Nevill & Van Der Linde 1997). Factors that may affect the abundance of *Culicoides* on farms include the types and number of livestock in the vicinity of the light trap, farming practises (e.g. methods and extent of irrigation, use of insecticides, storage conditions of animal dung, soil type and moisture (*i.e.* suitability for breeding sites) and climatic factors (*e.g.* wind speed) (Baylis, Meiswinkel & Venter 1999, Venter, Koekemoer & Paweska 2006). These factors may have varied between the years of sampling. Surveys in 1999 and 2004 reported much higher *C. imicola* abundance in the

Stellenbosch area of the Western Cape than that found in 1986 and 1984 to 1985 (Nevill et al. 1988, Venter, Nevill & Van Der Linde 1997, Venter, Koekemoer & Paweska 2006).

Although the midges caught in the study were shown to have been feeding on the horses, none of the *Culicoides* pools or individually tested midges tested positive for AHSV nucleic acid. In South Africa, attenuated viruses destined for use in vaccine may not produce a viraemia exceeding 10^3PFU/mL of blood in susceptible horses (Paweska, Prinsloo & Venter 2003). However, this testing is performed in a finite number of animals and it is therefore difficult to predict the effect on individual horses (Paweska, Prinsloo & Venter 2003). Additionally, the level of viraemia required for midge infection is unknown and midges with differing geographical distribution or genotype may differ in susceptibility to different isolates within an AHSV type (Paweska, Prinsloo & Venter 2003). Viral infection of, and replication in, the midge can also be affected by ambient temperature. At higher temperatures infection rate is higher and replication and transmission is quicker (Mellor & Hamblin 2004). This implies that there is a greater chance of vaccine circulation during the summer months (December, January and February). Indeed, outbreaks in the controlled area of the Western Cape Province have typically occurred from February to May (Sinclair, Buhrmann & Gummow 2006, Venter, Koekemoer & Paweska 2006, Grewar et al. 2013). According to the rules of the National Horse Racing Authority, foals must be vaccinated between the age of 6 and 12 months (National Horseracing Authority 2014). Since the earliest foals are born in August, the earliest that vaccination could commence would be February. This was discussed with the farm managers but was not possible due to operational commitments such as the National Yearling Sales. Nevertheless, perhaps this would have improved the design of the study.

A previous study has shown that only a small proportion of *Culicoides* became infected after ingesting vaccine virus under laboratory conditions (Paweska, Prinsloo & Venter 2003). This indicates that large numbers of *Culicoides* midges need to be present for a small proportion of these to become infected and possibly transmit vaccine virus from the small proportion of horses with a high enough viraemia. Additionally, in outbreak situations, arbovirus recovery rates from insect vectors collected in the field is usually low indicating a low infection prevalence (Chiang & Reeves 1962, Walter, Hildreth & Beaty 1980, Venter, Koekemoer & Paweska 2006). An AHSV field infection rate of less than 0.0001% was

documented in a *Culicoides* population over a six year period (Nevill, Erasmus & Venter 1992). In the AHSV outbreak situations of 1999 and 2004 in the Western Cape Province of South Africa, field infection rates in the dominant species, *C. imicola*, were found to be only 0.003% and 0.002%, respectively (Venter, Koekemoer & Paweska 2006). In these situations the abundance of *Culicoides* compensates for the low infection prevalence and results in transmission of AHSV (Venter, Koekemoer & Paweska 2006). This is possibly why the results of the outbreak provide more positive evidence of vaccine transmission than this study which was conducted over a much smaller area with lower numbers of horses and midges (World Organisation for Animal Health (OIE) 2014, Grewar 2014a, Grewar 2014b).

AHSV has been isolated from *C. imicola* and *C. bolitinos* in the field (Meiswinkel & Paweska 2003, Venter, Koekemoer & Paweska 2006). There is also evidence to support their susceptibility to oral infection with the majority of the AHSV vaccine strains currently in use in South Africa under laboratory conditions (Paweska, Prinsloo & Venter 2003). In the current study *C. imicola* was present in all analysed catches. It represented 23% of the *Culicoides* caught on Moutonshoek and 8% on Wilgerbosdrift making it the second and fifth most abundant species collected on each farm respectively. *Culicoides bolitinos*. was caught in five of the analysed catches and represented 4% of the *Culicoides* caught on Moutonshoek and 1% on Wilgerbosdrift. In previous studies, *C. imicola* was the most prolific species caught on both farms representing 80.9% of the *Culicoides* collected on Moutonshoek and 92.7% on Wilgerbosdrift. The lower prevalence of *C. imicola* and *C. bolitinos* in the present study may have contributed to the lack of vaccine transmission but their presence confirms the potential for transmission of AHSV and the LAV vaccine.

Although the study did not show the transmission of vaccine virus, the possibility of this occurring cannot be ignored due to the evidence available for BTV. BTV 2 vaccine virus (an LAV produced by OBP) was shown to be circulating in cattle and *Culicoides* midges in Italy in 2003 (Ferrari et al. 2005). An LAV strain of BTV 2 was found to be circulating naturally in the environment in Portugal in 2004 (Barros et al. 2007). In 2008 BTV 11 was detected for the first time in Belgium and it was suggested that it originated from an LAV strain (De Clercq et al. 2009). Also in 2008, BTV 6 was detected for the first time in Germany and the Netherlands. It was also closely related to the South African LAV strain

(Eschbaumer et al. 2010). Additionally, it is suspected that the AHSV outbreak in the Western Cape Province in 2014 was due to transmission of an AHSV strain derived from the AHSV-LAV (World Organisation for Animal Health (OIE) 2014, Grewar 2014a, Grewar 2014b). This study has shown that the behaviour of the currently available AHSV-LAV is unpredictable and not fully understood. This is significant as the vaccine is widely and regularly used in South Africa. Genetic sequencing of field samples that test positive for AHSV using the RT-qPCR with comparison to the published whole genome sequences of the AHSV types in the vaccine (Guthrie et al. 2015a, Guthrie et al. 2015b) could provide clearer answers on vaccine circulation in the environment. The AHSV-LAV does not seem to be the best means of control of AHS because, although it does provide protection, the risks, as seen with BTV, are substantial. However, this is the only vaccine currently registered and available for use in South Africa. The best alternatives seem to be recombinant vaccines as these would provide protection from the disease while simultaneously simplifying diagnostic testing. Disabled infectious single cycle (DISC) vaccines, as described for BTV, should also be considered (Matsuo et al. 2011, Celma et al. 2013, van de Water et al. 2015). Emphasis should be placed on the development of these vaccines as a matter of urgency.

CHAPTER 4

4. General Conclusions

This study has shown:

- AHSV nucleic acid (RNAaemia) was evident in a large proportion of the weanlings after vaccination with a commercial polyvalent AHSV-LAV using RT-qPCR assays.
- Multiple AHSV types were detected in individual weanlings using type-specific RT-qPCR assays following vaccination with a commercial polyvalent AHSV-LAV.
- RT-qPCR detected AHSV 1 and 3 on one of the farms after vaccination with AHSV-LAV Comb 2 which does not contain these types.

Areas of possible further investigation:

- Genetic sequencing of circulating AHSV to determine the LAV involvement in outbreak situations.
- Further development of a vaccine that involves fewer risks.

Concluding comments

This study has shown that RNAaemia can be detected in weanlings after vaccination with a commercial LAV under field conditions. The prevalence of AHSV types detected differs depending on whether AHSV-LAV Comb 1 or AHSV-LAV Comb 2 is administered as the first vaccination. The RNAaemia detected in weanlings following vaccination seems to be affected by age at which they are vaccinated due to the acquisition of maternal antibody if their dams are regularly vaccinated. AHSV types 1 and 3 were detected in weanlings following vaccination with AHSV-LAV Comb 2 only. Possible reasons for this occurrence include mechanical transmission, naturally circulating wild virus

or the presence of these AHSV types in the vaccine bottle. Multiple AHSV types were detected in individual weanlings which increases the chances of reassortment occurring.

None of the *Culicoides* pools or individually tested midges tested positive for AHSV. This supports the theory that large numbers of *Culicoides* midges need to be present for a small proportion of these to become infected and possibly transmit vaccine virus from the small proportion of horses with a high enough viraemia. In these situations the abundance of *Culicoides* compensates for the low infection prevelance and results in transmission of AHSV (Venter, Koekemoer & Paweska 2006).

Although the study did not show the transmission of vaccine virus, it reaffirms the fact that the behaviour of the LAV is not fully understood and needs further investigation. This is especially important in the light of the spread of BTV into areas where it previously did not occur and the suspicion that the AHSV outbreak in the Western Cape Province in 2014 was due to transmission of an AHSV strain derived from the AHSV-LAV.

CHAPTER 5

5. References

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Animal Ethics Committee

PROJECT TITLE	**Investigation of African horse sickness virus vaccine transmission in weanlings vaccinated with commercial modified live virus vaccine under field conditions**
PROJECT NUMBER	**V012-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. P Braithwaite**

STUDENT NUMBER (where applicable)	**----------**
DISSERTATION/THESIS SUBMITTED FOR	**Contract**

ANIMAL SPECIES	**Equine**	
NUMBER OF ANIMALS	**140**	
Approval period to use animals for research/testing purposes		**15 March 2014-31 December 2015**
SUPERVISOR	**Prof. AJ Guthrie**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 February 2014
CHAIRMAN: UP Animal Ethics Committee	Signature