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African horse sickness virus dynamics and host responses in naturally infected horses

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ABBREVIATIONS

AGID	Agar gel immunodiffusion
AHS	African horse sickness
AHSV	African horse sickness virus
BHK	Baby hamster kidney
CFT	Complement fixation test
CPE	Cytopathic effect
C_T	Cycle threshold
dsRNA	double stranded ribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EEV	Equine encephalosis virus
ELISA	Enzyme-linked immunosorbent assay
ERC	Equine Research Centre
Hgb	Haemoglobin
Ht	Haematocrit
ICU	Intensive care unit
IFA	Immunofluorescent assay
Lymph	Lymphocyte
Neut	Neutrophil
OBP	Onderstepoort Biological Products
OTAU	Onderstepoort Teaching Animal Unit
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
RCC	Red cell count
RT-qPCR	Reverse transcription quantitative PCR
SNT	Serum neutralisation test
$TCID_{50}$	50% Tissue culture infective dose
Temp	Temperature
VNT	Virus neutralisation test
WNV	West Nile virus

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SUMMARY

African horse sickness virus dynamics and host responses in naturally infected horses

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African horse sickness (AHS) is a life threatening disease of equids caused by African horse sickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*. The virus is transmitted to horses by midges (*Culicoides* spp.) and the disease is most prevalent during the time of year, and in areas where the *Culicoides spp.* are most abundant, namely in late summer in the summer rainfall areas of the country. Whilst the clinical signs and presentation of the disease were well documented by Sir Arnold Theiler (1921), very little is known or documented about AHSV dynamics or the clinical pathological and serological responses of horses to natural infection with AHSV.

This dissertation describes the history and current knowledge on AHS, and the methods and results of a prospective study on natural AHSV infection of horses, undertaken between 2009 and 2010 by the Equine Research Centre (ERC) at the University of Pretoria, Faculty of Veterinary Science, Onderstepoort. This study is the first documented study of its nature and included animals of various ages and therefore variable vaccination status. The objectives of the study were to describe the viral dynamics of AHSV infection in horses, to gain a better understanding of the clinical pathological and serological responses to natural AHS infection and to demonstrate early detection of AHS infection in horses under field conditions.

CHAPTER
1. General Introduction

African horse sickness (AHS) is a non-contagious disease of equids, which causes vascular injury that can result in four forms of disease; the pulmonary, cardiac, mixed or horse sickness fever forms (Guthrie & Quan 2009). It is an OIE-listed disease as it causes high mortality rates and has the potential for rapid spread (Quan, Lourens, MacLachlan, Gardner & Guthrie 2010). African horse sickness virus (AHSV) is endemic to sub-Saharan Africa with regular outbreaks occurring in southern Africa (Bourdin & Laurent 1974, Baylis, Mellor & Meiswinkel 1999). The disease was first noticed after the introduction of horses (which were not indigenous to southern Africa) into South Africa from Europe and the Far East (Henning 1956). Although deaths occur every year in southern Africa, major epidemics tend to occur approximately every 20 years and are associated with above average rainfall patterns (Bayley 1856, Guthrie 1996, Meiswinkel 1998) and may also be associated with the occurrence of the El Niño weather patterns (Baylis et al 1999). One of the most devastating recorded outbreaks in southern Africa occurred in 1854/55. It was estimated that up to 40% of the equine population of the Cape colony died (Bayley 1856, Henning 1956).

AHS has spread outside of Africa in the past. An outbreak in the Middle East and South-West Asia in 1960 led to the death of in excess of 300 000 horses (Howell 1960, Guthrie 1996). This outbreak introduced the "Horse Sickness Ban", which stopped export of horses out of Africa. Europe has also suffered outbreaks (Spain 1966 and 1987-90; Portugal 1989). The Spanish outbreak in 1966 was thought to have spread into Spain from Morocco (Hazrati 1967) possibly from AHSV infected

midges carried into Spain by large gusts of wind or by boat (Rodriguez, Hooghuis & Castano 1992). The 1987-90 outbreak was linked to zebra that were imported from Namibia and this outbreak subsequently spread to Portugal and Morocco (Lubroth 1988).

The recent emergence of multiple serotypes of bluetongue virus (BTV) in Europe since 1998 has created a great deal of concern. The change in climate is thought to be the main contributing factor; however changes in social factors and the increase in movement of virus-infected hosts and vectors may also contribute. Considering the similarities in epidemiology of bluetongue and AHS it is feared that an outbreak of AHS in Europe will follow (Purse, Mellor, Rogers, Samuel, Mertens & Baylis 2005, MacLachlan & Guthrie 2010)

The area around the Cape of Good Hope in South Africa has historically been free from AHS, with outbreaks due to the introduction of AHSV-positive horses from other provinces. In an effort to maintain this area as an AHS free zone for export purposes, movement control of equids into the area has been implemented by the South African State Veterinary Authorities since 1997 (Guthrie 1997). The Western Cape is currently divided into 3 zones; the Metropolitan Cape Town AHS free zone, the AHS surveillance zone, and the AHS protection zone (Figure 1). Horses moving into the AHS Controlled Area in the Western Cape Province from other provinces must have been vaccinated with the Onderstepoort Biological Products (OBP) polyvalent vaccine a minimum of 60 days prior to movement, a veterinarian must certify them healthy a minimum of 48 hours prior to movement, and a movement permit supplied by the State Veterinarian of their area must accompany them. Even with the current

movement controls in place, AHS outbreaks have occurred in the AHS Controlled Area.

An outbreak in the AHS protection zone in the Western Cape in 2006 (ProMed-MAIL 2006) was thought to have resulted from the movement of a subclinically AHSV-infected horse into the area. Because of this problem, this prospective study was undertaken to determine whether a recently developed reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay (Quan et al., 2010) could detect subclinical AHS cases. Furthermore, clinical pathological and serological responses of AHS positive cases were examined.
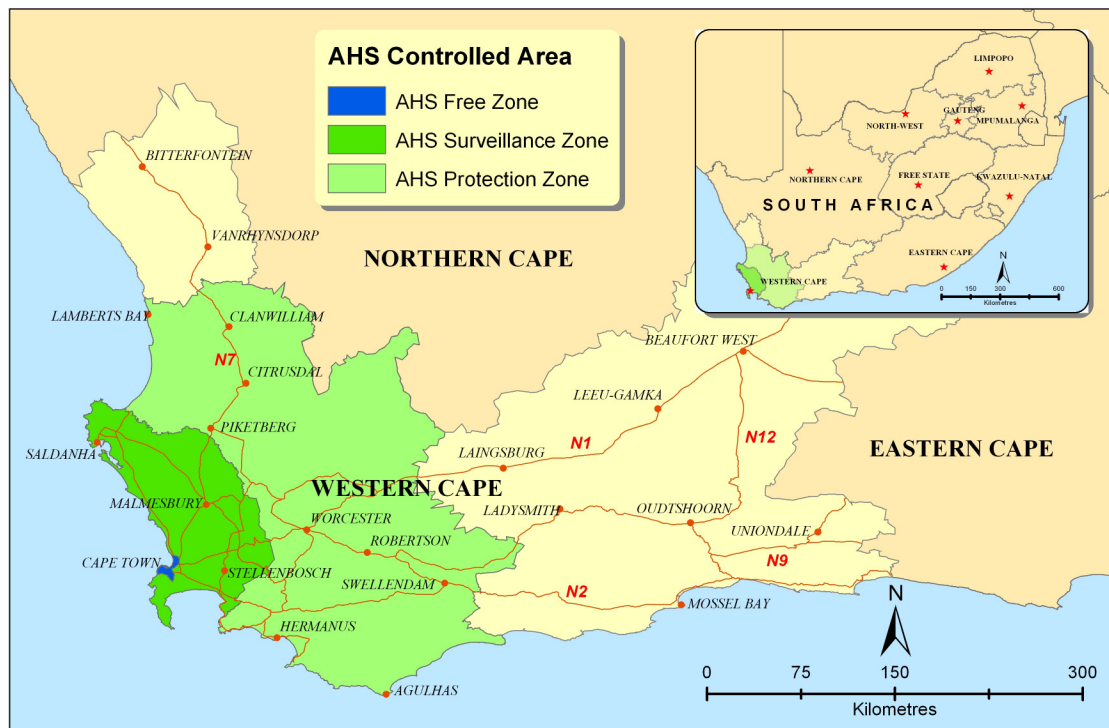
Figure 1. A map of the zones within the African horse sickness (AHS) Controlled Area of South Africa.

CHAPTER
2. Literature Review

2.1 Overview

AHS is an infectious, non-contagious, arthropod-borne disease caused by AHSV, a double-stranded ribonucleic acid (dsRNA) *Orbivirus* in the family *Reoviridae* (Coetzer & Guthrie 2004, Mellor & Hamblin 2004). Horses are the most susceptible equids, with infection often leading to high mortalities of up to 90% (Guthrie & Quan 2009). Donkeys and mules are also susceptible, but generally only develop a mild form of the disease (El Hasnaoui, El Harrak, Zientara, Laviada & Hamblin 1998, Fassi-Fihri, el Harrak & Fassi-Fehri 1998, Hamblin, Salt, Mellor, Graham, Smith & Wohlsein 1998). Zebras are highly resistant to the disease (Erasmus, Young, Pieterse & Boschoff 1978, Barnard 1998, Guthrie & Quan 2009). It has been reported that a continuous transmission cycle between zebras and the *Culicoides* midges occurs in the Kruger National Park (Barnard 1993). Under such conditions a large enough zebra population can act as a reservoir for the AHSV (Barnard 1998). Large donkey populations could play a similar role under the right climatic conditions (Hamblin et al 1998).

The virion is an unenveloped double-layered particle of about 70nm in diameter demonstrating icosehedral symmetry (Polson & Deeks 1963). The genome consists of ten double-stranded RNA segments (Oellermann 1970, Bremer 1990) that encodes for seven structural proteins (VP1-7) and four non-structural proteins (NS1-3 and NS3A). The core particle comprises two major proteins, VP3 and VP7, both of which are highly conserved (Mellor & Hamblin 2004, Quan et al 2010). There are nine antigenically distinct serotypes, with cross reactivity occurring between 1 and 2;

3 and 7; 5 and 8; and 6 and 9 (McIntosh 1958, Howell 1960). The AHSV is relatively stable at 4°C for approximately 6 months (Alexander 1935, House, House & Mebus 1992) but not at -25°C unless diluted with lactose-peptone-buffer solution (Ozawa 1965).

The virus is transmitted to horses by *Culicoides* spp., namely *Culicoides imicola* and *C. bolitinos*, and is most prevalent during the time of year, and in areas where, the *Culicoides* spp. are most abundant, namely in late summer in the summer rainfall areas of southern Africa (Du Toit 1944, Baylis, Meiswinkel & Venter 1999, Meiswinkel & Paweska 2003). The adult *Culicoides* become infected by taking blood meals from viraemic animals. The incubation period for the virus in the midges is approximately eight days, after which the virus localises in the salivary glands and is transmitted to the next host when the midge takes a blood meal (Lubroth 1988). *C. varipennis* has also been shown to be capable of transmitting the disease under experimental conditions, but is prevalent in the USA and not in southern Africa (Boorman, Mellor, Penn & Jennings 1975). Early and heavy rains followed by warm, dry weather favour the occurrence of AHS and therefore the first cases of AHS in southern Africa usually occur in late February, with the majority of cases occurring in March and April. Most *Culicoides* spp breed in damp soil rich in organic matter, however *C. bolitinos* breeds in bovine dung, and is therefore not as dependant on annual rainfall and soil-type (Venter, Koekemoer & Paweska 2006). Horses kept on open pasture, in low-lying wet areas, and not stabled at night are the most at risk of contracting the disease, as *Culicoides* midges are most active from sunset to sunrise, and do not readily enter buildings (Barnard 1997, Coetzer & Guthrie 2004). After the first frosts the disease disappears abruptly (Coetzer & Guthrie 2004).

Transovarial transmission of the virus and overwintering of *Culiciodes* has not been demonstrated (Lubroth 1988). A progressive change in climatic zones, and the subsequent spread of the vector of AHS, together with the increase of movement of horses around the world, has increased the risk of international spread of AHS (Stone-Marschat, Carville, Skowronek & Laegreid 1994, Purse et al 2005, Gale, Brouwer, Ramnial, Kelly, Kosmider, Fooks & Snary 2010).

2.2   Pathogenesis

After infection, initial multiplication of the virus occurs in the regional lymph nodes. Thereafter, a secondary viraemia occurs where the virus disseminates to the endothelial cells of the target organs, namely the lungs, spleen and other lymphoid organs (Mellor & Hamblin 2004, Guthrie & Quan 2009). Viral multiplication then occurs in these organs. Endothelial cell damage appears to be involved in the pathogenesis of AHS. The damage is thought to be due to ultra-structural changes in, and separation of, the endothelial cells (Laegreid, Burrage, Stone-Marschat & Skowronek 1992, Gomez-Villamandos, Sanchez, Carrasco, Laviada, Bautista, Martinez-Torrecuadrada, Sanchez-Vizcaino & Sierra 1999). This leads to effusions into body cavities, oedema and serosal and visceral haemorrhage (Guthrie & Quan 2009). However, one study showed that there was negligible damage to the pulmonary endothelial cells (Newsholme 1983). This has led to speculation that inflammatory mediators play a role in the pathogenesis of AHS. The virus is closely associated with erythrocytes in the blood (Theiler 1921). High concentrations of virus are found in the lymphoid tissue, and this might explain the lymphopaenia seen in experimental cases of AHS (Erasmus 1973, Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar & Laegreid 1995).

2.3   Clinical signs

The clinical symptoms of AHS have been well documented (Theiler 1921). The disease is characterised by pyrexia, oedema of the lungs, pleura and subcutaneous tissues, as well as petechiae and haemorrhages (Kazeem, Rufai, Ogunsan, Lombin, Enurah & Owolodun 2008). The incubation period was found, in experimental cases, to be on average five to seven days (Coetzer & Guthrie 2004). In a recent study where a horse was inoculated with a virulent field strain of AHSV, the incubation period was found to be seven days before dsRNA was detected by RT-qPCR (Quan et al 2010).

The viraemic period corresponds with the appearance of fever (Laegreid et al 1992), and can persist for between two to fourteen days (House et al 1992). This has been confirmed by viral isolation (Erasmus 1973, Sailleau, Moulay, Cruciere & Laegreid 1997). The exact period that the viraemia can extend beyond this time period is unknown, as viral isolation is not always sensitive enough to detect lower viral levels (Sailleau et al 1997). It has been shown that AHSV dsRNA , detected by RT-qPCR, peaked at approximately 15 days post-challenge and remained present for up to 97 days after initial exposure to the virus (Quan et al 2010). Further studies are necessary to determine the period during which horses are infectious to midges and the relationship between this and the persistence of viral nucleic acid detected using RT-qPCR. It has been shown that AHSV is associated with RBCs (Theiler 1921). A red blood cell has a life span of up to 145 days in circulation in the horse (Latimer, Mahaffey & Prasse 2003), and the persistence of RT-qPCR positive results could simply be related to the red cell life span.

There are four clinical forms of AHS. The peracute, or pulmonary form, otherwise referred to as "dunkop", is characterised by an incubation period of three to five days. The clinical signs are those of fever (39.5 – 40.5°C), followed by congestion of the mucous membranes. The animal becomes depressed, with dyspnoea, sweating and coughing a few hours prior to death. Some cases may also discharge copious amounts of frothy, serofibrinous fluid from the nostrils, which may occur only after death. Mortality is around 95%. Fully susceptible horses, such as foals that have lost their colostral immunity or unexposed horses, usually suffer from this form (House 1993, Mellor 1994, Coetzer & Guthrie 2004, Mellor & Hamblin 2004, Guthrie & Quan 2009). The subacute or cardiac form, otherwise referred to as "dikkop", has an incubation period of seven to fourteen days. Fever (38.5 – 39.5°C) and mucous membrane congestion are the initial signs, and later, subcutaneous and intermuscular oedema, particularly of the head and neck, occurs. Supraorbital swelling due to oedema is characteristic of the cardiac form. Petechiae may occur on the ventral surface of the tongue and conjunctivae and usually occur just prior to death. The presence of petechiae indicates a poor prognosis. Mortality is around 50%. The acute or "mixed" form is suspected to be the most common form, and presents with clinical signs of both the pulmonary and cardiac forms. "Horse sickness fever" form is a mild form with an incubation period of five to nine days. It is suspected to occur in partially immune animals with a low-grade fever and mild depression being the only noticeable symptoms. This form may be more prevalent as these mild symptoms are often not observed. Owners may not report these findings to a veterinarian, as the horse has usually recovered after being slightly "off" for a day or two, and they therefore do not seek veterinary attention. This is the most common form seen in zebras and donkeys, which are resistant to the development

of clinical signs (House 1993, Mellor 1994, Coetzer & Guthrie 2004, Mellor & Hamblin 2004, Kazeem et al 2008, Guthrie & Quan 2009).

2.4   Macroscopic pathology

On post mortem the outstanding feature is that of oedema. In the "dunkop" form there is marked pulmonary oedema, severe hydrothorax and petechiae. In the "dikkop" form the characteristic signs are that of a slightly yellowish, gelatinous oedema of the intermuscular connective tissues and subcutaneous tissues of the head and neck and it is often particularly severe around the ligamentum nuchae. A severe hydropericardium is often present. In the "mixed" form a mixture of the "dunkop" and "dikkop" changes are found (Kazeem et al 2008, Guthrie & Quan 2009).

2.5   Clinical pathology

Whilst the clinical signs and presentation of AHS have been well documented by Sir Arnold Theiler (1921), very little about the clinical pathology of the disease has been documented. Haematological abnormalities seen under experimental conditions include a leukopaenia (specifically a lymphopaenia and a neutropaenia), a thrombocytopaenia and an elevated haematocrit (Ht), red cell count (RCC) and haemoglobin (Hgb) concentration (Skowronek et al 1995). In the study carried out by Skowronek et al (1995) margination of leukocytes within pulmonary vessels and increased leukocyte numbers within capillaries were observed histologically and probably contributed to the leukopaenic state. Other contributors could be stress-induced corticosteroid release with subsequent lymphocyte sequestration and viral interference with leukocyte production (Latimer et al 2003). The polycythaemia seen

as an increase in Ht, RCC and Hgb, was most likely due to compromised endothelial cell barrier function and a subsequent shift in fluid from intravascular to extravascular compartments. Febrile dehydration may be a possible explanation for the polycythaemia. Haemostatic abnormalities included increased fibrin degradation products (FDPs), prolonged prothrombin, activated partial thromboplastin and thrombin clotting times (Skowronek et al 1995).

A thrombocytopaenia is a finding common to all forms of AHS (Skowronek et al 1995). There are four broad causes of a thrombocytopaenia: an increased consumption, sequestration, excessive loss or impaired production of thrombocytes (Latimer et al 2003). In AHS, increased consumption is the most likely cause of the thrombocytopaenia as thrombocytes adhere to the damaged endothelium seen in AHS (Laegreid et al 1992, Roth 1992, Segura, Monreal, Perez-Pujol, Pino, Ordinas, Brugues & Escolar 2006). This is not a feature unique to AHS as horses with other viral infections, such as equine Influenza show a decrease in thrombocyte count due to adherence of thrombocytes to damaged endothelium (Krumrych, Wisniewski & Dabrowska 1999). It is also postulated that vascular stasis resulting from haemoconcentration, as seen in experimental cases of AHS can also enhance thrombocyte binding. Disease outcome of AHS has been thought to be related to onset and duration of the thrombocytopaenia (Skowronek et al 1995).

In humans, many studies have shown the thrombocyte count to be of prognostic significance. Nijsten et al (2000) showed that non-survivors of certain surgeries had a lower rise in thrombocyte counts than survivors. The rate of change of the thrombocyte count over time from day two post-surgery onwards was shown to be much higher in survivors (Nijsten, ten Duis, Zijlstra, Porte, Zwaveling, Paling & The

2000). Strauss et al (2002) showed that mortality was higher in Intensive Care Unit (ICU) patients with a lower nadir in thrombocytopaenia and a drop in count greater than 30%. Some researchers have stated that the greater the decrease in thrombocyte count, the poorer the prognosis of the patient regardless of the absolute nadir of the thrombocyte counts (Vanderscheuren, De Weerdt, Malbrain, Vankersschaever, Frans, Wilmer & Bobbaers 2000). Others have stated that the lower the nadir, the poorer the prognosis (Stephan 1999). Most, however, agree that the daily change in thrombocyte count is a better prognostic indicator, although a thrombocytopaenia on its own at any given time is associated with an increased risk of mortality (Nijsten et al 2000, Akca, Haji-Michael, de Mendonça, Suter, Levi & Vincent 2002, Strauss, Wehler, Mehler, Kreutzer, Koebnick & Hahn 2002, Vandijk, Blot, De Waele, Hoste, Vandewoude & Decruyenaere 2010). It should be noted that in all the above cases, the patients died due to the effects of the disease causing the thrombocytopaenia, and not due to the thrombocytopaenia itself. So the thrombocytopaenia is a risk factor for mortality, rather than a cause of fatality in ICU patients. This may be extrapolated to AHS patients, as we know that horses with AHS rarely die from a thrombocytopaenia, but rather thrombocytopaenia is a symptom seen in horses with this disease. It is suspected that the thrombocytopaenia seen in AHS coincides with the increase in viral load, as seen in a recent vaccine trial done at the ERC (Guthrie, Quan, Lourens, Audonnet, Minke, Yao, He, Nordgren, Gardner & MacLachlan 2009).

In humans, thrombocyte aggregation at sites of inflammation may be responsible for impaired microcirculation and subsequent Multiple Organ Dysfunction Syndrome (MODS) and Acute Respiratory Distress Syndrome (ARDS) (Akca et al 2002). In

ARDS, a syndrome that has an outcome very similar to AHS, thrombocyte trapping occurs specifically in the lungs (Nijsten et al 2000, Akca et al 2002).

Thrombocyte numbers can be assessed by examination of a blood smear by a laboratory technician, or by an automated haematology machine (Byars, Davies & Divers 2003, Latimer et al 2003). The CELL-DYN® 3700 is a multiparameter automated haematology analyser used at the Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital. In this machine electrical impedance is used to measure the thrombocyte count as the cells traverse the aperture. It measures the changes in electrical current as the cell passes an aperture of known dimensions. A disadvantage with this technique is that two or more cells can pass through the aperture at the same time, and the machine will read this clump of cells as one cell. This will lead to a count that is falsely low (Anon. 2000). Thrombocyte clumping is a characteristic often seen with blood in EDTA tubes, which is the sample collection tube of choice for the CELL-DYN® 3700. With unexpectedly low thrombocyte counts one should confirm this count with a sodium citrate sample and manual counting (Byars et al 2003).

2.6   Control of AHS

The first live attenuated AHS vaccine was developed in the 1930's and has since been replaced by tissue culture attenuated vaccine viruses (Alexander & Du Toit 1934, House et al 1992, von Teichman & Smit 2008). Serotypes included in the vaccine are attenuated through multiple suckling mouse brains or cell culture passages followed by plaque purification to select mutants that are non-pathogenic but immunogenic (Erasmus 1966, von Teichman & Smit 2008). Although the vaccine

is relatively cheap, and on the whole provides adequate immunity, it has a number of limitations. It has the potential for variable attenuation and a possible lack of immunogenicity. There is a variable immune response in individual animals to each serotype, which may be due to over attenuation of certain serotypes, or interference between serotypes in the vaccine (Erasmus 1978, Coetzer & Guthrie 2004). There is also a possibility of reversion to virulence of the vaccine virus. The vaccine can interfere with laboratory diagnostic tests, as the vaccine virus cannot be distinguished from natural infection in the horse (House et al 1992, House 1993, MacLachlan, Balasuriya, Davies, Collier, Johnston, Ferraro & Guthrie 2007, Guthrie et al 2009).

A polyvalent cell culture attenuated vaccine is currently available commercially and in use from Onderstepoort Biological Products (OBP). Horses that have received three or more annual courses of vaccination are considered to be protected sufficiently (Erasmus 1978, von Teichman & Smit 2008, Guthrie & Quan 2009). Despite the rigorous use of vaccination to control AHS, cases of AHS occur annually in certain high-risk areas (including Onderstepoort). There are also anecdotal reports that vaccinated horses can become infected with AHSV without developing clinical signs of infection (Howell 1960). Such animals may provide a source of virus for vector midges and as such may well play a role in the spread of the disease if such animals are relocated during the infectious period.

The current vaccination schedule recommended by the manufacturers of the OBP polyvalent live attenuated vaccine is as follows (Guthrie 1996):

Initial vaccination: weanlings at approximately 6 months of age

Secondary vaccination: yearlings at approximately 12 months of age

Annual booster: All horses in high-risk areas should be vaccinated in late winter or spring (Sept-Nov).

The OBP polyvalent vaccine is made up of two components: AHS I and AHS II, with each component given at least 21 days apart. AHS I is a trivalent vaccine and contains serotypes 1, 3 and 4. AHS II is a quadrivalent vaccine, and contains serotypes 2, 6, 7 and 8. Serotype 8 provides cross-protection against serotype 5, and serotype 6 cross-protection against serotype 9 (von Teichman 2008). Although these vaccines should provide a theoretical lifelong immunity and a single dose should be sufficient, annual vaccination is advised to try to produce true polyvalent immunity in the face of variations in response to the vaccine. It has also been shown that wild-type virus infection appears to induce a more broadly cross-reactive immunity than administration of attenuated vaccine virus (Blackburn & Swanepoel 1988).

Inactivated vaccines have also been used in outbreaks of AHS in non-enzootic regions; however these vaccines are no longer available commercially. Inactivated vaccines have the advantage of being very safe, but require repeated immunisations because of loss of immunogenicity (Dubourget, Preaud, Detraz, Lacoste, Fabry, Erasmus & Lombard 1992, House 1993). Virus like particles (VLPs) produced from recombinant baculoviruses, a recombinant vaccinia vectored vaccine (Stone-Marschat, Moss, Burrage, Barber, Roy & Laegreid 1996) and a DNA vaccine, are among other vaccines that have been developed to combat AHSV infection in horses

(Guthrie et al 2009). VLPs are safe and have been shown to provide adequate protection experimentally, but have not been used in the field due to commercial production difficulties and cost as well as problems with long-term stability (Roy, Bishop, Howard, Aitchison & Erasmus 1996, Scanlen, Paweska, Veshoor & Van Dijk 2002, Guthrie et al 2009).

A recombinant canarypox-vectored vaccine against AHS serotype 4 has been developed recently (Guthrie et al 2009). It appears to be safe and produce solid protective immunity against this serotype, and has none of the disadvantages mentioned for live attenuated vaccines above. This vaccine was constructed using synthetic genes encoding the VP2 and VP5 proteins of AHSV-4 inserted into a recombinant canarypox virus vector.

Foals born to immune mares acquire passive immunity by ingestion of colostrum after birth. This immunity declines progressively as a result of catabolism of immunoglobulins to undetectable levels at four to six months of age (Alexander & Mason 1941, Piercy 1951, Coetzer & Guthrie 2004). Before immunity has declined, maternal antibodies interfere with production of antibodies in response to the vaccine. For this reason the foals are only vaccinated from six months of age.

Vaccination alone is not fully effective in the control of AHS. Managemental precautions need to be implemented as well. Stabling at night, especially from the time before sunset to after sunrise, is helpful, as this is the peak activity time for midges. The *Culicoides* spp. present in southern Africa do not readily enter buildings (Barnard 1997). Topical insect repellents and insecticides applied to the horse are

also effective. Control needs to be looked at as a holistic approach, rather than relying on one or the other control measures in isolation.

2.7   Diagnostic Techniques

Clinical signs, history and macroscopical lesions are usually sufficient to suggest a diagnosis of AHS; however some clinical signs and lesions are not specific for AHS. The clinical symptoms of AHS, especially the "horse sickness fever" form are very similar, if not identical to that of equine encephalosis virus (EEV). Some horses with EEV also exhibit the subcutaneous and intermuscular oedema, as well as the supraorbital swelling. The recumbancy and peracute death as well as the fever seen in cases of "dunkop" can be confused with the symptoms of West Nile virus (WNV). Terminal cases of WNV, however, usually exhibit typical neurological signs, which are not present in AHS. In some cases, pyrexia is the only symptom, and there are many differentials for this symptom. For these reasons laboratory confirmation is essential. A diagnosis can be obtained by identification of infectious virus, viral antigens or specific antibodies (Anon. 2008b). A number of such tests have been developed for the diagnosis of AHS.

2.7.1 Viral Isolation and Identification

Traditionally virus isolation with serotyping is used to provide a definitive diagnosis of AHS. Blood should be collected in heparin during the febrile stage, or lung, spleen and lymph node specimens collected at necropsy and kept at 4°C. High viral levels are usually found in the above organs of fatal cases (Coetzer & Guthrie 2004). Viral isolation may be obtained by inoculating the test sample onto a variety of cell cultures (e.g. BHK-21, Vero) or by intracerebral inoculation of suckling mice (Anon.

2008b). In cell culture, the cytopathic effect (CPE) of the virus is measured by refractivity and detachment of cells, and can appear two to eight days post-inoculation. Once it is clear that a virus has been isolated, it must be confirmed as AHSV, as the morphology and cytopathic effects of EEV are almost indistinguishable from AHSV (Crafford 2001). To a lesser extent the same can be said for WNV.

In this study, processed blood samples were inoculated onto a confluent monolayer of BHK-21 cells and incubated at 37°C. Cultures were observed daily for any CPE. When 100% of the cell monolayer showed CPE the cells and supernatant were harvested and identification was confirmed as AHSV by an indirect sandwich enzyme-linked immunosorbent assay (ELISA) (Hamblin, Mertens, Mellor, Burroughs & Crowther 1991). Cultures showing no CPE after three passages were classified as negative for AHSV (Quan, van Vuuren, Howell, Groenewald & Guthrie 2008).

ELISA tests make use of enzymes that have been coupled to antibody to mark immune complexes. The enzyme action on the substrate leads to a colour formation, which is quantifiable.

The following is a description of the indirect sandwich ELISA, as is performed by the Equine Virology Laboratory, Faculty of Veterinary Science, University of Pretoria for detection of AHSV (Hamblin et al 1991, Crafford 2001). Specific antibody (rabbit hyperimmune serum) is adsorbed to the ELISA plate. A test sample is added to the plate, allowing antibody–antigen complex formation. Antibody produced in a different host (guinea pig immune antiserum) is then added to the plate, which binds to the original antibody-antigen complexes allowing antibody-antigen-antibody complex formation. An enzyme-labelled species-specific antiserum (rabbit anti-guinea pig

antibody, conjugated to horseradish peroxidase) directed against the antibody of the second species is added. A chromogen (ortho-phenylene diamine) and substrate (0.05% H_2O_2) are added, which leads to a colour change in the presence of antibody-antigen-antibody-enzyme conjugate complexes. The reaction is stopped by adding H_2SO_4 after 5 – 10 minutes, and the absorbance value is measured spectrophotometrically. The plate is washed removing any unbound reagents and incubated at prescribed temperatures after each step. The cut-off value is the absorbance value obtained from the negative control. Any samples with an absorbance value lower than the cut-off are regarded as negative. Test samples with values greater than the cut-off are regarded as positive.

2.7.2 Virus Serotyping

Serotyping is done using a plaque inhibition neutralization test using type specific antisera. It can be used to identify unknown viruses with the use of a positive, known serum. It can be used to find the serotype of a virus that consists of several distinct serotypes, as is the case with AHSV (Anon. 2008b). Electrical insulating fish-spine beads filled with type-specific antiserum (produced in sheep) are used to indicate virus-antibody neutralisation on Vero cell monolayers inoculated with the test sample (Porterfield 1960). The fish-spine beads are placed on the surface of the inoculated Vero monolayer. A neutralisation zone around the bead is indicated by an absence of plaque formation. (Quan et al 2008).
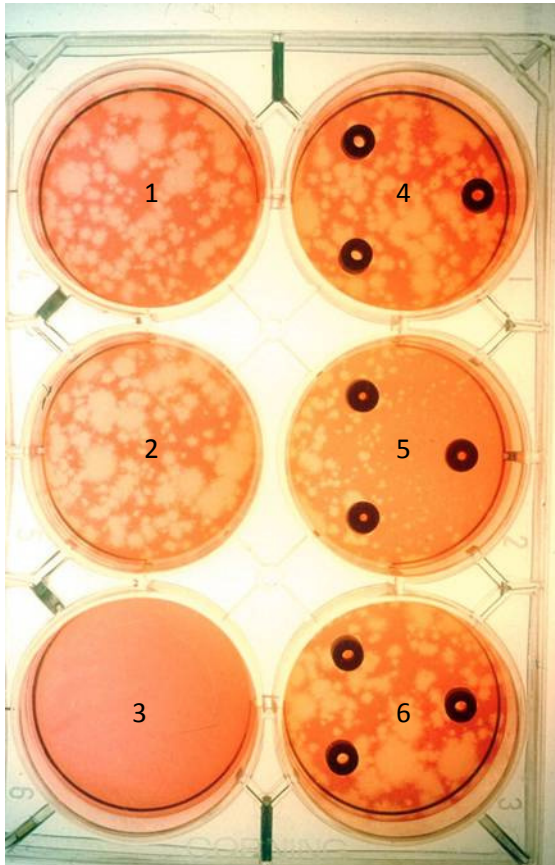
Figure 2. The plaque inhibition neutralization test: wells numbered 1-3 are the controls. Wells numbered 1 & 2 show viral plaque growth (multifocal pale areas) and well 3 is the negative control with no inoculated virus on a monolayer of Vero cells. Wells numbered 4-6 show monolayers with fish-spine beads (three beads per well) with antiserum to a different serotype in each bead. Well 5 shows a zone of inhibition around the right most bead where antibody neutralisation of AHSV has occurred.

2.7.3 Group-Specific Serology

Group specific antibodies can be detected using complement fixation tests (CFT), agar gel immunodiffusion (AGID) tests, indirect immunofluorescent antibody (IFA) tests and ELISA tests.

The CFT uses an immunological indicator system (a haemolytic system) to detect immune complex formation, and therefore group specific antibodies. The haemolytic system consists of red blood cells (RBC), normally harvested from sheep, and anti-red blood cell hyperimmune serum (rabbit serum). The test uses the concept that RBC's in the presence of hyperimmune serum will form immune complexes, which will trigger the complement cascade leading to lysis of the RBC's, which is a visible reaction.

The first step of the test is to incubate a known antigen (sucrose/ acetone extract of AHSV-infected mouse brain) with test and control (uninfected mouse brain) samples. Immune complexes form. Complement in the form of guinea pig serum is added. If immune complexes are present, the complement will be consumed. The haemolytic system is then added. If no complement is left, i.e. the immune complexes formed in step one, then no haemolysis will occur. If no immune complexes formed, then the haemolytic system will trigger the complement cascade, and haemolysis will occur (McIntosh 1956, Anon. 2008b).

The CFT detects primarily IgM antibodies, and is therefore ideal for detection of the primary antibody response (Crafford 2001). However some sera may have an anti-complementary effect and the use of this test is becoming less popular.

AGID tests utilise precipitation reactions in a semisolid medium. Through diffusion, optimal concentrations of antigen and antibody are brought together, and when the concentration of the complexes exceed that of the gel's capacity, the complexes precipitate out and form visible bands (Hamblin, Graham, Anderson & Crowther 1990, House, Mikiciuk & Berniger 1990). The antigen used in this test is crude and

non-specific lines are often obtained which can interfere with accurate diagnostics (House et al 1990).

The indirect IFA utilises a standardised antigen and fluorescent antiglobulin to determine the presence of a specific antibody. The fluorescent antiglobulin binds to antigen-antibody complexes and fluoresces. Non-specific fluorescence, especially in vaccinated horses, occurs often, which may make interpretation of results difficult and lead to false positive results (House et al 1990). For this reason, the test is better used as a screening test.

An indirect ELISA using either soluble AHSV antigen or a recombinant VP7 protein can be used. These assays detect IgG antibodies to either the soluble antigen or the VP7 protein. Compared to other assays the indirect ELISA using recombinant AHSV VP7 antigen, which is conserved among serotypes (Mellor & Hamblin 2004, Guthrie & Quan 2009), is more sensitive in detecting early immunological responses. It can be used to detect declining levels of maternal antibodies and has a good specificity (Maree & Paweska 2005). Other advantages of the use of VP7 are that it is stable and not infective (Anon. 2008b). It is considered a good screening test.

To perform the test antigen (AHSV recombinant VP7 protein) is adsorbed onto the ELISA plate. The test sample is added and antigen-antibody complexes form. An enzyme-conjugate (recombinant protein G conjugated with horseradish peroxidase) is added which binds to antigen-antibody complexes. The chromogen and substrate (tetra-methylbenzidine peroxidase substrate) is added, which leads to a colour change in the presence of antigen-antibody-enzyme complexes. The reaction is stopped by adding H_2SO_4 after 5 – 10 minutes, and the absorbance is measured

spectrophotometrically. The plate is washed removing any unbound reagents and incubated at prescribed temperatures after each step. The cut-off value is the absorbance value obtained with the negative control. Any samples with an absorbance value lower than the cut-off are regarded as negative. Test samples with values greater than the cut-off are regarded as positive (Crafford 2001, Maree & Paweska 2005).

A competitive ELISA is available for the detection of group-specific antibody (Hamblin et al 1990). This standardised assay measures the inhibition of guinea pig antisera (Crafford 2001). Antigen (BHK-21 infected with AHSV) is adsorbed onto the ELISA plate. The test sample and a known, specific, control antibody (guinea pig anti-AHS serum) is added. The test serum antibodies compete with the control antibodies for binding sites on the antigen. An enzyme-conjugated antiserum (rabbit anti-guinea pig horseradish peroxidase) directed against the control antibody is then added. Chromogen and substrate are added leading to a colour reaction. The plate is washed removing any unbound reagents and incubated at prescribed temperatures after each step. A colour reaction occurs in the presence of control antigen-antibody complexes. The weaker the change in colour the more test antigen-antibody complexes have formed.

2.7.4 Serotype-Specific Serology

Serotype specific antibodies can be detected using a serum neutralisation test (SNT). This test can be used to detect the presence of specific antibodies and to determine antibody titres in sera. Stock virus for each serotype are inoculated onto Vero cell monolayers in microtitre plates and serial dilutions of the test serum are

added and incubated for four to five days until CPE is noted. The presence of specific antibodies in the test serum inhibits the production of CPE. The antibody titres are recorded as the reciprocal of the highest final dilution of serum that provided at least 50% protection of the Vero cell monolayer. A titre greater than 10 indicates the serum is positive for that AHSV serotype antibodies. A four-fold increase in paired sample titres indicates seroconversion (Guthrie et al 2009). A disadvantage to this test is that as this test is serotype-specific, it cannot identify new serotypes (Crafford 2001).

It is important to note that all serological assays have a limited value for diagnosis of active infection, as many horses may die of AHS before a significant antibody response is noted (Rodriguez et al 1992, Sailleau et al 1997).

2.7.5 PCR

One of the disadvantages of viral isolation and viral neutralisation tests for serotyping is that it usually takes a minimum of two weeks to obtain a result (Hamblin et al 1991, Sailleau, Hamblin, Paweska & Zientara 2000) and this time constraint can be costly in the face of an outbreak situation, where a rapid, sensitive, specific and reliable test is needed. A number of polymerase chain reaction (PCR) assays have been developed for the detection of AHSV targeting the VP3 (Aradaib, Mohemmed, Sarr, Idris, Ali, Majid & Karrar 2006), VP7 (Zientara, Sailleau, Moulay, Plateau & Cruciere 1993, Zientara, Sailleau, Moulay, Wade-Evans & Cruciere 1995a), NS1 (Mizukoshi, Sakamoto, Iwata, Ueda, Kamada & Fukusho 1994), NS2 (Stone-Marschat et al 1994) or NS3 (Zientara, Sailleau, Moulay & Cruciere 1995b) genes. These tests have the potential to be rapid, sensitive and versatile, and can

supplement the older conventional methods. They can be used on specimens that do not contain live virus (Coetzer & Guthrie 2004, Agüero, Gómez-Tejedor, Cubillo, Rubio, Romero & Jiménez-Clavero 2008), that contain attenuated strains or strains of low virulence (Sailleau et al 1997) and can be used for earlier detection of viraemia than viral isolation (Stone-Marschat et al 1994). However none of these assays have been optimised for the detection of AHSV in blood from naturally infected horses (Quan et al 2010). The majority of AHSV assays are based on visualisation of the PCR product on agarose gel. Advantages of a real-time PCR over a gel-based PCR are that the real-time has a greater analytical sensitivity and specificity, is quicker to perform, and has a smaller potential for contamination (Hoffmann, Depner, Schirrmeier & Beer 2006, Quan et al 2010). A RT-qPCR has been developed recently in the Equine Research Centre (ERC) laboratory, University of Pretoria. This assay has been applied to samples collected from clinical field cases of AHS and has been applied to serial samples collected from animals experimentally infected with AHSV. This assay is unique in that it is designed from sequences of current circulating field virus strains (Quan et al 2010).

This RT-qPCR assay was used in this prospective study to quantify the AHSV viraemia. In brief, nucleic acid was extracted from 100 µl of blood and then subjected to RT-qPCR using specific primers and probes for the genes coding for the VP7 protein of AHSV. RNA purification was done using MagMAX™ Viral RNA Isolation kits (Ambion) and a Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) according to a protocol supplied by the manufacturer of the kit (Appendix 1). The sample underwent a lysis step and two washing steps and was finally eluted in 80 µl elution buffer. The eluate together with primers and probes (targeting AHSV VP7

genes and Xeno RNA) was heat-denatured. VetMax[TM]-Plus Multiplex One-Step RT-PCR kit (Lifetech) reagents were then added and a one step RT-qPCR performed on a StepOnePlus™ Real-Time PCR System (Lifetech) using a protocol as recommended by the manufacturer. This assay is a rapid, analytically sensitive and specific assay for detection of AHSV RNA (Quan et al 2010).

The Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) uses magnetic rods to collect the magnetic beads included in the MagMAX™ Viral RNA Isolation kit (Ambion) and enables high throughput RNA isolation. Traditional methods, such as phenol: chloroform extraction, are time consuming and difficult to automate and perform. High throughput techniques use glass fibre filters or magnetic beads. The magnetic beads provide higher purity RNA with more consistent recovery (Fang, Willis, Burrell, Evans, Haang, Xu & Bounpheng 2007). A guanidinium thiocyanate-based solution is used to solubilise cell membranes and inactivate nucleases, releasing the viral RNA. The magnetic beads with nucleic acid binding surfaces are then added, which bind the nucleic acid. The beads and RNA are captured on the magnets and washed to remove cellular debris and proteins. They are then washed again to remove residual binding solution. The RNA is then eluted in a low salt buffer solution, which releases the RNA from the magnetic beads (Anon. 2008a).

Xeno RNA control (Lifetech) is included as an internal control for the RT-qPCR, which undergoes independent amplification, increasing reliability of the test. A positive result for Xeno RNA implies that extraction of the RNA has been completed successfully and that no PCR inhibitors are present (Hoffman et al 2006). The inclusion of this internal control provides a method to identify false negative results due to unsuccessful extraction or the presence of PCR inhibitors. PCR inhibitors

include substances such as the porphyrin ring of haem found in haemoglobin of red blood cells, heparin and chelating agents such as EDTA (Barker, Trairat Banchongaksorn., Courval, Suwonkerd, Rimwungtragoon & Wirth 1992). These inhibitors are important to note, as the samples submitted for the RT-qPCR are generally either heparin or EDTA blood samples.

This RT-qPCR is currently being used routinely in conjunction with viral isolation and antigen-capture ELISA for AHS detection in the Equine Virology Laboratory, University of Pretoria.

CHAPTER
3. A prospective study on African horse sickness in naturally infected horses

3.1   Abstract

In this study, which started in February 2009, serum and EDTA blood samples were collected and rectal temperatures recorded weekly from approximately 50 Nooitgedacht horses resident in open camps at the Faculty of Veterinary Science, University of Pretoria at Onderstepoort. The horses ranged in age from foals a few days old at the start of the project, to horses 26 years of age. The EDTA samples were tested for the presence of AHSV dsRNA by a recently developed RT-qPCR (Quan et al 2010). Positive samples were evaluated further by measuring haematological parameters (specifically thrombocyte numbers). The clinical picture of the AHSV-positive horses was recorded, rectal temperatures monitored daily and clinical cases treated at the Onderstepoort Veterinary Academic Hospital (OVAH) with a standard protocol. In this study, it was shown that 12% of vaccinated horses in an AHS endemic area were infected subclinically with AHSV. The potential impact of such cases in the epidemiology of AHS warrants further investigation.

Keywords: AHS, RT-qPCR, thrombocytopaenia, subclinical AHS

3.2   Introduction

AHS is the most devastating of all equine diseases with a mortality rate of up to 90% in naïve horses (Guthrie & Quan 2009). Due to the nature of AHS, extremely strict quarantine measures are applied to horses or other equids to be exported from AHS-infected areas. AHS, therefore, has a severe negative economic impact on the

southern African equine industries. Foals born to immune mares acquire passive immunity by ingestion of colostrum after birth. Antibodies against AHSV decline progressively to undetectable levels by four to six months of age (Coetzer & Guthrie 2004). The clinical signs and presentation of infection with AHSV were documented comprehensively by Sir Arnold Theiler (Theiler 1921). The disease is characterised by pyrexia, oedema of the lungs, pleura and subcutaneous tissues, as well as petechiae and haemorrhages (Kazeem et al 2008). The incubation period is, on average, five to seven days in experimental cases (Coetzer & Guthrie 2004).

Sir Arnold Theiler established his laboratory at Onderstepoort in 1908 due to the occurrence of AHS in this area every year. It has been shown subsequently that there is an extremely large population of *C. imicola* in the area (Meiswinkel 1998). Whilst horses that are kept permanently at the Faculty of Veterinary Science of the University of Pretoria situated at Onderstepoort are vaccinated every year against AHSV, clinical cases of AHS still occur within this herd every year, and the area is notorious for the number of deaths that occur each year as a result of AHS.

In 2006, an outbreak of AHS occurred in the AHS protection zone in Robertson, Western Cape (ProMED-mail 2006). It was suspected that a subclinical, viraemic horse that had been transported from Gauteng into this area was the source of this outbreak. This outbreak emphasised the need for field studies of AHS infection in order to get a better understanding of the dynamics of the virus in vaccinated horses.

The recent development of a RT-qPCR assay (Quan et al 2010), that is capable of detecting viraemia prior to the development of any clinical signs, or possibly in the absence of clinical signs of AHS, provides us with a method for the early detection of

AHS cases. To date use of PCR has only been reported in experimental studies of AHS. This assay has been characterised using field strains of AHSV. The prospective study described here allowed us to identify cases of AHS earlier than other techniques currently available, and then to follow the viral dynamics, clinical, clinical pathological and serological responses of these horses to natural infections with AHSV. It was anticipated that these data would reveal which factors determine if vaccinated horses that were exposed to natural challenge with AHSV would develop clinical disease, or would only be subclinically infected.

The aim of our study was to follow a herd of horses in an AHS endemic area longitudinally during the AHS season. We aimed to study the dynamics of the virus in naturally infected horses under field conditions to establish if subclinical cases of AHS occur naturally under field conditions. We also aimed to document the dynamics of thrombocyte counts and RT-qPCR results in AHS cases.

3.3   Materials and Methods

Horses from the Onderstepoort Teaching Animal Unit (OTAU) herd (± 50) were used in this study (see Appendix 2). The AHS vaccination records for each horse included in the study were obtained prior to the commencement of sample collection and vaccination was done according to the OTAU protocol (see Appendix 3). All horses in the study, excluding newborn foals, had been vaccinated at least once with the OBP polyvalent vaccine. Rectal temperatures were recorded with a digital thermometer and EDTA and serum blood samples collected by jugular venepuncture from each horse on a weekly basis during the 2008/2009 and 2009/2010 summer seasons. The EDTA sample was tested for the presence of AHSV dsRNA with a

real-time RT-PCR (Quan et al 2010) and the serum was stored at -20℃ until needed. Samples were classified as positive on RT-qPCR if the fluorescence exceeded the threshold of 0.1 within a maximum of 40 cycles.

Nucleic acid was extracted from 100µl of whole blood using the MagMAX™ Viral RNA Isolation kit (Ambion) and a KingFisher 96 Magnetic Particle Processor (Thermo Scientific) according to the manufacturers protocol (Appendix 1). One hundred µl of whole blood was spiked with Xeno RNA control (Lifetech). Five µl of eluate was denatured together with the primers and probes (targeting AHSV VP7 genes and Xeno RNA) in a final volume of 10µl for one minute at 95ºC. The mixture was then kept on ice while VetMax™-Plus Multiplex One-Step RT-PCR kit (Lifetech) reagents were added. A one step RT-qPCR was then performed on a StepOnePlus™ Real-Time PCR System (Lifetech) using a final primer and probe concentration of 400nM and 180nM respectively, and a protocol recommended by the manufacturer.

Additional EDTA and heparin samples were collected, and the temperature and basic clinical symptoms for each horse recorded, on a daily basis for any horse that tested positive for AHSV in the routine RT-qPCR tests. Viral isolation on BHK-21 tissue culture was attempted from the heparinised blood samples (Quan et al 2008). A complete blood count was done at the Clinical Pathology Laboratory, OVAH, using the CELL-DYN®3700 multiparameter automated haematology analyser (Veterinary setting), and AHSV dsRNA levels determined by RT-qPCR from the EDTA sample. Serotype specific antibody to AHS was determined using serum neutralisation tests with appropriate reference antigens in microtitre plates as described for EEV antibody surveys (Anon. 2008b, Howell, Nurton, Nel, Lourens, Guthrie 2008).

Twofold dilutions (initial dilution of 1:5) of each test serum were incubated with $100TCID_{50}/100µl$ of each reference AHSV antigen (serotypes 1-9), added in equal volume to the serum dilutions. Samples were incubated at 37ºC (±2ºC) in 5% CO_2. A Vero cell suspension of 480 000 cells per ml was added in a volume of 80µl to each well. Plates were then incubated at 37ºC in 5% CO_2 for four to five days and CPE was recorded daily. The end-point was taken as the dilution at which the virus showed 50% CPE of monolayers inoculated with the dilution of the back titration of the virus representing $100TCID_{50}$. A serum neutralisation titre of ≥10 was considered positive, whereas a fourfold increase in titre was interpreted as a seroconversion to that specific serotype. Serogroup specific antibodies to AHS were determined using an indirect ELISA that detects antibodies to the VP7 core protein that is common to all AHSV serotypes, as described previously (Maree & Paweska 2005). The collection of samples ranged from approximately four weeks before to four weeks after the date that the horse initially tested RT-qPCR positive.

The duration of sampling from RT-qPCR positive animals was as follows: for non-pyrexic animals (i.e. temperatures not exceeding 39 °C at any sampling time), blood samples were collected and data recorded daily for five consecutive days. Thereafter, sampling was reduced to every Monday and Thursday until three consecutive samples were negative for AHSV by RT-qPCR or 30 days after the original positive sample was taken. For pyrexic animals (i.e. temperatures exceeding 39 °C at any sampling time), blood samples were collected and data recorded daily for five consecutive days after the drop of the temperature below 39 °C. Thereafter, sampling was reduced to every Monday and Thursday until three consecutive

samples were negative for AHSV by RT-qPCR or 120 days after the original positive sample was collected.

Heparin samples were only collected for the initial five consecutive days from both pyrexic and non-pyrexic horses. EDTA samples were only collected until the thrombocyte count had normalised, i.e. platelet counts above 117×10^9/L (Blood & Studdert 1999). Treatment of AHS positive cases showing clinical symptoms was done according to the OTAU and OVAH protocols.

3.4 Results

Of the 50 horses sampled during 2009 and 2010, seven tested positive for AHSV during the 2009 summer, and two during the 2010 summer. Of the nine laboratory AHSV positive cases, two were moderate clinical cases of "dikkop". One of these horses, "Kotie", a three-year-old mare, presented during the weekly sample collection with a fever of 40 °C, a thrombocyte count of 44.5×10^9/L and a RT-qPCR cycle threshold (C_T) value of 25.72 (a negative value being > 40) (Figure 3). During the next three days, moderate periorbital swelling developed, together with a mild increase in respiratory effort and a minimum thrombocyte count of 9.3×10^9/L was obtained. These symptoms subsided gradually over two weeks together with a decrease in viraemia and the thrombocyte count normalised six days after detection of AHSV dsRNA in the blood. AHSV dsRNA was present in the blood on RT-qPCR for more than 130 days after the spike in temperature.
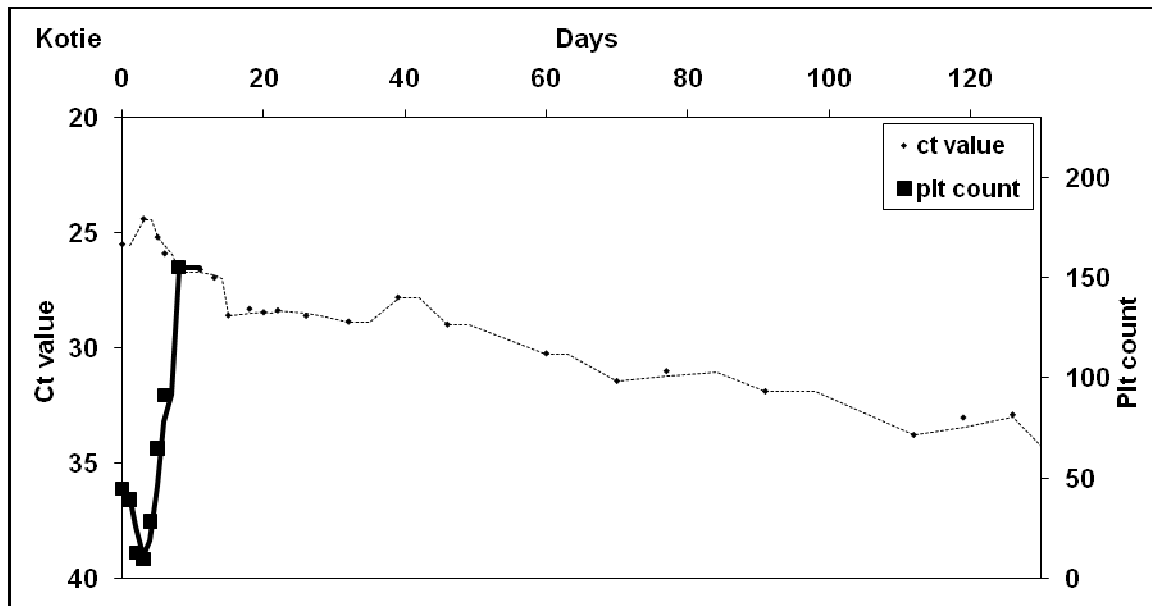
Figure 3. "Kotie": Temporal trend of viral load (C_T value) and thrombocyte count (Plt count – $\times 10^9$/L) after natural infection with AHSV.

"Jenny", a two-year-old mare, was a moderate clinical AHS case. She tested positive on RT-qPCR with a minimum C_T value of 26.96 over the next week and remained positive for more than 130 days (Figure 4). She was undergoing post-operative treatment for a coffin joint penetrating wound in the OVAH at the time she presented with AHS. She had a thrombocyte count of 42×10^9/L on the first day of sampling, which increased over the following four days until it was in the normal range. She developed similar symptoms to "Kotie" over a comparable time period.
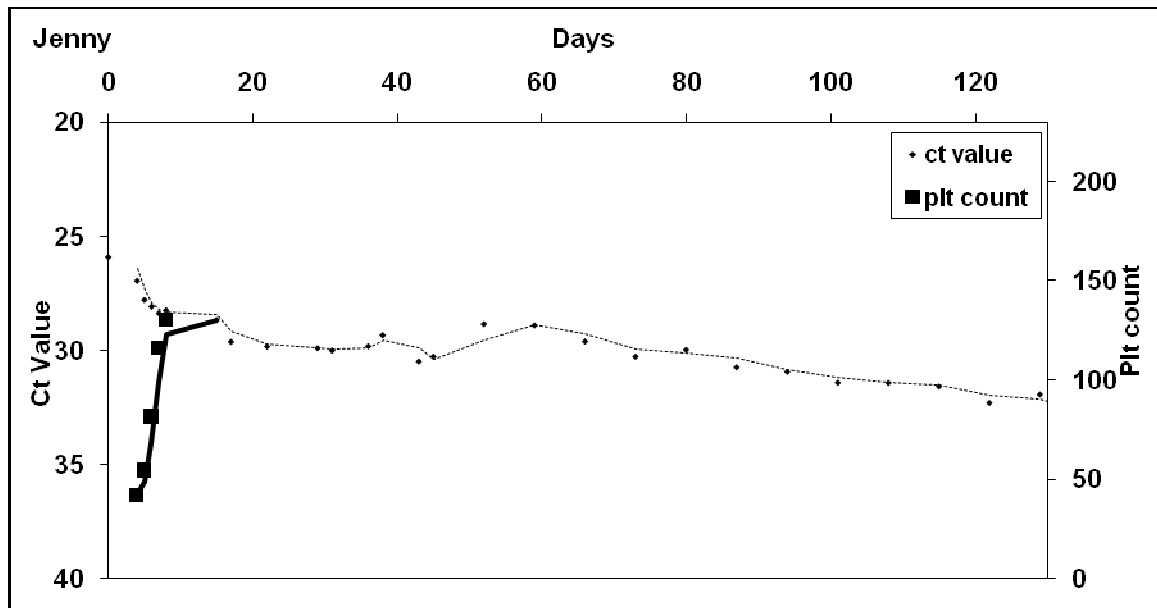
Figure 4. "Jenny": Temporal trend of viral load (C_T value) and thrombocyte count (Plt count – $\times 10^9$/L) after natural infection with AHSV.

One foal, "FoalV227", suffered from the "dunkop" form of AHS. A C_T value of 18.51 was obtained during the weekly sample testing even though the foal showed no clinical abnormalities at the time of collection (Table 1). The following day it was found collapsed in the camp showing severe dyspnoea, cyanotic mucous membranes and seizuring. It was euthanased later that day due to a lack of response to treatment. This foal had a thrombocyte count of 122×10^9/L on the day it was euthanased.

Table 1. "FoalV227": Thrombocyte count, C_T value and temperature values of a peracute case.

Sample Number	Collected	C_T	Plt Count $\times 10^9$/L	Temp
CW4113	30/04/2009	18.51		38.1
CWC4101	01/05/2009	19.34	122.0	37.3

There were two mild clinical cases, most likely the "horse sickness fever" form of the disease. The clinical signs observed were a slight increase in average body temperature, mild supraorbital swelling, and a drop in thrombocyte count. Analysis of samples from "Cazza" (Figure 5), a four-year-old mare, showed a minimum thrombocyte count of 57×10^9/L and a minimum C_T value of 26. She was RT-qPCR positive for 28 days. Analysis of samples from "Demi", a four-year-old mare, showed a minimum thrombocyte count of 64×10^9/L, and a minimum C_T of 27. She remained RT-qPCR positive for more than 60 days (Figure 5). The habitus of these two horses remained normal throughout this period, and these cases would probably not have been detected under field conditions.
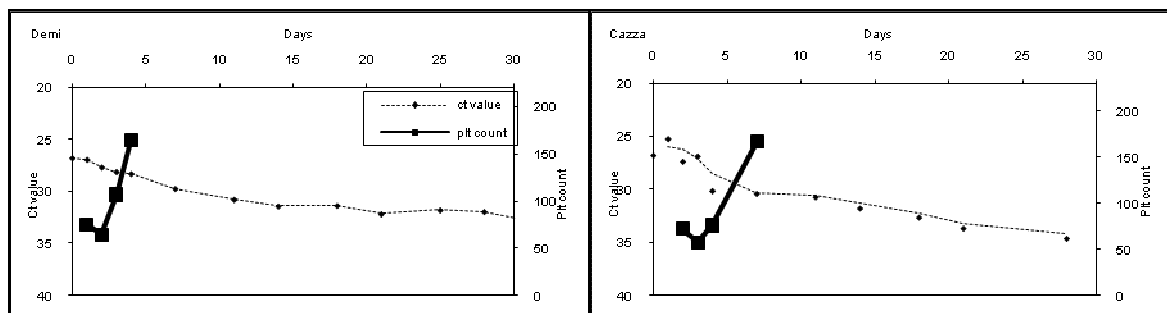
Figure 5. "Demi" and "Cazza" which presented as mild clinical cases of AHS. Temporal trend of viral load (C_T value) and thrombocyte count (Plt count – $\times 10^9$/L) after natural infection with AHSV.

There were four subclinical cases: "Zellie", "Rubilee" (Figure 6), "Jane" and "FoalV236". These horses showed no rise in rectal temperatures and their thrombocyte counts did not drop below the normal levels. They showed no change in their habitus, and no other clinical abnormalities. They remained real-time RT-qPCR positive for at least 14 days, but were negative by day 30.

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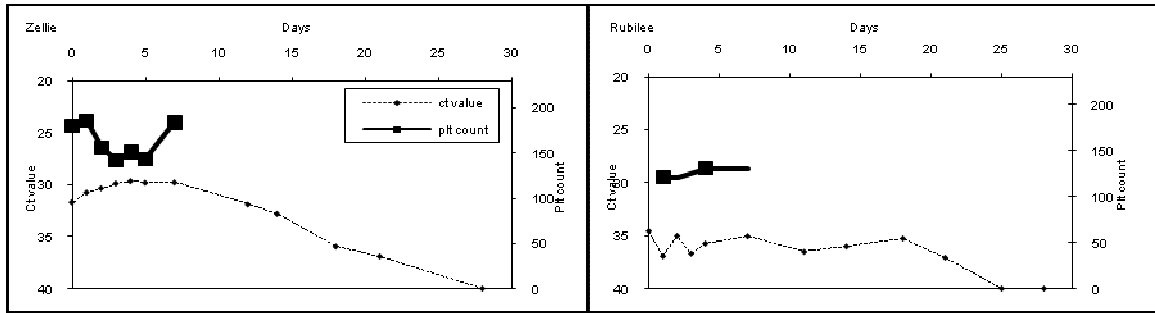
Figure 6. "Zellie" and "Rubilee" which presented as subclinical cases of AHS. Temporal trend of viral load (C_T value) and thrombocyte count (Plt count – $\times 10^9$/L) after natural infection with AHSV.

The changes in haematology results, besides thrombocyte count, did not reveal any consistent pattern. As seen in Table 2 below, most of the RT-qPCR positive cases suffered from a very mild absolute neutropaenia lasting for no more than one day. The exceptions were "Kotie", who had a concurrent *Theileria equi* infection, and "Zellie". A differential neutrophil count was not done. The CELL-DYN® 3700 was chosen due to its ease of use and speed in obtaining results. The ultimate goal was to get a fairly accurate thrombocyte count, which could be done using the CELL-DYN® 3700, which does not give a differential neutrophil count. The lymphocyte counts of all the horses, with the exception of "Kotie", were above the normal limit provided by the Clinical Pathology Laboratory. "Kotie" was lymphopaenic on day one of sampling, which resolved by day two, and had become a lymphocytosis by day seven, similar to the other horses.

Table 2. Haematology results from horses naturally infected with AHSV: cycle threshold (C_T) values, Haematocrit (Ht), Haemoglobin (Hgb), Red cell count (RCC), Neutrophil count (Neut), Lymphocyte count (Lymph) and temperature of each horse. (Normal values: C_T value: negative = 40; Ht = 0.24-0.44 L/L; Hgb = 80–140 g/L; RCC = $5.5\text{-}9.5 \times 10^{12}$/L; Neut = $3.54\text{-}7.08 \times 10^{9}$/L; Lymph = $1.8\text{-}3.6 \times 10^{9}$/L; Temp = < 39 ºC).

Horse Name	Days	C_T	Ht (L/L)	Hgb (g/L)	RCC($\times 10^{12}$/L)	Neut($\times 10^{9}$/L)	Lymph($\times 10^{9}$/L)	Temp(ºC)
Cazza	0	26.74						38.1
	1	25.17				5.02	3.13	38.7
	2	27.33	0.28	133	6.29	4.07	2.84	38.1
	3	26.84	0.28	103	6.31	3.28	3.31	36.8
	4		0.26	100	6.04	4.11	4.10	37.9
	7		0.29	108	6.60	6.16	4.74	36.5
Demi	0	26.85						38.6
	1	26.99	0.26	98	5.95	3.66	3.66	37.5
	2	27.71	0.25	94	5.77	4.16	3.64	37.0
	3	28.15	0.27	99	6.08	3.48	4.81	37.2
	4	28.33	0.24	92	5.52	5.49	4.22	37.5
Jane	0							37.6
	1	35.96	0.33	122	8.19	3.54	6.97	37.2
	2		0.31	110	7.57	3.49	4.54	37.6
	3		0.29	106	7.21	4.53	4.50	37.3
	4		0.30	110	7.51	4.50	4.31	37.0
Kotie	0		0.30	109	6.65	3.49	1.27	40.0
	1		0.29	108	6.58	2.46	3.20	38.3
	2		0.30	113	6.81	1.81	2.66	37.0
	3	24.40	0.33	120	7.36	1.87	3.37	37.2
	4		0.28	108	6.37	3.36	2.85	37.0
	5	25.20	0.28	102	6.19		3.68	37.2
	6	25.91	0.26	89	5.51	5.20	3.73	37.5
	7							37.4
	8	26.71	0.22	82	4.98	7.44	4.77	37.5
Rubilee	0	34.48						37.9
	1	36.90	0.29	105	6.71	3.26	9.14	37.0
	2	34.94						37.5
	3	36.65						37.5
	4	35.69	0.27	97	6.23	3.63	8.81	37.3
Jenny	0	26.96	0.23	76	5.33	4.91	4.21	37.8
	1	27.80	0.19	65	4.63	5.80	3.54	37.9
	2	28.09	0.19	70	4.61	5.52	5.75	37.3
	3	28.38	0.19	63	4.38	5.71	4.92	36.4
	4	28.25	0.21	72	4.95	6.44	5.81	37.9
Zellie	0	31.69	0.37	126	7.73	1.65	2.86	37.2
	1	30.77	0.34	117	7.08	2.86	2.73	38.3
	2	30.35	0.32	115	7.11	3.35	2.47	38.9
	3	29.89	0.29	105	6.04	2.47	2.14	38.1
	4	29.66	0.36	121	7.54	2.10	3.33	37.8
	5	29.81	0.32	105	6.62	2.46	2.37	37.3
	7		0.35	117	7.36	2.82	2.96	36.9
FoalV236	0	30.18						38.1
	1		0.30	113	9.39	4.80	8.05	37.8
	2	32.67						38.2
	3	33.71						37.5
	4		0.35	125	10.47	4.13	7.29	37.3
FoalV227	0	18.51						38.1
	1	19.34	0.43	152	11.50	7.18	5.75	37.3

"Jenny" and "Kotie" were the only two horses whose haematocrit values were outside the normal range. "Kotie" had a haematocrit value of 22 L/L on day seven, which is in the low normal range, but "Jenny" was anaemic for a few days with her haematocrit value dropping to a minimum of 19 L/L. No cause for the anaemia could be found. Shortly before "FoalV227" was euthanased, haematology values of 11.5×10^{12}/L RCC, 152 g/L haemoglobin concentration and 0.43 L/L haematocrit were obtained from his/her blood samples. This was the only case of the RT-qPCR positive horses that had a polycythaemia.

Of the nine RT-qPCR positive cases, only two had a recorded temperature greater than 39.0°C. "Kotie" and "Jenny" had a fever of 40.0°C and 40.1°C, respectively. The rest showed mild increases in rectal temperature, but all remaining below 39.0°C. The temperatures fluctuated from day to day, as seen in Figure 7, and no correlation with the C_T value was evident.
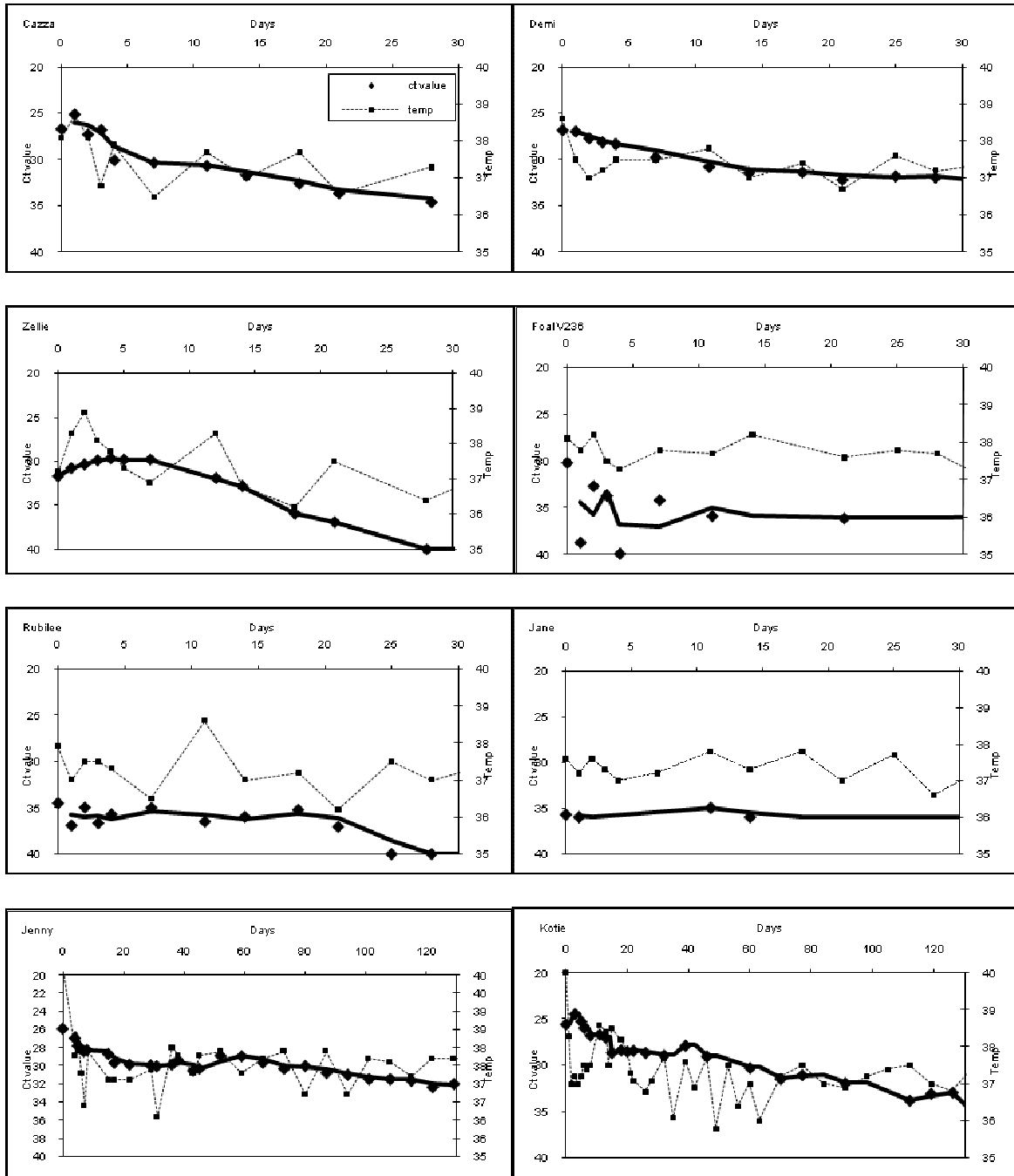
Figure 7. Temporal trend of C_T values and rectal temperatures of horses naturally infected with AHSV.

No isolates were obtained for any of the nine positive cases, and therefore no serotype could be obtained by the plaque inhibition test.

The results obtained from the SNTs, showing the serotype specific antibody response, and indirect ELISAs, showing the group specific antibody response, are shown in Table 3. Serial samples were submitted from each RT-qPCR positive case. The highlighted sample of each horse in the table indicates the sample that was taken at the time that the horse first tested RT-PCR positive. A titre greater than or equal to 2.5 was considered positive for AHSV antibodies.

Table 3. Serum neutralisation test and indirect ELISA (group specific) test results for RT-qPCR positive horses. These results are from serial serum samples collected before and after the horses were shown to be RT-qPCR positive. (A titre ≥ 2.5 is considered positive on indirect ELISA). The highlighted sample of each horse in the table indicates the sample that was taken at the time that the horse first tested RT-PCR positive.

Sample number	Date	Horse	Serum neutralization test results									I-ELISA
			AHS 1	AHS 2	AHS 3	AHS 4	AHS 5	AHS 6	AHS 7	AHS 8	AHS 9	
1	2/4/2009	Cazza	80	28	112	112	0	640	224	112	112	34.3
2	16/4/2009		224	28	56	160	0	640	448	160	112	34.8
3	30/4/2009		321	224	448	112	10	896	1792	320	896	49.4
4	14/5/2009		1280	3584	224	448	224	1280	2560	1792	896	53.8
5	28/5/2009		2560	1792	160	320	160	1280	1280	1792	640	58.2
6	19/3/2009	Demi	448	112	160	80	0	320	3584	320	224	28.3
7	2/4/2009		1280	160	320	80	0	320	7168	320	224	23.1
8	16/4/2009		1280	224	320	1280	28	320	7168	448	448	42.8
9	30/4/2009		320	80	320	640	20	448	2560	160	320	40.0
10	14/5/2009		896	320	320	10240	80	1280	>20480	640	640	55.6
11	19/3/2009	Jane	160	80	448	112	0	896	640	160	160	44.2
12	2/4/2009		640	80	56	80	0	224	640	112	160	39.6
13	16/4/2009		160	160	112	112	0	448	640	160	224	46.7
14	30/4/2009		1280	320	160	640	224	1280	7168	1792	1280	54.7
15	14/5/2009		448	224	448	112	0	448	896	160	320	37.4
16	26/2/2009	Kotie	112	40	28	56	0	80	80	56	40	15.3
17	12/3/2009		56	20	28	56	0	80	80	80	28	14.9
18	26/3/2009		112	40	40	56	0	56	112	56	40	18.2
19	9/4/2009		3584	5120	896	640	160	2560	2560	640	640	41.2
20	23/4/2009		2560	10240	896	640	224	3584	7168	1280	896	44.4
21	19/3/2009	Rubilee	28	20	20	56	0	112	80	40	14	16.6
22	2/4/2009		112	56	224	160	224	896	224	896	448	32.8
23	16/4/2009		224	80	320	320	224	1792	1792	1792	640	40.7
24	30/4/2009		320	112	160	448	28	160	1280	224	320	39.4
25	14/5/2009		40	10	320	112	80	1280	640	448	320	39.6
26	2/4/2009	FoalV227	10	0	10	0	0	0	80	28	40	28.0
27	16/4/2009		40	0	0	0	0	0	112	40	28	23.8
28	30/4/2009		160	56	112	112	20	448	448	160	320	36.7
29	19/3/2009	FoalV236	80	28	40	56	0	14	112	20	28	18.2
30	2/4/2009		40	0	28	56	0	40	112	28	80	10.5
31	16/4/2009		112	0	28	40	0	28	160	20	80	15.5
32	30/4/2009		1280	640	640	112	56	1792	1792	320	640	42.5
33	14/5/2009		10	0	0	10	0	0	40	0	20	9.8
34	28/5/2009	Jenny	0	0	0	0	10	10	10	10	0	18.5
35	4/12/2009		0	0	0	10	224	40	160	320	112	17.3
36	8/12/2009		28	112	112	56	1280	448	896	5120	640	37.5
37	7/1/2009		10	0	28	20	320	640	320	2560	640	41.9
38	11/2/2010	Zellie	224	112	320	224	14	1280	320	160	1280	35.8
39	25/2/2010		320	160	224	224	28	1280	640	160	1280	34.7
40	11/3/2010		320	80	320	160	20	1280	896	160	1280	34.0
41	25/3/2010		1280	448	448	224	40	5120	448	320	896	38.7
42	8/4/2010		3584	1280	160	224	56	3584	1792	320	1280	44.7

3.5 Discussion

A novel and important finding from this study was the detection of subclinical cases of AHS (detection of AHSV by real-time RT-PCR in the absence of clinical signs). This RT-qPCR assay has been applied to samples collected from clinical cases of AHS and has been applied to serial samples collected from animals infected experimentally with AHSV. In an experimental case, a horse that had never been vaccinated against AHSV and was challenged with a virulent AHSV strain, tested positive for AHSV dsRNA using an RT-qPCR assay seven days after challenge and remained positive for up to 97 days post challenge. This animal was pyrexic with a fever in excess of 39°C from day 12 to day 14 post-challenge. The viraemia was thus detectable by RT-qPCR approximately five days prior to the animal becoming pyrexic (Quan et al 2010).

A total of nine cases of AHS were identified in the 50 horses included in this study (18%). Of these, three (6%) presented as clinical AHS and six (12%) were mild clinical or subclinical cases. The minimum C_T values of these mild clinical to subclinical cases ranged from 26 to 34, and similar values were often obtained from blood samples collected from mild clinical AHS cases. The level of the viraemia required for horses to infect midges is not known. The appreciable viraemia detected in the mild clinical and subclinical cases suggest that these animals may have a viraemia sufficient to infect midges and such cases pose a severe risk for introduction of AHS into an area if they are moved. The data from these four subclinical cases provide support for the hypothesis that horses that have been vaccinated against AHSV can be subclinically infected with AHSV if challenged under field conditions.

As has been reported in experimental cases of AHS, the RT-qPCR detected AHSV in horses prior to the onset of clinical signs (Quan et al 2010). In this prospective study, this was particularly noticeable in "FoalV227" that was clinically normal with a C_T of 18 the day prior to its euthanasia. The most likely explanation for the fact that the thrombocyte count was not below normal in this foal is that it was euthanased before a drop in thrombocyte count could be realised.

The lymphocytosis seen in most of the cases could be explained by chronic antigenic stimulation due to the viral infection of AHSV. There is also the possibility that the lymphocytosis is due to some other factor than AHS. The lymphopaenia seen in "Kotie" is a more expected result in a viraemia (Skowronek et al 1995, Latimer et al 2003).

The anaemia seen in the case of "Kotie" was most likely due to the concurrent *T. equi* infection rather than being related to the AHS, which may also have been a cause of the neutropaenia and lymphopaenia seen commonly in inflammation and infection (Latimer et al 2003). This may also have aggravated the thrombocytopaenia (De Waal & Van Heerden 2004). The aetiology of the anaemia seen in "Jenny", which was more severe, is not known. She was being treated for a coffin joint laceration at the time of development of AHS symptoms, which may have caused anaemia of chronic inflammation (Latimer et al 2003). There was no evidence of *T. equi* found on a blood smear; however a *T. equi* infection may be difficult to find on blood smear as a parasitaemia can be very low especially in chronic cases (De Waal & Van Heerden 2004). Concurrent *T. equi* infections with AHS are commonly seen (Coetzer & Guthrie 2004).

The polycythaemia reported in experimental cases of AHS (Skowronek et al 1995) was not seen here except for "FoalV227". It is possible that the other horses did not suffer fluid shifts significant enough for these changes to be noted. "FoalV227" presented with severe lung oedema following euthanasia on post mortem, and it is likely the fluid shift from intravascular to extravascular was severe enough to cause haemoconcentration and therefore a polycythaemia. This foal was four months old when it contracted AHS, had not been vaccinated against AHSV was at an age where colostral immunity was waning and was most likely fully susceptible to AHSV infection (Alexander & Mason 1941, Piercy 1951).

The erythron and leukron of the RT-qPCR positive cases in this study did not compare with the experimental study of Skowronek *et al.* (1995). This may be due to field strains not being as virulent as laboratory strains of the virus. In addition, the experimental horses used by Skowronek *et al.* were completely susceptible to AHSV, having never been vaccinated or exposed to natural infection, whereas the Nooitgedacht horses used in this study had been vaccinated yearly, and had, most likely, been exposed naturally to field viruses. These Nooitgedacht horses should have been fully protected against AHSV.

Horses with AHS often present with a decreased thrombocyte count (Skowronek et al 1995). In this study, the thrombocyte count was inversely related to viral load which agrees with the results found by Guthrie *et al.* (2009). A minimum thrombocyte count of 9×10^9/L was obtained from "Kotie's" samples. This very low value did not accompany any signs of a bleeding tendency such as petechiation. It is possible that this value may have been lower than reported by the CELL-DYN® 3700 due to thrombocyte clumping which is often seen in EDTA samples (Byars et al 2003), but a

similar result was obtained using manual thrombocyte counting of a citrated blood sample.

In all cases that survived, AHSV derived nucleic acid could be detected for extended periods in circulation. In the clinical cases, AHSV dsRNA was detected for more than 130 days. Mild clinical to subclinical cases were AHSV dsRNA positive for 30 to 40 days. AHSV can be isolated from the blood of experimentally infected horses by viral isolation for up to 21 days (Coetzer & Guthrie 2004). In this study AHS nucleic acid was detected in the blood for periods considerably longer than this. It has been shown that bluetongue virus, an *Orbivirus* closely related to AHSV, is erythrocyte associated through binding of the virus to glycophorin A on erythrocyte membranes (Hassan & Roy 1999). The lifespan in circulation of an erythrocyte can be up to 145 days (Latimer et al 2003), and it is likely that the RT-qPCR detected erythrocyte-associated AHSV dsRNA, which was not necessarily infectious. The period which horses can be infectious to midges is not known but may extend beyond the current estimated viraemic period. This warrants further investigation.

The rectal temperature data were very variable (Figure 7), and did not appear to be a predictive or prognostic indicator of AHSV infection. There does not appear to be any correlation between the C_T value and the rectal temperature of the horse.

Virus was not isolated from any of the RT-qPCR positive samples collected in this study. It may be that the viral load was too low for viral isolation to be effective (Stone-Marschat et al 1994). The virus may have failed to adapt and replicate in tissue culture, and therefore no isolate was obtained (Hamblin et al 1991). Some of the samples were also stored at -20°C for a few days prior to attempted isolation,

which may have had a negative effect on the virus viability (Ozawa 1965). As these horses were vaccinated, it is possible that AHSV was bound by neutralising antibodies, making viral isolation difficult, as anti-AHSV antibodies will interfere with viral replication. In the case of the foals, maternal antibodies may have interfered with the binding of the virus to the surface receptors of tissue culture cells. McIntosh showed that in these cases, when the blood of the infected horse is injected into a susceptible horse with no neutralising antibodies, that horse would succumb to disease (McIntosh 1958).

This study has shown that while serology may be of use in experimental cases, where exposure to the virus can be controlled, it is of limited use in the diagnosis of AHS in a herd of equids kept under field conditions. The SNT results showed a very broad response to multiple serotypes, which were already high at the time the horses tested RT-qPCR positive in some cases. It has been shown that natural infection of horses with wild-type virus strains induces a more broadly cross-reactive immunity than that observed by attenuated vaccine strains (Blackburn & Swanepoel 1988). This could explain the reason for the multiple serotype seroconversions. Some of the horses already showed strong seroconversion before testing RT-qPCR positive as well as after. Others showed a decrease in titres in the four weeks after testing RT-qPCR positive. No distinct patterns were observed in the indirect ELISA test results. It should be noted that the results obtained in these nine RT-qPCR positive horses on indirect ELISA showed no appreciable difference to those of known RT-qPCR negative horses with similar sampling intervals (Appendix 4). The titres in both RT-qPCR positive and negative horses were consistently high from sample to sample, with all titres over the cut-off value of 2.5. This could be attributed to yearly

vaccination together with natural exposure to AHSV. This study has shown that these serological tests are of little diagnostic use in vaccinated horses that are subsequently exposed to natural challenge with AHSV. The RT-qPCR, however, provided results that are rapidly available and easily interpreted, whereas the serological tests could not compete in terms of accuracy, sensitivity and ease of interpretation.

In this study we were able to establish a pattern in the thrombocyte counts of infected horses. This drop in thrombocyte count along with factors such as the minimum C_T value may be useful as prognostic indicators and should be further investigated. It was shown that AHS cases could be diagnosed before the detection of clinical signs. We have seen that horses may possibly remain viraemic and therefore infectious to midges for greater periods than originally estimated. Most importantly, we have demonstrated that naturally infected, subclinical cases of AHS do occur and this critical finding may influence the way we manage the risk of AHS associated with movement of horses. These risks require further investigation.

CHAPTER
4. General Conclusions

This study has shown:

- Subclinical cases of AHS occurred in vaccinated, naturally infected horses in endemic areas.

- Positive AHS cases could be diagnosed using RT-qPCR before clinical signs were detected.

- The period that a naturally infected horse can remain RT-qPCR positive for was up to 120 days.

- The degree of thrombocytopaenia in AHS cases was appears to be related to the C_T value.

Questions still to be investigated:

- Can subclinical cases of AHS infect *Culicoides* midges?

- What level of viraemia is needed to infect these midges?

- Are these subclinical AHS cases a risk for the movement of horses in AHS restricted areas?

Concluding comments:

This study has shown that early diagnosis and detection of subclinical cases of AHS can be achieved with RT-qPCR. No virus isolates were obtained from blood samples collected from any of these cases demonstrating that the RT-qPCR is more sensitive than the conventional viral isolation method. The extended period of time that these cases remain RT-qPCR positive, can be of use in the surveillance of AHS, as

exposure can be confirmed after the period of viraemia, and past the normal period where viral isolation will no longer be sensitive in detecting AHSV. No virus isolates were obtained from any of the RT-qPCR positive samples. The indirect ELISA, a current test for AHS used in international trade, gave results that were difficult to interpret and of limited significance. The results of the SNTs were just as inconclusive, making interpretation near to impossible. This shows that for rapid, sensitive, routine diagnostics and surveillance, the RT-qPCR is an extremely reliable and attractive alternative to tests currently approved for International Trade Purposes by the World Organisation for Animal Health (OIE).

CHAPTER
5. References

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APPENDIX 1

Standard Operating Procedure - African horse sickness virus real-time RT-PCR assay

Modified from the MAGMAX Blood Total Nucleic Acid Purification Protocol

Version 4.0 June 03, 2009

A. WELL PREPARATION

Place the following 5X MagMAX™-96 Viral RNA Isolation Kit (AMB1836-5) reagents on MagMAX™ -96 Deep well Plates:

- Plate 1 – Sample plate: add 100 µl blood sample to each well. Add 20ul of bead mix (see B.1. below) to sample, centrifuge, and shake sample plate for 1min at max setting on a IKA TTS3D shaker (Merck). Add 400µl of Lysis Solution Mix (see B.2. below) to each sample.
- Plates 2 and 3 – Wash Solution 1 (see B.3. below): 300 µl/well.
- Plates 4 and 5 – Wash Solution 2 (see B.4. below): 450 µl/well.

Place the following reagent on Kingfisher 96 plate (200ul):

- Plate 6 – Elution buffer: 80 µl/well.

Add two positive and one negative control samples to sample plate with lysis solution.

B. REAGENT PREPARATION

Always include 10% coverage to accommodate pippetting imprecision.

1. Bead Mix (per sample)

RNA binding beads	10 µl
Lysis/Binding enhancer	10 µl

Store on ice.

2. Lysis Solution Mix (per sample):

Lysis Binding solution	200 µl
Carrier RNA	2 µl
Xeno RNA	2 µl

Mix well, then add:

Isopropanol	200 µl

3. Wash Solution 1

Add 60ml 100% Isopropanol to Wash Solution 1 Concentrate Bottle, and mix well. Store at room temperature.

4. Wash Solution 2

Add 160ml 100% Ethanol to the Wash Solution 2 Concentrate Bottle, and mix well. Store at 4°C.

Run the programme "AM1836 DW Blood100v2" on the Kingfisher.

C. RT-qPCR

Using a 0.1ml PCR plate, add 5 µl of Primer Probe Mix (see below) to each well.

Primer Probe Mix (per sample)

25X VP7 primer/probe mix	1 µl
25X Xeno primer/probe mix	1 µl
molecular grade water	3 µl

Add 5 µl of extracted RNA to each well and cover plate with plate foil. Spin briefly in the centrifuge to collect the mixture at the bottom of the well. In a thermal cycler, denature samples at 95°C for 1 min. Place plate immediately on ice. Using the VetMAX™-Plus Multiplex One Step RT-PCR Kit (Applied Biosystems – 4515330), make a PCR Mix (see below).

PCR Mix (per sample)

2X Multiplex buffer	12.5µl
10X Enzyme mix	2.5µl

To the denatured RNA/primer/probe mix, add 15 µl of PCR Mix. Cover plate with an optical adhesive cover and scrape down with plastic scraper. Place in StepOne Plus machine.

APPENDIX 2a:

Table 4. Horse List 2009: A list of the horses included in the study in 2009, their birth date,

age (years), sex (C – colt, M – mare, F – filly, S - stallion) and brand number.

Animal ID	Animal Name	Birth Date	Age	Sex	Brand No.
1	Bakama	10/02/1999	8	S	JJ6
3	Mischa	01/11/1991	17	M	AP6
4	Pauline	02/12/1984	24	M	U35
5	QT	24/09/1984	24	M	V89
7	Karamel	05/04/1994	14	M	V170
8	Alexis	16/12/1994	14	M	V172
9	Frances	09/02/1995	13	M	V174
10	Cindy	16/03/1995	13	M	V181
11	Lamay	31/08/1996	12	M	V189
12	Aspaai	04/11/1996	12	M	JP24
13	Bonita	01/12/1996	12	M	JJ4
14	Ariane	02/01/1998	10	M	V204
15	Crispian	02/09/1998	10	M	V212
16	Maxie	17/12/1998	10	M	V214
17	Jennifer	02/12/1999	9	M	V220
18	Jody Lee	07/12/1999	9	M	V221
19	Chellie	03/02/2000	8	M	V224
20	Larika	11/12/2000	8	M	V226
21	Warden	12/12/2000	8	M	V227
22	Countess	22/12/2000	8	M	V229
23	Anita	27/08/2000	8	M	JJ9
24	Katie	01/07/2001	7	M	VN5
25	Zellie	09/12/2002	6	M	V233
26	Lo-Anne	23/12/2002	6	M	V236
27	Ingrid	12/01/2003	5	M	V237
28	Wilgerus	11/08/2003	5	M	WT83
29	Francisca	24/12/2003	5	M	JJ14
30	Cazza	04/12/2004	4	M	V248
31	Demi	10/01/2005	3	M	V252
32	Adri	13/01/2005	3	M	JJ21
33	Bianca	14/11/2003	5	M	JJ13
34	Jane	06/03/2005	3	M	JJ22
35	Kotie	25/09/2005	3	M	JJ25
36	Rubilee	20/01/2006	2	M	V260
37	Casey	04/12/2006	2	M	V261
38	Penny Lee	18/12/2006	2	M	V263
39	Caroll	27/02/2007	1	M	V264
40	V224Foal	22/12/2008		C	V279
41	V227Foal	15/12/2008		C	died
42	V212Foal	14/12/2008		C	V278
43	V236Foal	11/12/2008		C	
44	V189Foal	22/01/2009		F	V283
45	U35Foal	18/01/2009		F	V282
46	V174Foal	13/12/2008		F	V276
47	VN5Foal	18/01/2009		F	V280
48	V181Foal	11/12/2008		C	V275
49	WT83Foal	13/12/2008		F	V277
50	V204Foal	20/03/2009		F	V284
53	Laura			M	V203
54	Jenny	07/12/2007	1	M	V271

Table 5. Horse List 2010: A list of the horses included in the study in 2010, their birth date,

age (years), sex (C – colt, M – mare, F – filly, S - stallion) and brand number.

Animal ID	Animal Name	Birth Date	Age	Sex	Brand No.
1	Bakama	10/02/1999	9	S	JJ6
5	QT	24/09/1984	25	M	V89
7	Karamel	05/04/1994	24	M	V170
8	Alexis	16/12/1994	15	M	V172
9	Frances	09/02/1995	14	M	V174
10	Cindy	16/03/1995	14	M	V181
11	Lamay	31/08/1996	13	M	V189
12	Aspaai	04/11/1996	13	M	JP24
13	Bonita	01/12/1996	13	M	JJ4
14	Ariane	02/01/1998	11	M	V204
15	Crispian	02/09/1998	11	M	V212
16	Maxie	17/12/1998	11	M	V214
17	Jennifer	02/12/1999	10	M	V220
18	Jody-Lee	07/12/1999	10	M	V221
19	Chellie	03/02/2000	9	M	V224
20	Larika	11/12/2000	9	M	V226
21	Warden	12/12/2000	9	M	V227
22	Countess	22/12/2000	9	M	V229
23	Anita	27/08/2000	9	M	JJ9
24	Katie	01/07/2001	8	M	VN5
25	Zellie	09/12/2002	7	M	V233
26	Lo-Anne	23/12/2002	7	M	V236
27	Ingrid	12/01/2003	6	M	V237
28	Wilgerus	11/08/2003	6	M	WT83
29	Francisca	24/12/2003	6	M	JJ14
30	Cazza	04/12/2004	5	M	V248
31	Demi	10/01/2005	4	M	V252
32	Adri	13/01/2005	4	M	JJ21
33	Bianca	14/11/2003	6	M	JJ13
34	Jane	06/03/2005	4	M	JJ22
35	Kotie	25/09/2005	4	M	JJ25
36	Rubilee	20/01/2006	3	M	V260
37	Casey	04/12/2006	3	M	V261
38	Penny Lee	18/12/2006	3	M	V263
39	Caroll	27/02/2007	2	M	V264
40	V224Foal	22/12/2008	1	C	V279
42	V212Foal	14/12/2008	1	C	V278
44	V189Foal	22/01/2009	1	F	V283
45	U35Foal	18/01/2009	1	F	V282
47	VN5Foal	18/01/2009	1	F	V280
48	V181Foal	11/12/2008	1	C	V275
49	WT83Foal	13/12/2008	1	F	V277
50	V204Foal	20/03/2009	1	F	V284
51	V226Foal	09/06/2009	0.5	C	
52	V172Foal	08/02/2010		C	
53	Laura			M	V203
54	Jenny	07/12/2007	2	M	V271
55	V284Foal	13/02/2010		F	
56	JP24Foal	13/02/2010		C	
57	V212Foal	20/02/2010		C	

APPENDIX 3

Table 6. OTAU AHS Vaccination Protocol.

Vaccine	Vaccination Date	Batch	Expiry date
AHS I	13/11/2008	Batch 174	01/07/2009
AHS II	15/12/2008	Batch 208	01/08/2009
AHS I	01/12/2009	Batch 184	01/08/2011
AHS II	22/12/2009	Batch 220	01/10/2011

APPENDIX 4

Table 7. Indirect ELISA results from five RT-qPCR negative horses. These results are from samples collected at similar intervals to the RT-qPCR positive horses shown in Table 6, however each sample tested here was confirmed negative on RT-qPCR.

Sample	Date	Horse	I-ELISA
1	2/4/2009		38.000
2	16/4/2009		43.000
3	30/4/2009	V233	27.000
4	14/5/2009		51.900
5	28/5/2009		37.700
6	2/4/2009		40.300
7	16/4/2009		34.000
8	30/4/2009	V236	40.000
9	14/5/2009		52.000
10	28/5/2009		39.000
11	2/4/2009		35.000
12	16/4/2009		37.000
13	30/4/2009	V237	36.000
14	14/5/2009		50.900
15	28/5/2009		36.400
16	2/4/2009		33.000
17	16/4/2009		35.500
18	30/4/2009	WT83	37.000
19	14/5/2009		25.700
20	28/5/2009		35.700
21	2/4/2009		37.000
22	16/4/2009		39.000
23	30/4/2009	JJ14	34.000
24	14/5/2009		43.000
25	28/5/2009		40.600