A COMPARISON OF EQUINE ORBIVIRUS DYNAMICS ON TWO EQUINE ESTABLISHMENTS ON THE EAST RAND, GAUTENG PROVINCE, SOUTH AFRICA

By

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DECLARATION

I hereby certify that this research is the result of my own investigation. Where use was made of the work of others, it has been duly acknowledged in the text. The results in this dissertation have not been submitted, in whole or in part, for a degree at any other tertiary institute.

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I hereby release this dissertation for examination in my capacity as supervisor.

Estelle H Venter

This dissertation forms part of the requirements for a modular web-based MSc degree research project in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. The research project carries a weight of approximately 80 - 100 credits, and is therefore smaller than projects required for a research-based MSc degree with a weight of 240 credits. It would be appreciated if reviewers could evaluate the dissertation in that context.

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ABBREVIATIONS

AGID	Agar gel immunodiffusion
AHS	African horse sickness
AHSV	African horse sickness virus
BTV	Bluetongue virus
CFT	Complement fixation test
CPE	Cytopathic effect
C_T	Cycle threshold
DAFF	Department of Agriculture, Forestry and Fisheries
DEET	N,N-diethyl-3-methylbenzamide
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EC	External control
EE	Equine encephalosis
EEV	Equine encephalosis virus
ELISA	Enzyme-linked immunosorbent assay
ERC	Equine Research Centre
IFA	Immunofluorescent assay
OIE	World Organisation for Animal Health
OBP	Onderstepoort Biological Products
PCR	Polymerase chain reaction
RT-qPCR	Reverse transcription quantitative PCR
SNT	Serum neutralisation test
VNT	Virus neutralisation test
VGL	Veterinary Genetic Laboratory
VP	Viral protein

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SUMMARY

A comparison of equine orbivirus dynamics on two equine establishments on the East Rand, Gauteng Province, South Africa

By

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African horse sickness (AHS) is a non-contagious viral disease transmitted by arthropod vectors namely *Culicoides (Avaritia) imicola Kieffer* and *Culicoides (Avaritia) bolitinos Meiswinkel* endemic to sub-Saharan Africa. The disease affects all equine species, where its severity increases in horses foreign to Africa. Currently, vaccination is the only means of controlling the disease.

African horse sickness poses a great risk to South African equines, not only due to the high mortality rate, but also due to the large scale restrictions implemented on the movement of horses for breeding or competition and on the international exportation of horses by the Department of Agriculture, Forestry and Fisheries (DAFF) and the World Organisation for Animal Health (OIE).

A prospective study was undertaken between 2013 and 2014 by the Department of Veterinary Tropical Diseases and the Equine Research Centre (ERC), Faculty of Veterinary Science (FVS), University of Pretoria to determine the presence of *Culicoides* midges, the vector of the African horse sickness virus (AHSV) and the prevalence of disease at two equine establishments on the East Rand, Gauteng Province, South Africa.

The two establishments differed extremely, when looking at infrastructure, management and vaccination protocols, this being the primary reason for their inclusion into the study.

In the study, which started in December 2013, EDTA blood samples were collected and rectal temperatures recorded every 14 days over six months, from 28 Friesian / Lusitano and Appaloosa horses both resident in stables and open camps at the two

establishments. The horses ranged in age from yearlings to four years. The EDTA samples were tested for the presence of AHSV and equine encephalosis virus (EEV) dsRNA by RT-qPCR (Quan *et al.* 2010).

The clinical picture of the horses was recorded and rectal temperatures monitored for presentation of clinical cases caused by both viruses. It was shown that a total of nine (32%) cases of AHSV and five (18%) cases of EEV were identified in the 28 horses included in this study, where 89% of the horses had been vaccinated against AHS.

As part of the risk assessment at each establishment it was essential to monitor the presence of the known vectors of AHSV. Therefore the conventional down-draught Onderstepoort black-light trap was operated overnight at various intervals throughout the study. The infection rate using RT-qPCR of the collected *Culicoides* midges was lower than the previous assumptions made by the owner and consulting veterinarians based on the mortality rate during the previous AHS season. Both AHSV and EEV were detected in separate single pools of collected midges. The low number of positive midges found in this study during 2014 could be explained by the occurrence of both diseases followed by the very active midge season of 2013. It is hypothesized that the prevalence of these diseases is dependent on seasonal patterns where a build-up of virus must reach a critical level after which spilling over will occur into associated equine populations (Venter *et al.* 2014).

The present study also investigated the relationship between prevention strategies; primarily vaccination with a registered vaccine and the incidence of both diseases, where it shows that the prevalence of disease is dependent on the various prevention strategies implemented at each establishment.

The presence of subclinical infection as seen in this study requires further investigation as it has a major impact on the movement of equines and the possible introduction of disease into naïve populations. The analysis of EE in the study, which is more prevalent than AHS, however does not cause severe disease, assists in the evaluation of wild-type virus transmission, as there is no commercial vaccine is available for EE. The presence of the virus assists in the study of the virus/host dynamics, natural maintenance cycles and the transmission of orbiviruses amongst South African horses. (Venter *et al.* 1999).

CHAPTER 1

1.1 INTRODUCTION

The name orbivirus was proposed in 1971 to describe a group of arthropod-borne viruses with a unique morphology. Viruses within the genus *Orbivirus,* family *Reoviridae* are complex non-enveloped double-stranded RNA viruses containing 10 segments (Roy *et. al* 1994). An electron microscopy study revealed that their physico-chemical properties as well as their characteristic appearance were sufficiently distinct to form their own taxonomic group where their name reflected the especially large doughnut-shaped capsomeres seen on the surface of virus particles (Gorman *et al.* 1983).

Transmission of orbiviruses to vertebrate hosts is primarily by arthropod vectors, which depending on the individual virus can be exclusively by *Culicoides* midges. Midges play a significant role in the transmission of the African horse sickness virus (AHSV), bluetongue virus (BTV) and equine encephalosis virus (EEV).

The two diseases affecting equines namely African horse sickness (AHS) and equine encephalosis (EE) play a significant role in equine veterinary science in South Africa.

The present study investigated the dynamics of these equine orbiviruses and the relationship of prophylactic strategies to the incidence of equine orbiviral infection.

Due to the principal importance to equines, the study included the assessment of *Culicoides* spp directly implicated with the epidemiology of both the viruses.

From the study, the data obtained from the two contrasting scenarios will add to the existing scientific knowledge of the disease, virus and vectors and aid in the identification of managerial aspects that may reduce the prevalence of equine diseases.

1.2 LITERATURE REVIEW

1.2.1 African horse sickness

1.2.1.1 History

African horse sickness is an infectious, non-contagious viral disease found to infect all equines. Although more severe in horses, other equid species including donkeys and mules present with a mild form of this disease (Binepal *et al.* 1992). Zebra on the other hand show no clinical signs and are infected subclinically. The disease itself is enzootic to sub-Saharan Africa; however AHS has occurred outside the region on several occasions causing considerable economic loss to international equine industries (Lord *et al*. 1997, Sailleau *et al.* 2000, Maree & Paweska 2005).

African horse sickness made its first known historical reference in an epidemic that occurred in Yemen 1327 (Moule 1896, cited by Wetzell *et al.* 1970, Coetzer & Guthrie 2004).

In South Africa AHS was only documented 60 years after the initial introduction of horses into the country during 1657 (Mellor & Hamblin 2004). During 1719, the disease then referred to as ‘perrezieke’ or ‘pardeziekte’ claimed the lives of approximately 1700 equines (Coetzer & Erasmus 1994). It was claimed by Bayley (1856) (cited by Guthrie & Quan 2009) that in the 1854 - 1855 outbreak; approximately 70 000 horses died, where this was at the time about 40% of the equine population in the Cape Colony.

Historically the Cape of Good Hope (Cape Town) was listed as the only area free of the disease and it was said that this was due to the rainfall pattern of the area. This is where a major link was made between rainfall, climate and the presence of the disease (Baylis *et al.* 1999).

African horse sickness was also then one of the main reasons for the establishment of the Veterinary Institute at Onderstepoort in 1908 by Sir Arnold Theiler, where it was considered to be an ancient disease (Wentzel *et al.* 1970).

1.2.1.2 Aetiology

There are nine antigenically distinct serotypes of AHSV (McIntosh 1958; Howell 1962 Barnard 1993). Of the nine serotypes, types 1 to 8 are typically found and restricted to sub-Saharan Africa, whereas type 9 has been more widespread and responsible for epidemics outside the endemic area of Africa including; Spain, Tunisia and Morocco (Mellor & Hamblin 2004).

Overall the virus is composed of an inner and outer capsid, a core, and a nucleoprotein complex. The virion is unenveloped and about 70 nm in diameter encapsulated by a double-layered icosahedral shell composed of 32 capsomeres (Bremer 1976; Van Dijk & Huismans 1980). The genome comprises 10 double stranded RNA segments, each of which encodes at least one polypeptide (Roy *et al.* 1996). Seven structural proteins known as viral proteins (VP1 – VP7) exist and form the double-shelled virus particle, and there are at least 5 non-structural proteins (NS1 – NS3/NS3a, NS4) (Belhouchet *et al*. 2011). The inner capsid proteins VP3 and VP7 are serogroup-specific antigens, while the outer capsid protein (VP2) harbours serotype specific antigenic epitopes that segregate a particular serogroup into distinct serotypes (Van Niekerk *et al.* 2003).

The virus is fairly resistance to temperature and the OIE (2013) has reported that in the presence of protein, the virus will remain infective after heating at 55–75°C for 10 minutes. The virus is stable at higher pH levels (6-12), where its optimal pH for survival is 7.0–8.5. The AHSV quickly becomes inactivated below a pH level of 6, especially due to the process of rigor mortis (OIE 2013).

1.2.1.3 Epidemiology

African horse sickness is endemic to tropical and sub-tropical regions of southern Africa where the disease itself was believed to be confined to sub-Saharan Africa except for occasional excursions into North Africa or the Arabian Peninsula (Hamblin *et al.* 1998).

However, during the periods 1959 to 1991, periodic outbreaks of AHSV occurred out of Africa and spread across Saudi Arabia, Syria, Lebanon, Jordan, Iraq, Turkey, Cyprus, Iran, Afghanistan, Pakistan, India and Spain (Mellor & Hamblin 2004) making it quite evident that the disease was able to breach its borders creating a global concern.

The virus can infect all equids (Lord *et al.* 1997). Zebra, the natural hosts of the disease show no clinical signs but serve as amplifying hosts of the virus (Mellor & Hamblin 2004) where a continuous transmission cycle between vectors and Zebra occurs in the Kruger National Park (Barnard 1993). Large populations of donkeys can play a similar role to zebra and therefore increase the transmission of AHSV together with the surrounding vectors (Hamblin *et al.* 1998). Antibodies to AHSV have been reported in a number of other species where seropositive herbivores include camels (*Camelus dromedaries*), sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), African elephants (*Loxodonta africana*), black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*); however it is not clear whether wildlife other than zebra play any key role in the epidemiology of the disease (Binepal *et al.* 1992, Simpkin 2008).

Dogs too have been infected by the AHSV, although this is usually by ingestion of infected horse meat and therefore plays a non-specific role in the epidemiology of the disease (Mellor & Hamblin 2004).

1.2.2 Clinical signs

Horses infected with AHSV may manifest four clinical forms. These forms differ in their intensity, mortality rate, symptoms and prognosis (Simpkin 2008). Infection with the AHSV may present with similar clinical signs to that of closely related viral infections such as infection with EEV and therefore laboratory diagnosis is recommended for correct virus identification (Guthrie & Quan 2009, Simpkin 2008).

a) “Dikkop” or sub-acute cardiac form

Usually occurs during the late stage of infection and presents remarkable subcutaneous swelling of the head, predominately of the supraorbital fossae, where the skin rises well above the level of the zygomatic arch (Figure 1a). Incubation is usually 5 – 7 days followed by fever of 39 – 41°C for 3 – 4 days. Oedema (Figure 1b), which usually only starts at the decline of fever, later spreads to the conjunctiva, lips, cheeks, tongue and intermandibular space and in some extending right down the neck to the chest. Dyspnoea and cyanosis may be evident (Guthrie & Quan 2009). A mortality rate of up to 50% can be seen with this form of AHS (Coetzer & Guthrie 2004).

Figure 1a: Notable filling of the supraorbital fossae in a horse infected with African horse sickness virus. © Institute for Animal Health, Pirbright.

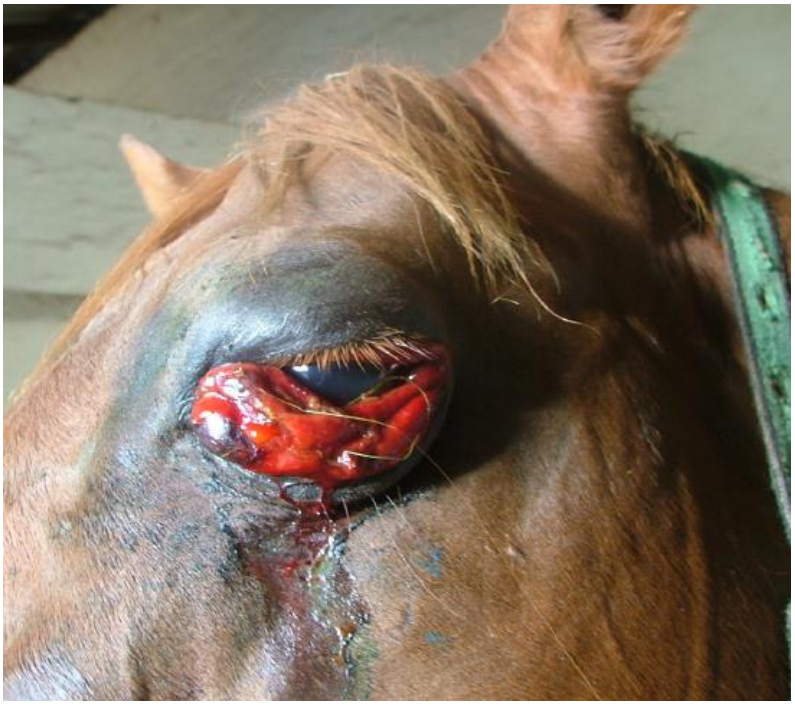

Figure 1b: Severe conjuctival oedema and hyperaemia with haemorrhage in an African horse sickness virus infected horse. © UC Davis.

b) “Dunkop” or pulmonary form

This form is an acute respiratory form. Infection of this form usually occurs when susceptible equids are infected (foals or serologically naive populations) (Simpkin 2008). Incubation is usually 3 – 4 days with a sudden rise in temperature for 2 days ranging between 40 – 41°C (Guthrie & Quan 2009). This is followed by respiratory distress, severe dyspnoea and coughing followed by a frothy discharge from the nostrils (Figure 2) and onset of death usually follows within a few hours. A mortality rate of >95% can be seen.

Figure 2: Notable frothy discharge from the nostrils post mortem due to pulmonary form of African horse sickness. © Research Gate.

c) “Mixed” form

The mixed form is the most common form of AHS. In this form both pulmonary and cardiac forms are present, however one more dominant over the other. Mortality rates for this form of the disease are usually around 70% and death will normally occur 3 – 6 days after the initial fever (Mellor & Hamblin 2004).

d) Subclinical (fever) form

This is the milder form of all four and may not be diagnosed clinically. Incubation is approximately 5 – 9 days followed by a fever that increases gradually over 4 – 5 days up to 40°C, and then drops following normal recovery. Mortality is rare (Coetzer & Guthrie 2004).

1.3 EQUINE ENCHEPHALOSIS

1.3.1 History

Sir Arnold Theiler referred to the disease during the early 1900s as ‘equine ephemeral fever’; today it is termed equine encephalosis. The term "encephalosis" was defined as any form of organic disease or dysfunction of the brain (Anon.1986, cited by Howell *et al.* 2002). The disease is a mild or subclinical *Orbivirus* infection transmitted by the same *Culicoides* spp. that plays a significant role in the transmission of the AHSV (Maclachlan & Guthrie 2010).

Equine encephalosis was first isolated in South Africa in 1967 and since then subsequent serological studies have shown that approximately 60% of donkeys, zebras and horses in South Africa are seropositive (Howell *et al.* 2002).

1.3.2 Aetiology

The disease is caused by the EEV and shows typical morphological and physio-chemical characteristics to other viruses within the genus *Orbivirus*. Seven non-cross reactive serotypes have been identified and its morphology resembles that of the AHSV and BTV (Howell *et al.* 2002).

1.3.3 Epidemiology

Although very little is known about the epidemiology of EE (Lord *et al.* 2002), EEV shares similar hosts and vectors to AHSV. EEV infections where clinical signs have been noted have been seen in all breeds and ages of horses; most cases are, however, subclinical (Lord *et al.* 2002; Mildenberg *et al.* 2009).

1.3.4 Clinical signs

Subclinical infection with EEV is usually very common. Diagnosis of animals showing clinical signs is therefore only achieved by detection of the virus in blood or tissues using virus isolation and the polymerase chain reaction (PCR) in order to differentiate it from the mild forms of AHS (Guthrie & Quan 2009).

Large populations of equines typically show no specific signs of illness; however, signs that may occur as seen in Israel include that of fever, listlessness and inappitance (Mildenberg *et al.* 2009).

In a small population of equines, severe nervous complications have been reported when infected with the EEV. Signs include frenzy, convulsions, and uncontrolled running into objects (Guthrie & Quan 2009).

1.4 ORBIVIRAL TRANSMISSION

Culicoides spp. (Diptera: Ceratopogonidae) are haematophagous arthropods (Figure 3), 1 – 3 mm in size (Venter *et al.* 1997, Page *et al*. 2009). These small biting flies are associated with the transmission of several pathogens of economic and veterinary importance with a worldwide distribution (Meiswinkel *et al.* 2000, Page *et al*. 2009, Scheffer *et al.* 2012, Venter *et al.* 2012).

Approximately 1500 *Culicoides* spp. have been identified worldwide (Scheffer *et al.* 2012), of which more than 100 species occur in South Africa (Nevill *et al.* 1992b), where in general, the most abundant species and of principal concern to equines is *Culicoides (Avaritia) imicola* Kieffer (Figure 4), followed by *Culicoides (Avaritia) bolitinos* Meiswinkel. Both species are implicated in the transmission of the AHS and EE viruses.

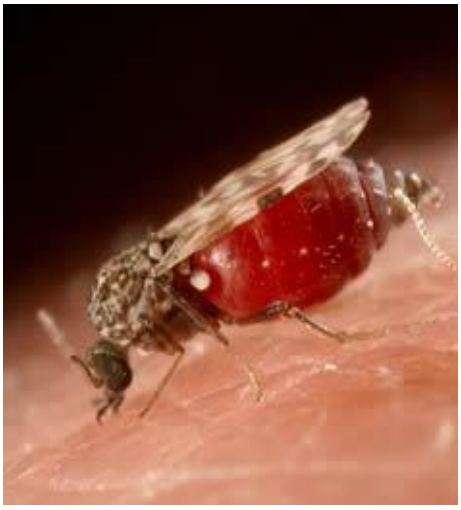

Figure 3: Blood fed *Culicoides spp*. © Institute for Animal Health UK.

Figure 4: *Culicoides (Avaritia) imicola* Kieffer © G.J. Venter.

Distribution and seasonality is dependent on the climatic conditions of the specific area (Venter *et al.* 1996; Venter *et al.* 2014) and midges are found to be abundant in the warm wet summer months, feeding during the twilight periods and at night (Meiswinkel *et al.* 2000). Mechanical aspiration of horses at Onderstepoort has shown, however, that *Culicoides* females of different species can be collected from horses before sunset (Scheffer *et al.* 2012). Meiswinkel *et al.* (2000) described the complete life cycle of midges as a 3 – 4 week period under favourable conditions therefore developing several generations within a single season. Female midges are the known blood suckers and form a crucial part in the epidemiology of both AHS and EE. Both viruses have been isolated from *Culicoides* spp. in various geographical distribution studies (Lord *et al.* 2002).

For maturation of eggs the adult female has to take a blood meal and therefore becomes infected when ingesting a blood meal from a viraemic animal (Venter *et al.* 1997), where the incubation period of the virus within the midge is approximately 8 days. The virus is then localised to the salivary gland of the midge and transmitted to the next host upon a blood feed (Lubroth 1988). The interval between virus ingestion and the ability of the midge to transmit the virus is known as extrinsic incubation period. In the absence of transovarial transmission (Venter *et al.* 1991; Venter *et al*. 2014), competent *Culicoides* females only become infected after feeding on a viraemic host.

The recent studies of Venter *et al.* (2014), added to the findings by Nevill (1971) and Venter *et al.* (1997), where, adult midges are present throughout winter and that the deceptive impression where no detectable cases of viraemia, disease or seroconversion in the host are found in winter, the virus did not circulate all year long in endemic areas, is in fact incorrect and therefore does not need to be re-introduced annually from the more subtropical regions.

Venter *et al.* (2014) discovered that despite a drastic decline in numbers during the colder seasons, adult *Culicoides* midges of several species remained present and that continued blood meals were taken and breeding continued in the Gauteng Province of South Africa. Therefore the apparent absence of orbiviruses during the colder months could be ascribed to the relatively low midge numbers, therefore low infection prevalence, all resulting from lower life cycle developmental rates of the infected midges due to the unfavourable conditions (Venter *et al*. 2014).

The importance of this finding is that adult *Culicoides* midges potentially are present in all seasons. Outbreaks of AHS and EE can therefore commence as soon as population numbers have again reached a critical level thus confirming Barnard (1993) that the continued circulation of insect borne viruses depends on the constant availability of susceptible hosts and competent vectors in sufficient numbers.

1.5 PREVENTION AND CONTROL

Since no curative treatment is available for both AHS and EE, vaccination (AHS only) and vector control are normally performed as prevention and control measures. These diseases are treated symptomatically although with AHSV infections; symptomatic treatment is usually inadequate to prevent equines succumbing to the virus (Simpkin 2008). As EEV infection is usually subclinical and no vaccine has been developed, vector control methods as used for the prevention of AHS will limit vector exposure and therefore limit viral infection in susceptible equines (Guthrie & Quan 2009).

1.5.1 African horse sickness vaccines

The first vaccination “serum-virus method” against AHS was developed in the 1930s by the inoculation of animals with serum from recovered animals and two isolates of virus which differed in virulence (Burrage & Laegreid 1994). Early vaccinations gave good immune responses but this sometimes led to side effects with significant morbidity and mortality and therefore have subsequently been replaced by live tissue cultured attenuated vaccines (House *et al.* 1990, Burrage & Laegreid 1994, Weyer *et al.* 2013).

Currently the polyvalent, attenuated live virus (ALV) vaccine available commercially from Onderstepoort Biological Products (OBP) is the only registered vaccine available in South Africa for protection against AHS. The vaccine 1 and 2 combination contains AHSV serotypes 1, 2, 3, 4, 6, 7 and 8 (Mellor & Hamblin 2004). African horsesickness virus serotype 5 is not included in the vaccine as it has been reported to cause severe reactions that resulted in the deaths of the vaccinated horses, furthermore, AHSV serotype 9 was excluded from the vaccine due to its low virulence and that it is rarely present in South Africa and there is cross protection by AHS serotype 6 (Mellor & Hamblin 2004).

The manufacturers, OBP, have set vaccination guidelines (Table 1) and the vaccination programme should begin before the onset of substantial rainfall, creating a favourable environment for the midge vectors.

Table 1: African horse sickness vaccination protocol set out by the manufacturer.

Vaccination	Period
Initial vaccination	Weanling at approximately six months of age
Booster vaccination	Yearling at approximately twelve months of age
Annual vaccination	All equines late winter – spring (Aug – Nov)

Recently a new recombinant canarypox vectored vaccine was developed which indicated promising preliminary results. The ALVAC®-AHSV vaccine will provide sufficient immunization for equines against the AHSV (Guthrie *et al.* 2009).

Although new vaccination research and vaccine development is constantly a strong research topic, the current AHS vaccination alone does not provide complete control and therefore in conjunction, strict management protocols need to be implemented.

1.5.2 Management of vectors

The reduction of vectors will reduce the potential risk of the disease by drastically reducing the ability of the vector having contact with equines. Management of vector exposure can be considered a control method by preventing midges from biting horses. Prophylactic interventions such as the control of animal movement, stabling and repellents will prevent or limit midge and horse interaction (Meiswinkel *et al.* 2000; Page *et al*. 2009).

1.5.2.1 Stabling

As *Culicoides* midges are crepuscular or nocturnal insects where their activity peaks during twilight; stabling equines during this period will help reduce contact between the midge vector and therefore the virus (Meiswinkel *et al.* 2000, Simpkin 2008). Whilst stabling equines is a recommended control method, *Culicoides* midges are known to be endophilic, entering stables, therefore adequate closure methods are also required (Meiswinkel *et al.* 2000). The use of meshing (80% shadecloth) across all open fronted stables was described by Page *et al*. (2009), where a 14 fold reduction of *Culicoides* entering stables was reported.

1.5.2.2 Movement of equines

African horsesickness is a controlled animal disease in South Africa because of the high potential for national and international spread. The area surrounding the Cape of Good Hope (Cape Town and surrounding area) has been historically free of AHS, therefore an effort has been made to control the movement of equines by the state veterinary authorities since 1997 (Guthrie 1997, Weyer *et al.* 2013) as an outbreak in the AHS controlled area in the Western Cape Province will have a significant impact on affected properties and on the exportation of horses from the *AHS Free Zone* (Figure 5). Therefore strict guidelines and control protocols have been given in the OIE International Animal Health Code and in the South African / European legislation regarding the movement of equines into the controlled zone of the Western Cape to curb the spread of the disease (DAFF 2015).

Movement of animals locally is also a concern, when horses are moved to and from stable yards for the purpose of competition. Simpkin (2008) suggested that testing and control of movement in endemic areas is logistically hard; however the responsibility lies with the owners to monitor virus activity in the specific competitive areas and by taking extra precautions when traveling to an area where potential cases have been reported.

Movement of zebra, mules and donkeys should only occur when seasonal insect activity is at its lowest and authorization is granted by appropriate veterinary authority.

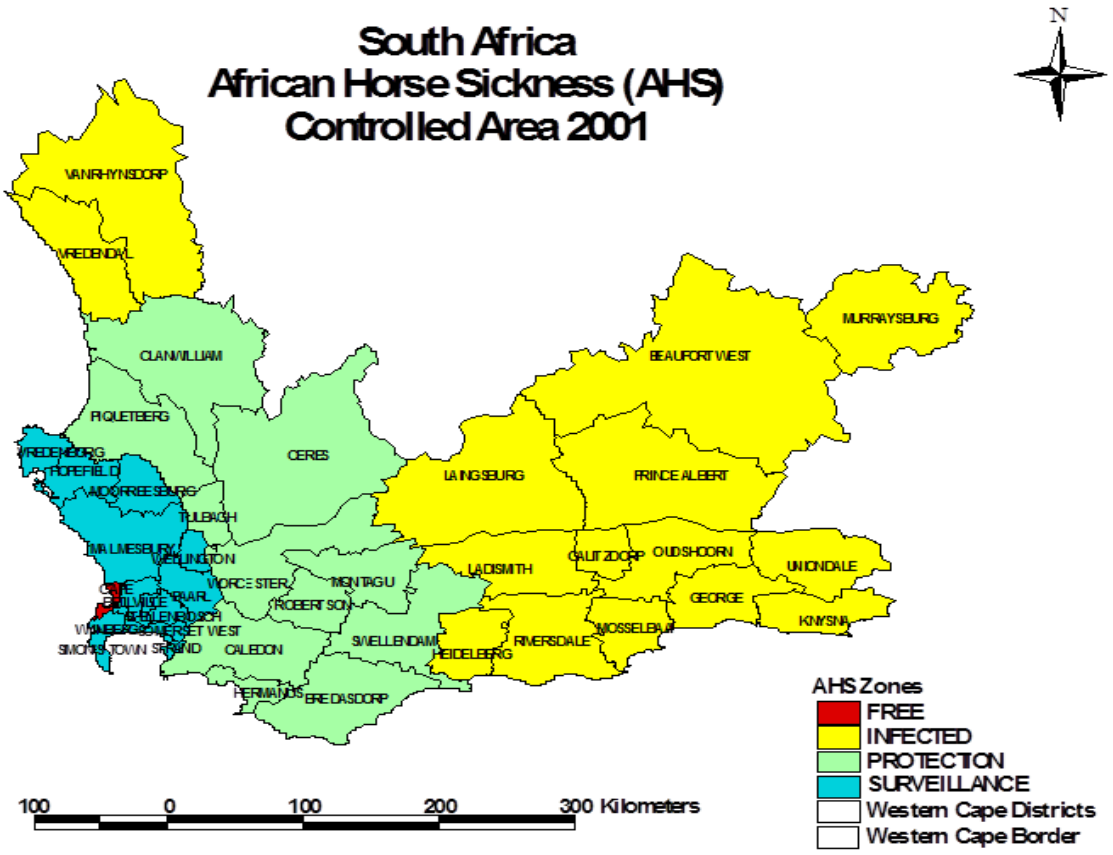

Figure 5: African Horse sickness controlled area, South Africa. © DAFF 2015.

1.5.2.3 Repellents

Natural and synthetic repellents have long been used to repel biting arthropods.

1) N,N-diethyl-3-methylbenzamide

N,N-diethyl-3-methylbenzamide (DEET) is often used as the “Gold Standard” when assessing insect repellents (Page *et al*. 2009). It is used as a repellent for a number of biting insects but most commonly against mosquito bites in humans, as no commercially manufactured products containing DEET are available in South Africa for registered for horses (Simpkin 2008).

2) Pyrethrins and pyrethroids

Pyrethrins are natural insecticides derived from *Chrysanthemum cinerariaefolium* and related species while pyrethroids are synthetically developed pyrethrins developed solely to enhance the efficacy of the substance (Page *et al*. 2009). Cypermethrin is a good example of a pyrethroid registered for the use on equine animals in South Africa.

3) Essential oils

Plant derivatives in the form of essential oils have been reported to have the ability to repel insects with a short period of protection. Page *et al*. (2009) reported that although poorly studied many essential oil repellents have a sweet smell and this is said to mask the natural body odours and those from gaseous exchange that attract biting insects to horses.

1.5.2.4 Alternative control measures

There are many more methods currently being used to repel midges and though no single preventative or control measure provides 100% protection on its own, the greater the number of preventative measures in place, will reduce the risk of infection with either AHSV or EEV.

1.6 LABORATORY DIAGNOSTICS

The epidemiology, clinical signs and gross lesions of AHS are often sufficiently specific to allow for a provisional diagnosis to be made. However, specific lesions have been seen not to be specific to AHS alone. Multiple symptoms found in horses suffering from various forms of AHS resemble that of EEV and West Nile virus infections. For this reason laboratory diagnosis is essential, and various tests have therefore been developed to facilitate a definitive diagnosis (Weyer *et al.* 2013).

1.6.1 Viral isolation

Viral isolation has been traditionally used to obtain a definitive diagnosis for AHSV by inoculating the test sample onto a variety cell cultures monitoring for any cytopathic effect (CPE). Once a virus has been isolated, confirmation that it is either ASHV or EEV is required, as their morphology and CPE are almost indistinguishable (Crafford 2001). This can be achieved by using an enzyme linked immunosorbant assay (ELISA) or serotyping of the viruses using a virus neutralisation test (VNT) (Hamblin *et al.* 1991).

1.6.2 Virus serotyping

Serotyping is done using plaque inhibition neutralising tests using specific antisera to the different serotypes, in this case AHSV and EEV, which have multiple serotypes. Porterfield (1960, cited by Weyer *et al.* 2013) described the process whereby fishspine beads are filled with type specific antiserum used to indicate virus-antibody neutralisation on test sample inoculated on Vero cell monolayers. Absence of plaque formation around the bead is an indication of antibody neutralization.

1.6.3 Group specific serology

Group-specific serology uses group-specific antibodies against viruses to determine the seroprevalence.

1.6.3.1 Complement fixation testing

The complement fixation test (CFT) has been used extensively in the past, specifically in detecting an immunological immune complex formation. Currently its use is decreasing and has been replaced in many laboratories with molecular tests with higher sensitivity and degree of standardisation (OIE 2013). The CFT is a useful tool in endemic areas for the demonstration and titration of a group-specific IgM antibody response following a recent infection or vaccination (Crafford 2001).

1.6.3.2 Agar gel immunodiffusion

Using semisolid mediums, precipitations through diffusions bring optimal concentrations of antibody and antigen together. Once the concentration of the complex has exceeded that of the gel's capacity, the complex precipitates out forming visible bands (Hamblin *et al.* 1990). There is however concern about the lack of sensitivity of the test, which is dependent on the concentration of antibody present in the test serum when looking at the AHSV (Laviada *et al.* 1997; Zientara *et al.* 1994; Crafford 2001).

1.6.3.3 Indirect immunoflouresent antibody test

Specific antibodies are determined by using standardised antigen and fluorescent antiglobulin. Binding of the antiglobulin fluorescent to the antibody-antigen complex causes it to fluoresce. The Indirect immunoflouresent antibody test (IFA) is a rapid and inexpensive test for identification of AHSV both for control and surveillance purposes, however non-specific fluorescence, can occur in vaccinated horses, leading to false positives (House *et al*. 1990).

1.6.3.4 Indirect enzyme-linked immunosorbant assay

The recombinant VP7 has been used as an antigen for the determination of AHSV antibody with a high degree of sensitivity and specificity (Laviada *et al.* 1992; Wade-Evans *et al.* 1993). These assays detect immunoglubin G (IgG) antibodies to either the antigens or VP7. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with domestic equines. The method described by Maree & Paweska (2004) uses IgG as a conjugate that also reacts with zebra serum.

1.6.4 Serotype-specific serology

The detection of sero-specific antibodies with the use of a serum neutralisation test (SNT) also determining specific antibody titres in sera. The SNT may have additional value in epidemiological surveillance and transmission studies in endemic areas where multiple serotypes are most likely present (OIE 2013). The SNT is serotype-specific and can be used to differentiate between the antibodies produced against each of the nine antigenically distinct serotypes of AHSV. The SNT is considered to be highly sensitive and specific and it does not cross-react with other *Orbivirus* serogroups. Although

functional the SNT is not a common diagnostic tool for routine testing as it is extremely time consuming.

1.6.5 Nucleic acid methods

The polymerase chain reaction (PCR) is widely used as the diagnostic test of choice in the identification of AHSV. It is a rapid, sensitive test for early detection of viraemia determining the amount of a target sequence that is present in a sample and is used extensively for routine diagnosis of many animal infectious diseases.

Conventional gel-based PCR has been an essential diagnostic tool in the surveillance and control of disease, although now it is being replaced by real-time PCR in diagnostic laboratories where significant advantages include; higher sensitivity and reduced processing time and lower risk of contamination is found compared to conventional gel-based PCR (Fernández-Pinero *et al.* 2009).

A number of RT-qPCRs have been reported for the diagnosis of AHS, detecting either individual serotypes or all serotypes of the virus (Sailleau *et al.* 2000; Aguero *et al.* 2008; Fernández-Pinero *et al.* 2009; Quan *et al.* 2010).

Another advantage of these new approaches is that they can be applied to specimens from clinical cases that do not contain live virus (Guthrie *et al.* 2013).

For the purpose of this study a quantitative reverse transcription duplex PCR (RT-qPCR) developed by the ERC (Quan *et al.* 2010) was used to quantify AHSV and EEV viraemia, where blood and homogenised tissue and midge samples are subjected to RT-qPCR using specific primers and Taqman® probes that targets specific segment genes, coding for the VP7 of the AHSV and EEV (Quan *et al.* 2010; Rathogwa 2014).

The RT-qPCR assay detects highly conserved regions within the genes of the AHSV (S8 and S9) and EEV (S7) which encode the VP7 structural protein and NS2 non-structural protein (Quan *et al.* 2010, Rathogwa *et al.* 2014). The assay was to quantify AHSV / EEV viraemia. This RT-qPCR in conjunction with viral isolation and antigen-capture ELISA is being routinely used for the diagnosis of AHS at the Veterinary Genetic Laboratory (VGL), FVS, University of Pretoria.

1.7 OBJECTIVE

The objectives of this study were:

- To compare the prevalence of AHSV and EEV on two equine farms under different management scenarios.
- To establish a relationship between the occurrences of disease to either managerial or environmental conditions that contributes to the occurrence of AHS / EEV.
- To advise on measures to limit the occurrence of disease on each establishment.

CHAPTER 2

MATERIALS AND METHODS

2.1 IDENTIFICATION OF EQUINE ESTABLISHMENTS

Two establishments on the East Rand, Gauteng Province, South Africa (Figure 6) were selected to participate in the project. The criteria used in this selection was closely linked to but not limited to; management strategies, stabling facilities, vector control and vaccination procedures.

Establishment A**:** A Friesian / Lusitano Stud with strict management strategies, vaccination protocols and a very well equipped equine facility. GPS co-ordinates S 26 05.095 E 28 23.401 (GPS coordinates indicate the closest airfield to the establishment).

Establishment B: An Appaloosa Stud, the farm is old and in need of facility upgrades. Stabling facilities are limited and vaccination protocols followed are haphazard. GPS co-ordinates S 25 59.338 E 28 25.089 (GPS coordinates indicate the closest airfield to the establishment).

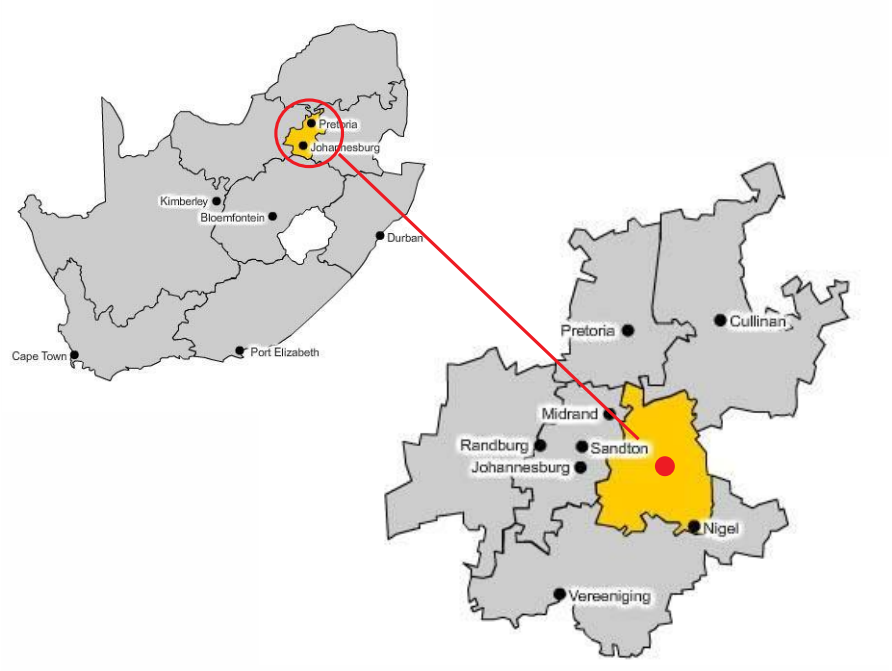

Figure 6: Location of the two establishments on the East Rand, Gauteng Province, South Africa.

2.2 STUDY PERIOD

The study / sampling period ran from November 2013 to May 2014 seen as the peak seasonal occurrence of both AHS and EE in the area.

2.3 EXPERIMENTAL PROCEDURE

2.3.1 Selection of equines

Fifteen young horses from each equine establishment, approximately 1 – 4 years of age, were selected to participate in the project (n = 30). Each selected horse was scanned for a micro identification chip for reliable identification throughout the project; for those that did not have, a chip was provided. Furthermore all horses were allocated project numbers for presentation of results (Tables 2a & b).

Table 2a: List of participating equines at Establishment B.

NUMBER	EQUINE NAME	MICROCHIP ID NUMBER
1	SURVIVOR	710098100127723
2	CHESTNUT GELDING	710098100130148
3	BAY FOAL 2013	710098100126509
4	BAY FOAL 2013 (COLT)	710098100130556
5	BAY FOAL #4	710098100125858
6	NEAR LEOPARD FILLY 2011	710098100127433
7	HALF LEOPARD SPOT FILLY 2012	710098100126782
8	GREY FILLY 2010	710098100130251
9	NEAR LEOPARD COLT	710098100130777
10	NEAR LEOPARD SPOT COLT 2013	710098100129432
11	NEAR LEOPARD FILLY 2012	710098100130507
12	CHESTNUT FILLY 2011 (NO EYE)	710098100129464
13	CHESTNUT MARE 2010	710098100127763

Table 2b: List of participating equines at Establishment A.

NUMBER	EQUINE NAME	MICROCHIP ID NUMBER
14	FEDERATO QS	985141000622394
15	HINDER VAN QUANTUM	978000001359835
16	HIGHLANDER QS	985141000621846
17	D'ARTANYAN VAN QUANTUM	978000001363518
18	FURAGO QS	978000001357750
19	GALILEO OF QUANTUM	041781A953
20	HINDRIK VAN QUANTUM	978000001354445
21	HAYLEY QS	710098100130555
22	JASMINE VAN QUANTUM	985141000622400
23	HAZEL	985141000622384
24	ILLUSTRADOR	985141000622341
25	RUBIE VAN QUANTUM	985141000622414
26	KONDOR VAN QUANTUM	985141000622471
27	KYANITE VAN QUANTUM	985141000622337
28	HELIODOR	985141000622467

A basic history was established for each horse including recent illnesses and treatments, vaccination history, nutritional status, body condition score and stabling facilities. Data sheets were created for each equine and the microchip barcodes, histories, clinical findings, and collection dates were recorded.

At the onset of sampling the number of horses participating from each establishment changed. These changes were due to illness / deaths prior to project approval and / or equine sales. Establishment A therefore had 15 participating horses and establishment B, 13 participating horses (n = 28)

Of the 28 participating horses, 25 were vaccinated with a registered AHS vaccine (Table 3). Three equines had no form of vaccination prior to the start of the study.

Table 3: Vaccination status of equines participating in the study.

Establishment	Combination 1 Vaccination Date	OBP Batch Vaccine Combo 1	Combination 2 Vaccination Date	OBP Batch Vaccine Combo 2
A	27/11/2013	107	13/12/2013	239
B	01/11/2013	108	22/11/2013	240

2.3.2 Sampling and data collection from equines

Participating equines were clinically examined for any signs related to AHS or EE and all findings recorded. Following visual examination, rectal temperatures were taken and recorded for each equine by digital thermometer.

Blood samples were drawn from each equine by jugular venepuncture in ethylene diamine tetra-acetic acid (EDTA), heparin and serum vacutainer tubes, where they were uniquely barcoded corresponding to each equines data sheet. Clinical examination together with EDTA blood collection from each equine occurred twice a month, all efforts we made to collect samples before 8am. Blood collection in serum and heparin vacutainers was drawn every third visit or at any sign of illness. Following collection, all blood samples were stored at 4°C at the FVS, University of Pretoria (Table 4).

Table 4: Blood or tissue collection schedule at each establishment.

Establishment A	Establishment B
2013-12-27	2013-12-20
2014-01-10	2014-01-03
2014-01-25	2014-01-25
2014-02-07	2014-02-05
2014-02-24	2014-02-14
2014-03-07	2014-02-28
2014-03-20	2014-03-14
2014-04-11	2014-03-28
2014-04-25	2014-04-11
2014-05-09	2014-04-25
2014-05-23	2014-05-09
-	2014-05-24
-	2014-05-30

2.3.3 Collection of *Culicoides* midges

Midges were collected by placing the 220V Onderstepoort downdraft suction light traps operating with an 8W UV-light tube (ARC-Institute of Agricultural Engineering, South Africa) (Figure 7).

Insects entering the light trap were collected in plastic containers containing 200 ml water and 0.2 ml Savlon solution (chlorhexidine gluconate 0.3 g/100 ml and cetrimide 3.0 g/100 ml) (Johnsons & Johnson, South Africa) as previously described (Venter *et al.* 2009b; Scheffer *et al.* 2012).

Light traps were operated at regular intervals installed approximately 1.5 m – 1.8 m above ground level and as close to the host animals as possible (stabling and paddock facilities) throughout the collection period (Figure 8). The light traps were set to operate from 6 pm – 6 am on the selected days. No other trapping devices were operated in the immediate vicinity of the horses and the light trap.

Figure 7: 220V Onderstepoort downdraft suction light traps with collection container (ARC-Institute of Agricultural Engineering, South Africa). © G.J. Venter.

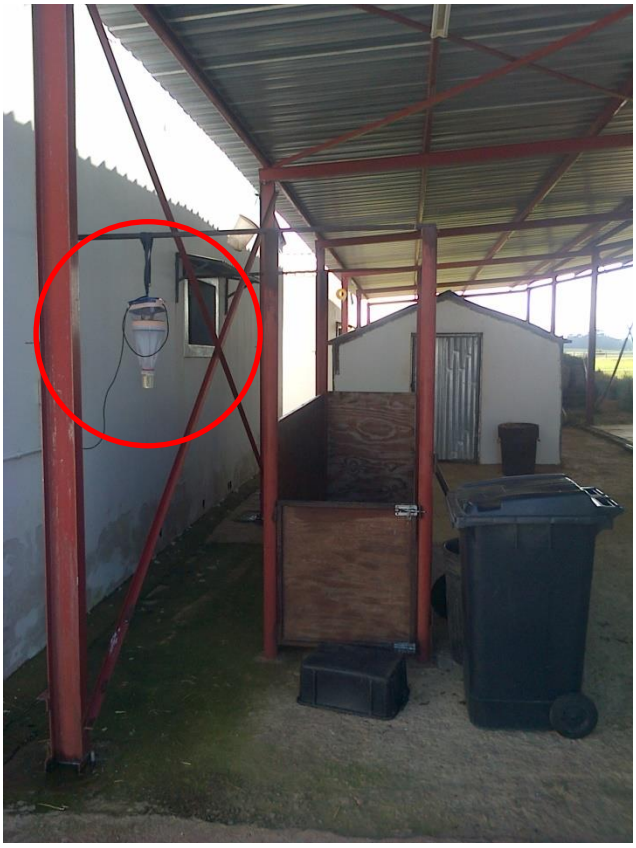

Figure 8: Light trap in place adjacent to the stable block at Establishment A.

Insect collections were stored in 70% ethanol at 4°C until transferred to the Entomology Section of the ARC-Onderstepoort Veterinary Institute (ARC-OVI) for assistance in midge separation and identification to allow for RT-qPCR processing at the ERC, FVS. Separation procedures followed were adaptations of the published data of Venter *et al*. (2009a; 2009b).

2.3.4 *Culicoides* midge sorting and identification

Culicoides midge identification and numbers were determined under a stereo microscope and the insects were segregated according to relevant species, gender and parity status. Males, nulliparous, parous, and blood-fed females were differentiated. As the preliminary studies of Scheffer *et al.* (2012) demonstrated that the RT-qPCR used in this study could detect a single infected midge in a pool of 200 *Culicoides* midges, sorted collections were grouped into pools of 200. For larger collections, containing primarily nulliparous *Culicoides* only a single pool of 200 was used for analysis.

All pools contained only *C. imicola* and *C. bolitinos*, and all other insects including non-specific *Culicoides* species were discarded.

2.3.5 Analysis of collected samples

2.3.5.1 Analysis of blood samples by RT-qPCR

A quantitative reverse transcription duplex PCR (RT-qPCR) developed by Quan *et al.* (2010) was used, where blood and homogenised tissue and midge samples are subjected to RT-qPCR using specific primers and Taqman® probes that targets specific segment genes, coding for the VP7 of the AHSV and EEV (Quan *et al*. 2010, Rathogwa *et al.* 2014). A volume of 100 µl of blood was added to 20 µl of a magnetic bead mix (MagMAX™ RNA/DNA kit, Applied Biosystems) and centrifuged at 1000 rpm (BOECO centrifuge) for approximately 1 minute. Following centrifugation, 400 µl of lysis binding solution (AM8500) was added to each well and centrifuged again for approximately 1 minute (this binding solution included 20,000 copies of XenoRNA, supplied as part of the VetMax™-Plus one-step RT-PCR Kit (Applied Biosystems PN 4415328).

Following the centrifugation process the supernatant was used for RNA extraction in the Kingfisher automated purification system (ThermoFisher Scientific). After extraction, the elution plate was removed, covered and transferred to the freezer for ‘snap’ cooling at -20°C for approximately 5 minutes.

Using the Step One Plus Real Time PCR System (Applied Biosystems), 5 µl of the RNA extract was transferred to thin-walled PCR wells (Kingfisher 96 well), together with 5 µl of the primer-probe-mix where the final primer and probe concentration was 400 nM and 180 nM respectively (25X VP7 primer/probe mix, 25X Xeno primer/probe mix) (Applied Biosystems PN: 4445067) and 15 µl of the PCR master mix as described by Quan *et al.* (2010).

Thereafter the one step RT-qPCR was performed where the method described by Quan *et al.* (2010) was adapted by Guthrie *et al.* (2013) with the addition of propriety Xeno4 primers and probes to target XenoRNA as a synthetic external control (EC). The AHSV assay; forward (AGA GCT CTT GTG CTA GCA GCC T) and reverse (GAA CCG ACG CGA CAC TAA TGA) primer concentrations were 200nM and the probe (FAM-TGC ACG GTC ACC GCT-MGB) concentration was 120nM. For the Xeno4 (EC) assay both primers had a concentration of 250 nM where the probe concentration was 200nM, labelled VIC.

2.3.5.2 Analysis of pooled *Culicoides* collections by RT-qPCR

Pools of midges were transferred to Eppendorf tubes to allow for evaporation of the 70% ethanol in which they were stored. Once evaporated, 500 µl of phosphate buffer saline (PBS) was added to each tube containing pooled midges. The pools were then transferred to MagNA Lyser Green Beads Tubes (Roche Products, South Africa) to be homogenized twice in a MagNA lyser (45 seconds at 7000 rpm), followed by centrifugation for 2 minutes at 2800 rpm (BOECO centrifuge).

The procedure following centrifugation was the same as used for blood samples (See Section 2.3.5.1, using 100 µl of homogenised midge solution per extraction (Quan *et al.* 2010; Guthrie *et al.* 2013).

CHAPTER 3

RESULTS

3.1 BLOOD COLLECTION DATA

Of the 28 horses (Tables 5a & b) sampled during 2013 and 2014, six tested positive for AHSV (blue), two tested positive for EEV (green) and three tested positive for both AHSV and EEV (yellow). For a RT-qPCR test to be considered positive with a 95% limit of detection, the C_T value of the represented samples should be <37.14 and <38.42 for that of AHSV and EEV, respectively. In most cases the horses remained AHSV or EEV RT-qPCR positive for at least 14 days but were negative by day 30 following detection of either disease.

Table 5a: Positive equines at Establishment B.

NUMBER	EQUINE NAME
1	SURVIVOR
2	CHESTNUT GELDING
3	BAY FOAL 2013
4	BAY FOAL 2013 (COLT)
5	BAY FOAL #4
6	NEAR LEOPARD FILLY 2011
7	HALF LEOPARD SPOT FILLY 2012
8	GREY FILLY 2010
9	NEAR LEOPARD COLT
10	NEAR LEOPARD SPOT COLT 2013
11	NEAR LEOPARD FILLY 2012
12	CHESTNUT FILLY 2011 (NO EYE)
13	CHESTNUT MARE 2010

Table 5b: Positive equines at Establishment A.

NUMBER	EQUINE NAME
14	FEDERATO QS
15	HINDER VAN QUANTUM
16	HIGHLANDER QS
17	D'ARTANYAN VAN QUANTUM
18	FURAGO QS
19	GALILEO OF QUANTUM
20	HINDRIK VAN QUANTUM
21	HAYLEY QS
22	JASMINE VAN QUANTUM
23	HAZEL
24	ILLUSTRADOR
25	RUBIE VAN QUANTUM
26	KONDOR VAN QUANTUM
27	KYANITE VAN QUANTUM
28	HELIODOR

All positive cases were subclinical and showed no change to their habitus with no rise in rectal temperature appearing clinically normal on the day of sampling.

Positive cases found at both Establishment A and B are represented by Figures 9 – 12 and Figures 13 – 19 respectively, where the sequential trend of C_T values and rectal temperature can be seen after natural infection AHSV or EEV.

On two separate occasions equines numbered 3 and 5 presented with an elevated rectal temperature of 39.3 °C and 40.3 °C respectively during sampling. Both equines presented with acute fever, inappetence and poor exercise tolerance. However on both occasions both AHSV and EEV RT-qPCR tests were negative. Both equines at sampling presented with severe tick infestation and a differential diagnosis was given as equine piroplasmosis. Treatment followed by intramuscular injection of Forray® 65, Imidocard dipropionate 12% m/v (MSD Animal Health) at 2.4mg/kg live weight. Both equines recovered quickly and were found to be clinically normal at the next sampling period.

All equine RT-qPCR data, both positive and negative can be seen in Appendix 1.

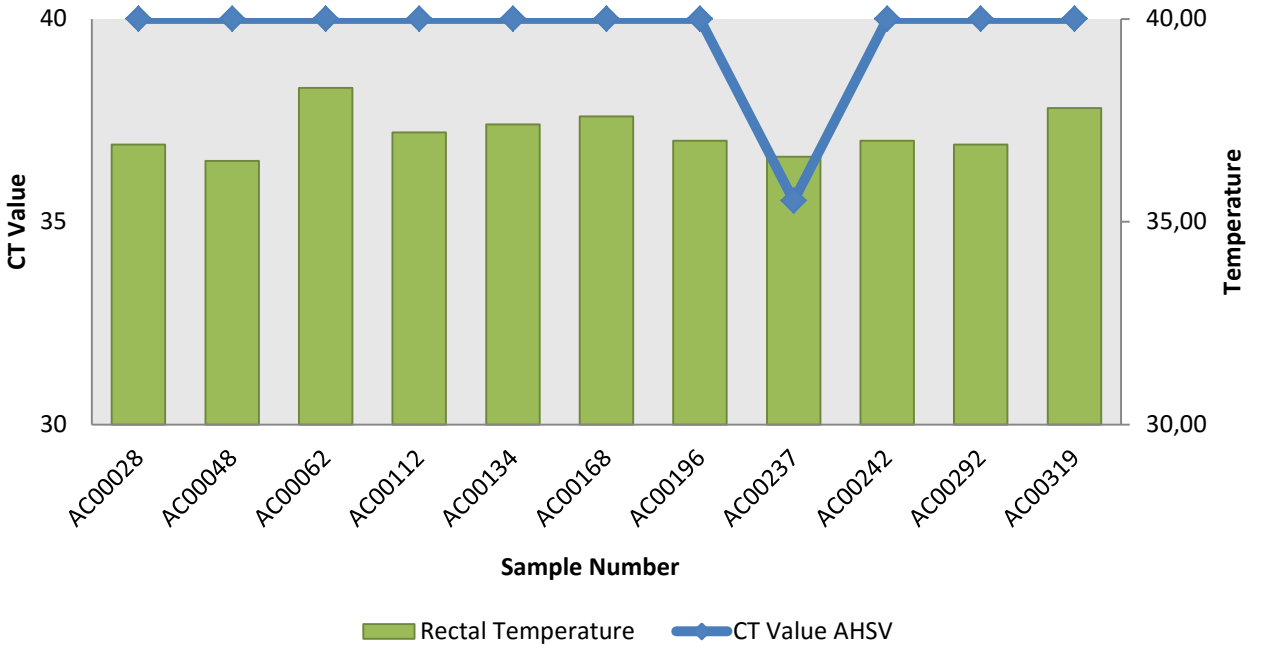

Figure 9: Equine 28 – Sequential trend of C_T values and rectal temperature after natural infection AHSV.

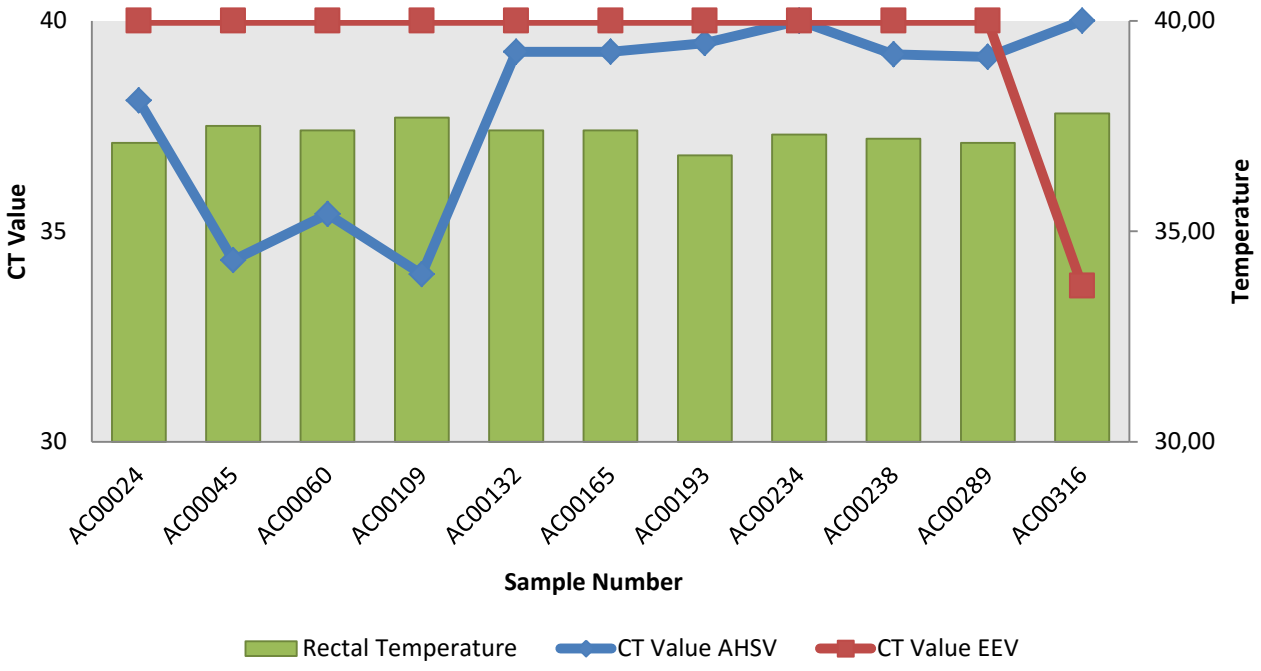

Figure 10: Equine 24 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV and EEV.

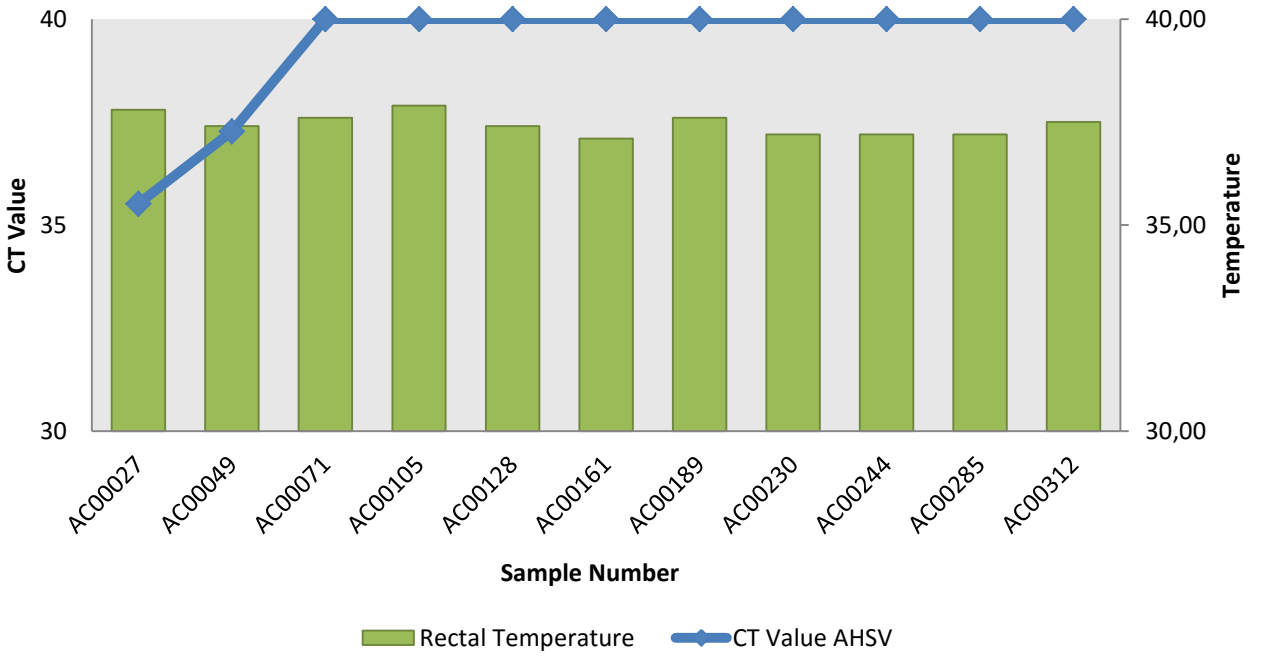

Figure 11: Equine 27 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV.

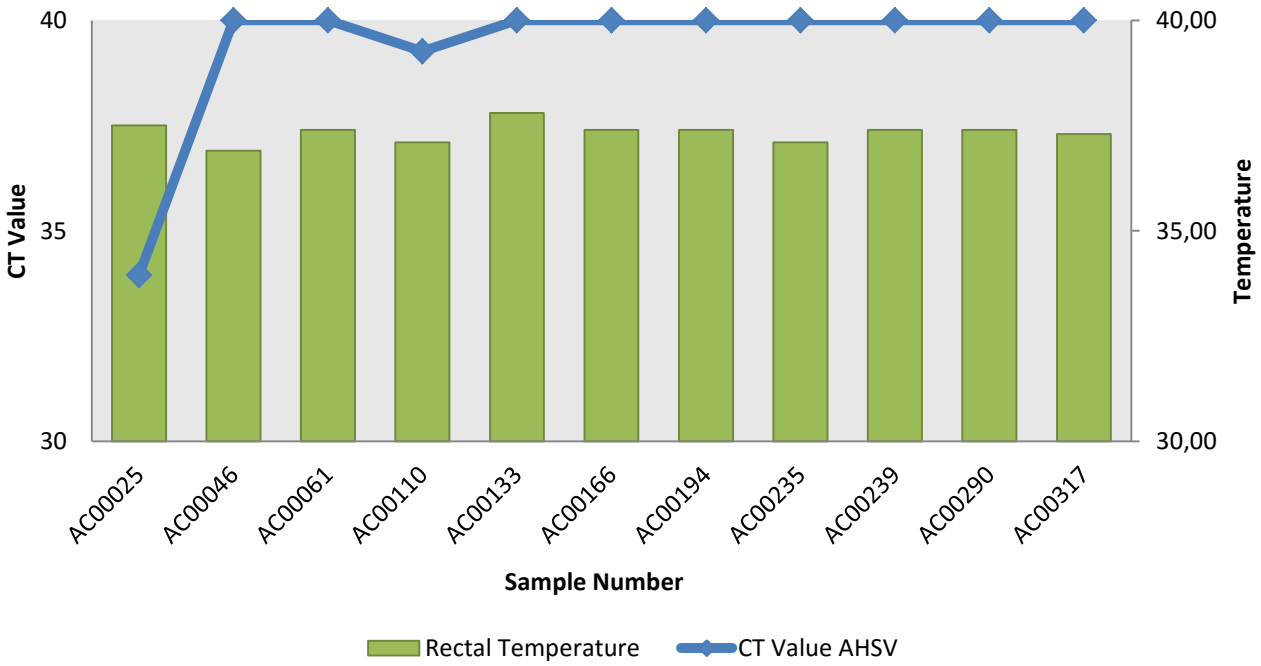

Figure 12: Equine 25 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV.

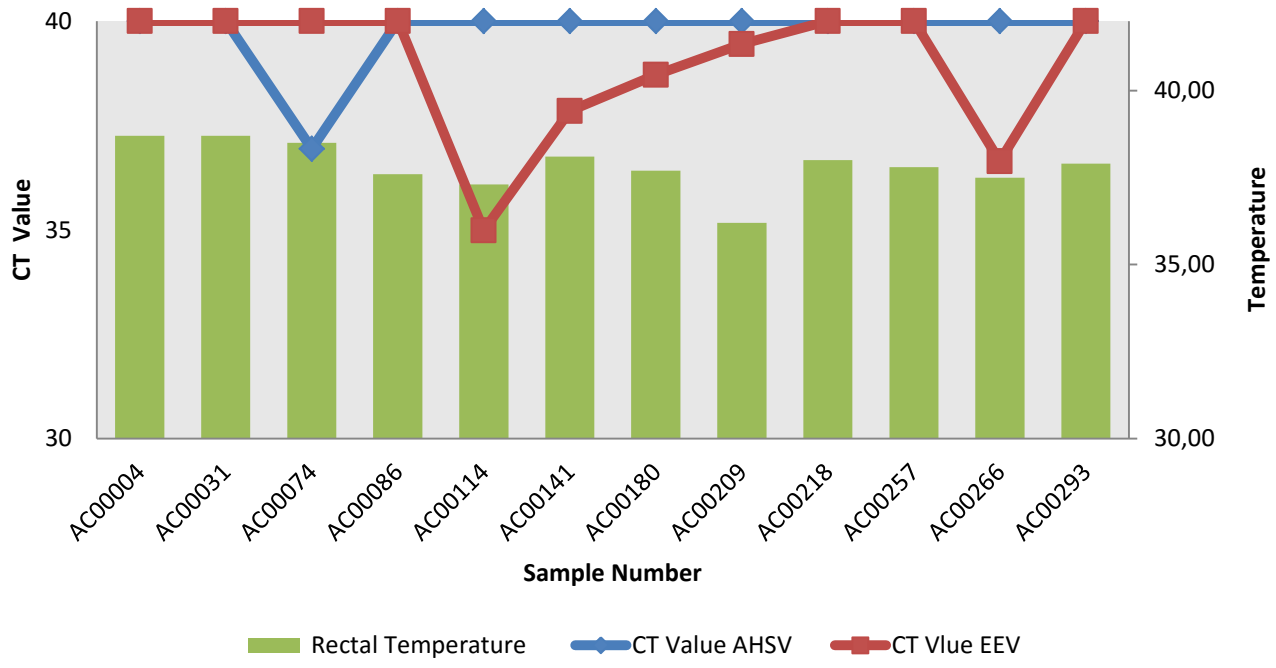

Figure 13: Equine 4 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV and EEV.

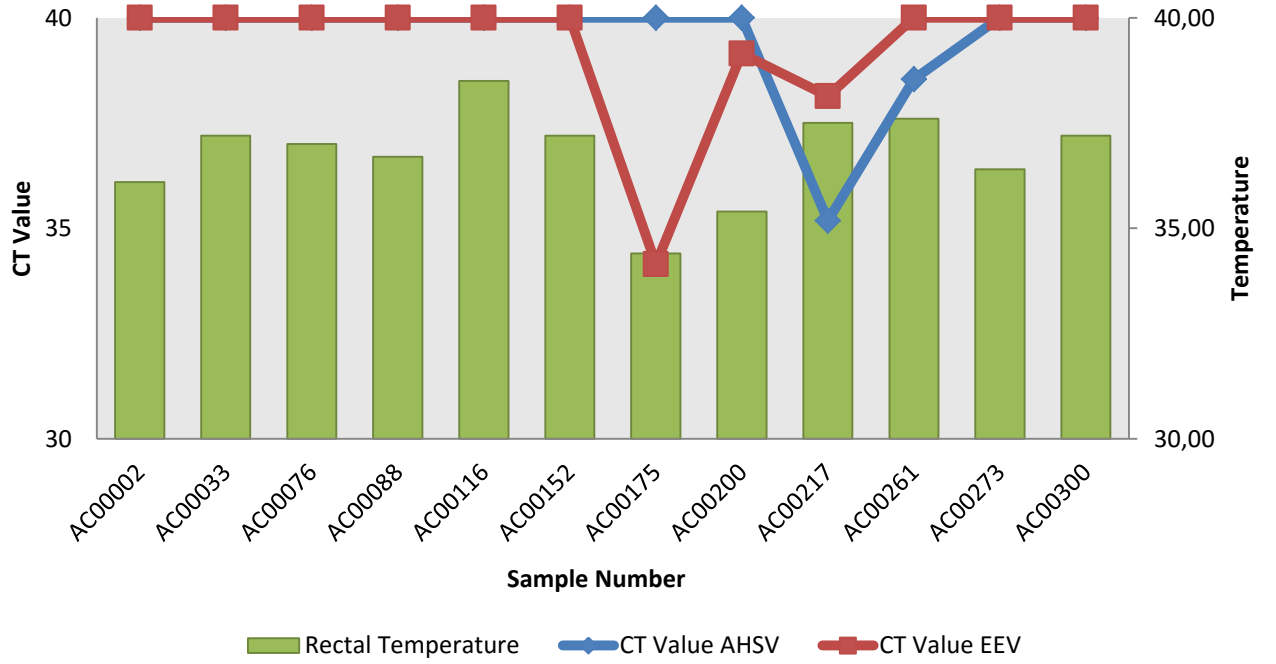

Figure 14: Equine 2 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV and EEV.

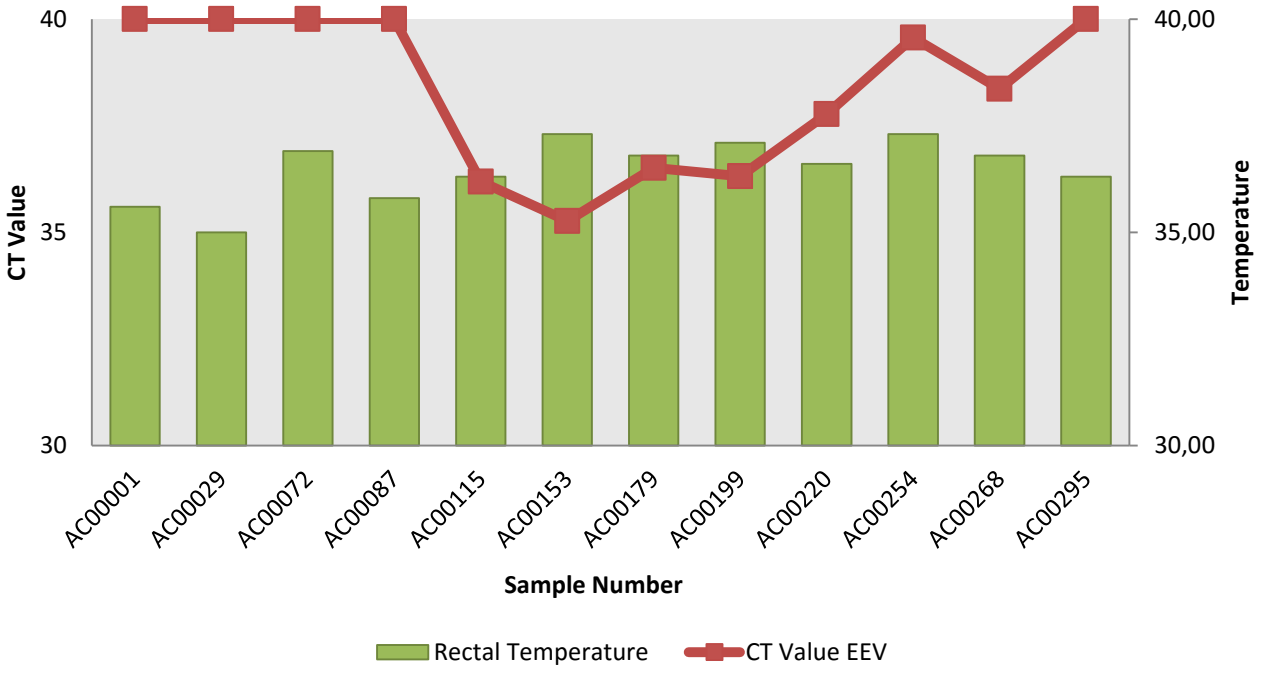

Figure 15: Equine 1 – Sequential trend of C_T values and rectal temperature after natural infection with EEV.

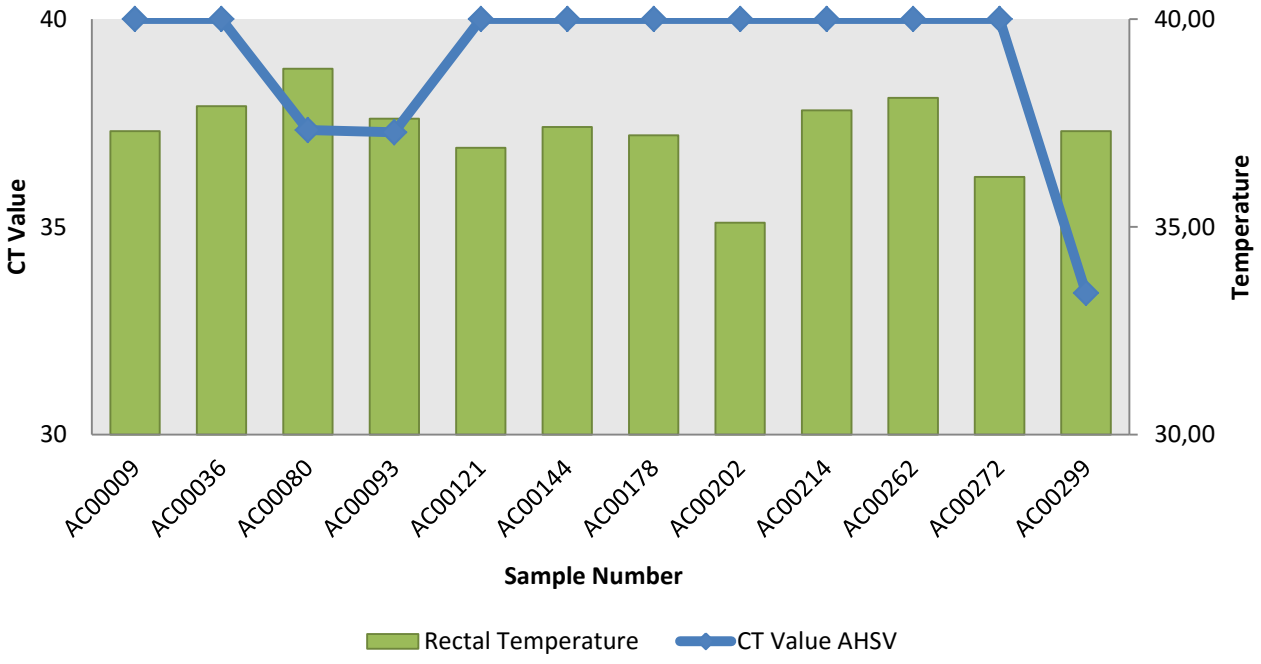

Figure 16: Equine 9 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV.

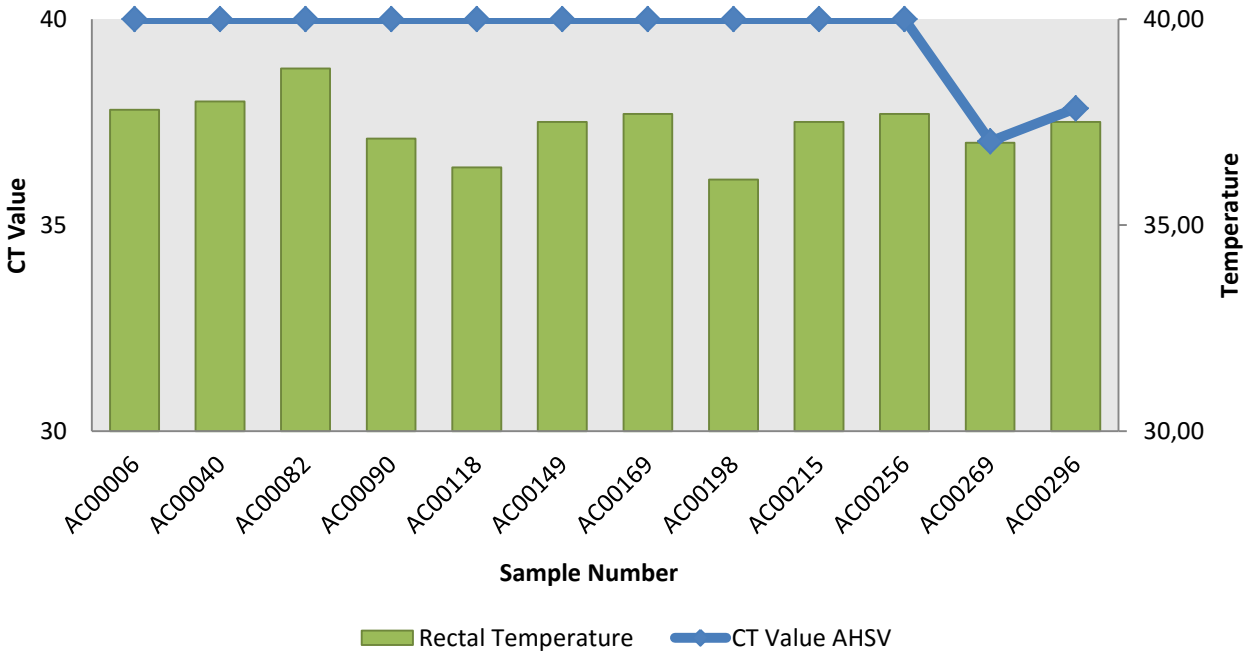

Figure 17: Equine 6 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV.

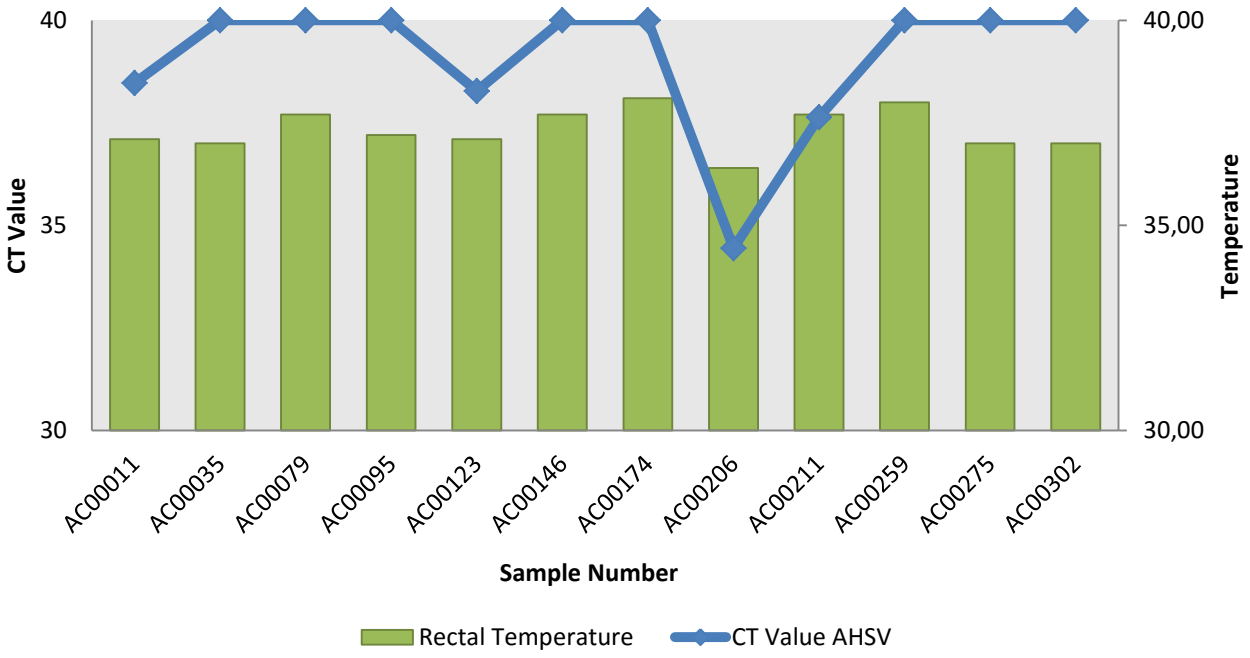

Figure 18: Equine 11 – Sequential trend of C_T values and rectal temperature after natural infection with AHSV.

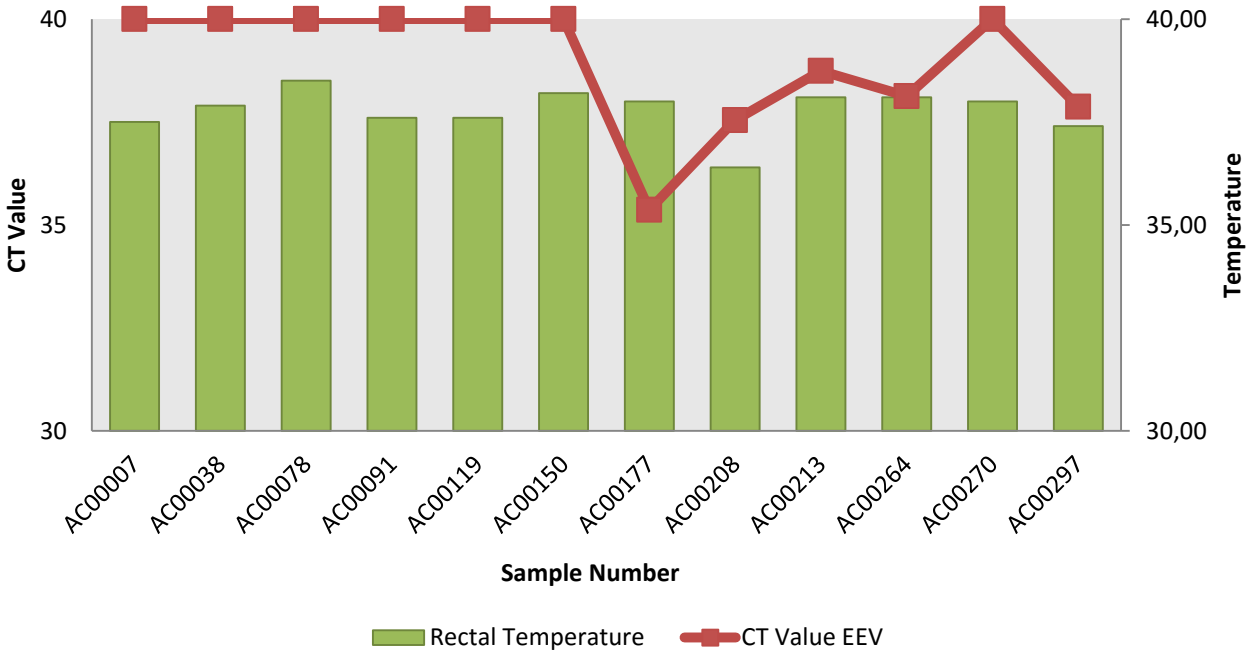

Figure 19: Equine 7 – Sequential trend of C_T values and rectal temperature after natural infection with EEV.

3.2 RT-qPCR DIAGNOSTIC CONTROL EVALUATION.

In an effort to accurately evaluate all nucleic acid tests, all diagnostic tests included both positive and negative controls. Control C_T values can be seen in Tables 6 (AHSV) and 7 (EEV).

Table 6: RT-qPCR control values for AHSV.

Sample Run	Negative Blood C_T value	Negative H_20 C_T value	Negative PCR C_T value	Low Positive C_T value	High Positive C_T value	Positive Blood C_T value
AC01	40	40	40	27,4	27,1	26,9
AC02	40	40	40	35,7	25,4	24,7
AC03	40	40	40	28,7	24,6	24,3
AC04	40	40	40	31,7	27,7	27,2
AC05	40	40	40	28,1	24,0	23,7

Table 7: RT-qPCR control values for EEV.

Sample Run	Negative Blood C_T value	Negative H_20 C_T value	Negative PCR C_T value	Low Positive C_T value	High Positive C_T value	Positive Blood C_T value
AC01	40	40	40	25,5	28,4	28,3
AC02	40	40	40	29,7	28,5	27,9
AC03	40	40	40	30	27	30,4
AC04	40	40	40	30,6	30,9	30,3
AC05	40	40	40	31,4	27,3	27

3.3 MIDGES COLLECTION DATA

The total sum of collected *C. imicola and C. bolitinos* midges trapped at both establishments during the collection period was ***11157***.

Sample numbers, collection dates and pool counts for both establishments can be seen in Table 8.

Table 8: Total number of collected midges per catch.

Sample Number	Collection Date	Combined Pooled Count (*C. imicola, C. bolitinos*)
ACM0001	27/12/2014	116
ACM0002	27/12/2014	12
ACM0003	10/01/2014	152
ACM0004	10/01/2014	276
ACM0005	25/01/2014	524
ACM0006	25/01/2014	287
ACM0007	05/02/2014	38
ACM0008	08/02/2014	819
ACM0009	14/02/2014	36
ACM0010	24/02/2014	2658
ACM0011	01/03/2014	94
ACM0012	22/03/2014	805
ACM0013	27/03/2014	887
ACM0014	10/04/2014	198
ACM0015	10/04/2014	203
ACM0016	24/04/2014	2053
ACM0017	26/04/2014	25
ACM0018	09/05/2014	1068
ACM0019	09/05/2014	906

The total number of pooled *C. imicola* / *C. bolitinos* collected throughout the six month study period at each establishment can be seen in Figure 20.

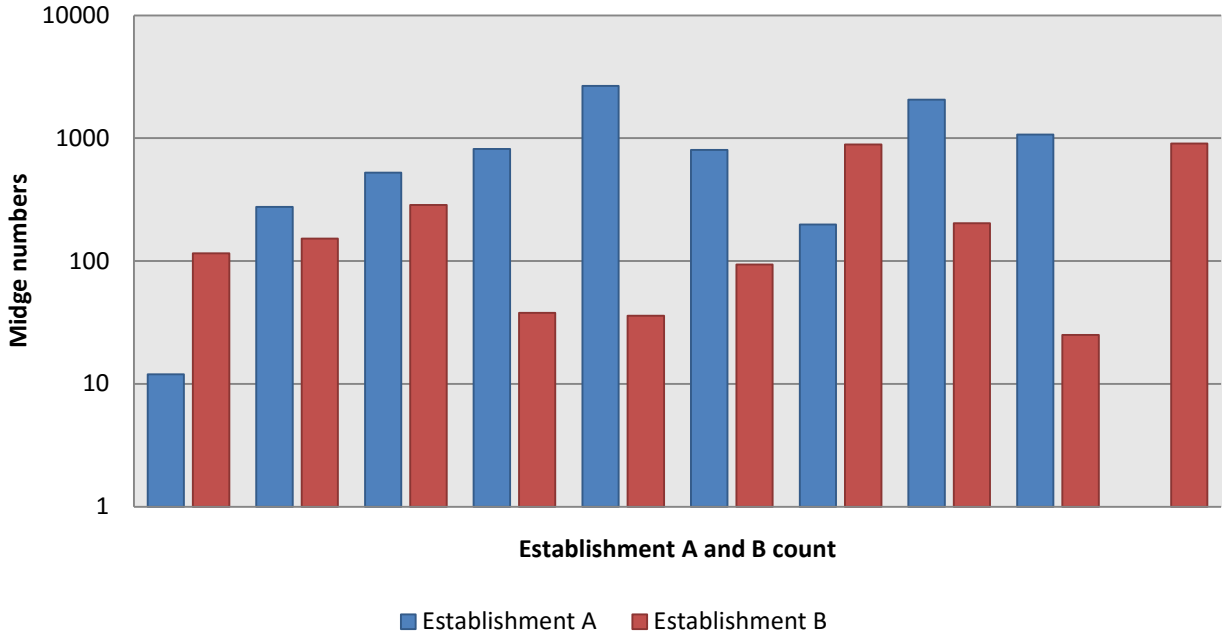

Figure 20: Comparison of pool midge numbers at the participating establishments.

Of the 91 pooled *Culicoides* midge collections, only two pools (2.2%; 2/91) revealed positive results by RT-qPCR for the presence of the equine orbiviruses tested for, one positive for the presence of AHSV (Yellow) and the other EEV (Green) (Tables 9 and 10). The applied RT-qPCR detected AHSV and EEV RNA; this was clearly evident in the C_T values obtained, it could not be determined whether this was due to the viruses having replicated in one positive midge, or because a large number of midges were positive in the 2 pools.

Table 9: C_T value of *Culicoides* midge pools tested for AHSV and EEV by RT-qPCR collected from Establishment A.

Pool Sample Number	Collection Date	Gender & Parity (*C. imicola*, *C. bolitinos*)	C_T Value AHS	AHS Status	C_T Value EEV	EEV Status
ACM0002a	27/12/2014	Parous Females	40	Negative	40	Negative
ACM0002b		Nulliparous Females	40	Negative	40	Negative
ACM0002d		Males	40	Negative	40	Negative
ACM0004a	10/01/2014	Parous Females	40	Negative	40	Negative
ACM0004b		Nulliparous Females	40	Negative	40	Negative
ACM0004d		Males	40	Negative	40	Negative
ACM0005a1	25/01/2014	Parous Females	40	Negative	40	Negative
ACM0005a2		Parous Females	40	Negative	40	Negative
ACM0005b		Nulliparous Females	40	Negative	40	Negative
ACM0005c		Blood fed Females	40	Negative	40	Negative
ACM0005d		Males	40	Negative	40	Negative
ACM0008a1	08/02/2014	Parous Females	40	Negative	40	Negative
ACM0008a2		Parous Females	40	Negative	40	Negative
ACM0008a3		Parous Females	40	Negative	40	Negative
ACM0008b		Nulliparous Females	40	Negative	40	Negative
ACM0008c		Blood fed Females	40	Negative	40	Negative
ACM0008d		Males	40	Negative	40	Negative
ACM0010a1	24/02/2014	Parous Females	40	Negative	40	Negative
ACM0010a2		Parous Females	40	Negative	40	Negative
ACM0010a3		Parous Females	40	Negative	40	Negative
ACM0010a4		Parous Females	40	Negative	40	Negative
ACM0010a5		Parous Females	40	Negative	40	Negative
ACM0010a6		Parous Females	40	Negative	40	Negative
ACM0010a7		Parous Females	40	Negative	40	Negative
ACM0010a8		Parous Females	40	Negative	40	Negative
ACM0010a9		Parous Females	40	Negative	40	Negative
ACM0010a10		Parous Females	40	Negative	40	Negative
ACM0010a11		Parous Females	40	Negative	40	Negative
ACM0010b		Nulliparous Females	40	Negative	40	Negative
ACM0010c		Blood fed Females	40	Negative	40	Negative

ACM0010d		Males	40	Negative	40	Negative
ACM0012a1	22/03/2014	Parous Females	40	Negative	**34.10302734**	**Positive**
ACM0012a2		Parous Females	40	Negative	40	Negative
ACM0012a3		Parous Females	40	Negative	40	Negative
ACM0012b		Nulliparous Females	40	Negative	40	Negative
ACM0012c		Blood fed Females	40	Negative	40	Negative
ACM0012d		Males	40	Negative	40	Negative
ACM0014a	10/04/2014	Parous Females	40	Negative	40	Negative
ACM0014b		Nulliparous Females	40	Negative	40	Negative
ACM0016a1	24/04/2014	Parous Females	40	Negative	40	Negative
ACM0016a2		Parous Females	40	Negative	40	Negative
ACM0016a3		Parous Females	40	Negative	40	Negative
ACM0016a4		Parous Females	40	Negative	40	Negative
ACM0016a5		Parous Females	40	Negative	40	Negative
ACM0016a6		Parous Females	40	Negative	40	Negative
ACM0016b		Nulliparous Females	40	Negative	40	Negative
ACM0016d		Males	40	Negative	40	Negative
ACM0018a1	09/05/2014	Parous Females	40	Negative	40	Negative
ACM0018a2		Parous Females	40	Negative	40	Negative
ACM0018a3		Parous Females	40	Negative	40	Negative
ACM0018a4		Parous Females	40	Negative	40	Negative
ACM0018a5		Parous Females	40	Negative	40	Negative
ACM0018b		Nulliparous Females	40	Negative	40	Negative
ACM0018c		Blood fed Females	40	Negative	40	Negative
ACM0018d		Males	40	Negative	40	Negative

Table 10: C_T value of *Culicoides* midge pools tested for AHSV and EEV by RT-qPCR collected from Establishment B.

Pool Sample Number	Collection Date	Gender & Parity (*C. imicola*, *C. bolitinos*)	C_T Value AHS	AHS Status	C_T Value EEV	EEV Status
ACM0001a	27/12/2014	Parous Females	40	Negative	40	Negative
ACM0001b		Nulliparous Females	40	Negative	40	Negative
ACM0001c		Blood fed Females	40	Negative	40	Negative
ACM0001d		Males	40	Negative	40	Negative
ACM0003a	10/01/2014	Parous Females	40	Negative	40	Negative
ACM0003b		Nulliparous Females	40	Negative	40	Negative
ACM0003c		Blood fed Females	40	Negative	40	Negative
ACM0003d		Males	40	Negative	40	Negative
ACM0006a	25/01/2014	Parous Females	40	Negative	40	Negative
ACM0006b		Nulliparous Females	40	Negative	40	Negative
ACM0006c		Blood fed Females	40	Negative	40	Negative
ACM0007a	05/02/2014	Parous Females	40	Negative	40	Negative
ACM0007c		Blood fed Females	40	Negative	40	Negative
ACM0009a	14/02/2014	Parous Females	40	Negative	40	Negative
ACM0009b		Nulliparous Females	40	Negative	40	Negative
ACM0009d		Males	40	Negative	40	Negative
ACM0011a	01/03/2014	Parous Females	40	Negative	40	Negative
ACM0011b		Nulliparous Females	40	Negative	40	Negative
ACM0013a1	27/03/2014	Parous Females	40	Negative	40	Negative
ACM0013a2		Parous Females	40	Negative	40	Negative
ACM0013a3		Parous Females	40	Negative	40	Negative
ACM0013a4		Parous Females	40	Negative	40	Negative
ACM0013b		Nulliparous Females	40	Negative	40	Negative
ACM0013c		Blood fed Females	40	Negative	40	Negative
ACM0013d		Males	40	Negative	40	Negative
ACM0015a	10/04/2014	Parous Females	40	Negative	40	Negative
ACM0015b		Nulliparous Females	40	Negative	40	Negative

ACM0017a	26/04/2014	Parous Females	40	Negative	40	Negative
ACM0017b		Nulliparous Females	40	Negative	40	Negative
ACM0019a1	09/05/2014	Parous Females	40	Negative	40	Negative
ACM0019a1		Parous Females	40	Negative	40	Negative
ACM0019a2		Parous Females	40	Negative	40	Negative
ACM0019a3		Parous Females	**37.16783524**	**Positive**	40	Negative
ACM0019b4		Nulliparous Females	40	Negative	40	Negative
ACM0019c		Blood fed Females	40	Negative	40	Negative
ACM0019d		Males	40	Negative	40	Negative

CHAPTER 4

DISCUSSION AND CONCLUSION

African horse sickness is a controlled disease in terms of the Animal Diseases Act (Act No 35 of 1984) in South Africa. It affects all equids with horses being the most susceptible and often suffering high mortalities. The disease has a significant effect on the equine industry and international equine trade. It is controlled by annual vaccination using the vaccine produced at OBP, the only registered vaccine available in South Africa.

The control of the disease seems an irremediable task at present, therefore South African horse owners in spite of vaccination need to implement appropriate alternatives for effective management and prevention of infection by the AHSV.

This led to the investigation into equine orbiviral dynamics, evaluating the infection rates of AHSV on two equine establishments with contrasting infrastructure and management protocols; where a number of different prophylactic methods were used, aimed at reducing the likelihood of AHS infection. In addition to AHSV, EEV was also included in the study as a useful tool when evaluating equine orbivirus dynamics as infection can only be by means of wild-type virus as no commercial vaccine is currently available.

Upon initial pursuit, Establishment A was found to be the model establishment (Control Establishment), where a collection of biosecurity measures were followed for the prevention of most infectious agents of concern. Good record keeping and reporting systems were in place for any abnormality. Rectal temperatures were taken twice daily and insect repellent (Deltab ® - MSD Animal Health) was applied regularly. Insect proof accommodation was of primary concern together with eliminating the insect breeding sites through general establishment cleanliness (Figure 21).

Vaccination protocols followed were those recommended by the vaccine manufacturers and that of the regular visiting private veterinarians. Artificial wind was created as a preventative measure using ceiling fans, and insect light traps were installed in stables in an attempt to lower midge numbers in stable blocks due to the midge's natural endophilic habits (Figure 22).

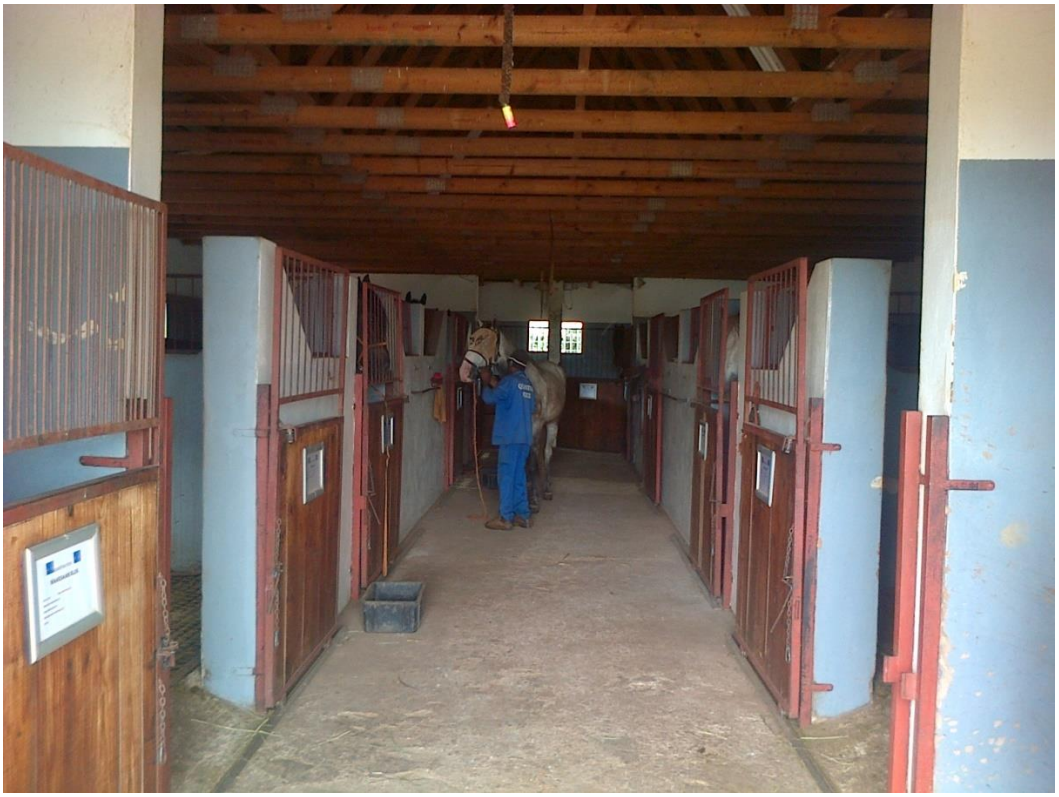

Figure 21: Stable cleanliness of Establishment A.

Figure 22: Insect control Establishment A.

In contrast, Establishment B was an old farming enterprise with a poor infrastructure and dilapidated stabling facilities (Figure 23). Preventative measures were rarely practiced and the equines lived out, free to roam the farm including those low lying water catchment areas providing an adequate breading habitat for the associated vectors.

The prevalence of AHS recorded during the present study was lower than in previous reports, where according to the owner of Establishment B; AHS claimed the lives of 8 (27%) horses during the 2013 season. This was confirmed by the local veterinarian (Packer, G.C. pers comm, 30/11/2013), suggesting that fewer animals were immune during the previous establishment outbreaks.

Vaccination was not part of the management regime in the previous seasons; although prior to the start of the study after consultation with veterinary officials, a decision was made by the management of Establishment B to vaccinate the herd to circumvent the previous season's mortality rates, together with confining the herd to paddocks during the evenings with frequent use of an insect repellent (CyLance® – Bayer Animal Health). Upon further investigation after consultation with the farm management, only 89% of the horses involved in the study had actually received the vaccination.

Figure 23: Infrastructure Establishment B.

When evaluating the blood samples, a total of nine (32%) cases of AHSV and five (18%) cases of EEV were identified in the 28 horses included in this study. Of the positive AHS cases; five occurred at Establishment A and four at Establishment B whereas positive cases of EEV at Establishment A and B amounted to one and four, respectively. An important finding from this study was that all positive cases were subclinical for both viruses; where the minimum C_T value was 33.4 for AHS and 33.7 for EEV.

Since vaccination was practiced on both establishments, this supports the hypothesis described by Weyer *et al.* (2013) that vaccinated horses can still become infected subclinically, producing a detectable viraemia using PCR. Viraemic horses infected under normal field conditions may be a source of virus, infecting midges, ergo pose risk for the transmission of the AHSV into naïve population if they are moved (Weyer *et al*. 2013).

In order to avoid infection of arthropod vectors, only attenuated viruses that generate titres $<10^3$ $TCID_{50}$/ml in vaccinated animals are acceptable for vaccine production (Paweska *et al.* 2003), however the possible circulation of AHS vaccine virus in the field, poses several problems that must be considered when vaccination occurs with the only registered vaccine using live attenuated strains. There is a distinct need to distinguish between wild-type and vaccine virus where this will broaden the understanding of the current epidemiological situation, as was found with the active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy (Ferrari *et al.* 2005). Without further investigation severe economic losses will be the likely outcome which will continually increase.

When evaluating the safety of vaccines, the active circulation of attenuated live virus would pose problems including the possible reversion to virulence, an eventuality marked as critical, as described by the South African Veterinary Association in 2015 with the recent 2014 Porterville AHS outbreak; Where according to the South African Veterinary Association, the outbreak was likely due to recombination of AHS vaccine strains which were successfully transmitted by *Culicoides* midges to susceptible equine populations. The potential of reassortment with other AHSV strains, due to the simultaneous circulation of wild and vaccine strains warrants further investigation in AHSV endemic areas. The level of the viraemia required for horses to infect midges is not yet known (Venter *et al*. 2000; Venter & Paweska 2007; Weyer *et al.* 2013).

In an attempt to further understand the low disease seasonal prevalence at the participating establishments; midge collections were evaluated involving detection of virus in pools of collected midges. Entomological risk assessments of vector-borne diseases obtained through surveys influence decision making when implementing the best control measure/s by establishing effective qualitative and quantitative detection of all potential viruses transmitted by *Culicoides* midges (Venter *et al.* 2012).

Collection procedures closely followed that of Venter *et al.* (2009b), where in a comparative study in South Africa, the 220 V down-draught Onderstepoort black-light trap proved to collect significantly more *Culicoides* midges under field conditions than other traps (Rieb, mini-CDC, Pirbright and BG-sentinel).

Light trap results need to be linked to what is known about the *Culicoides* spp.; biology, capacity and competence and is essential for accessing the percentage of female *Culicoides* that could actually transmit virus.

Due to many events that must occur in order to allow for the successful transmission of viruses i.e. the variety of climatic conditions, the physiological status; including mesenteronal infection escape barriers, dissemination barriers, transovarial transmission barriers, and salivary gland infection escape barriers and the host seeking ability of the C*ulicoides* females, the likelihood of one individual midge fulfilling all these criteria is extremely low. Compensation for this is usually by the considerable midge population sizes (Venter *et al.* 2012).

In the present study, no AHSV was detected in the 55 *Culicoides* pools collected from Establishment A, however at Establishment B; AHSV was detected in one of the 36 pools resulting in a field infection prevalence of 2.7 %.

When evaluating EE, one pool of the 55 *Culicoides* pools collected from Establishment A detected EEV with a field infection prevalence of 1.8 % opposed to none detected in the 36 *Culicoides* pools collected from Establishment B.

During an outbreak analysis of AHSV and EEV infection in 1999 by Venter *et al.* (2006), the field infection prevalence was found to be 0.003% and 0.038%, respectively. Although the results from this study represent a higher infection percentage than those published by Venter *et al.* (2006) as did the results of Scheffer *et al.* (2012). It was suggested that the increased sensitivity of the tests used by Scheffer *et al.* (2012), the same tests used in this study is a likely response for the observed differences together with the fact that the collection areas differed in seasonal rainfall patterns, although *C. bolitinos* is less dependent on annual precipitation than seen in *C. imicola* (Venter *et al.* 2006).

The data collected concurred with recent observations of Venter (2015) (Venter, GJ [OVI-Entomology] pers comm, 29/10/2015) that the general prevalence of the diseases was lower during the study period than that experienced during previous seasons.

The fact that AHSV and EEV could be identified from field-collected midge pools in the study confirms that vector competent populations are present in and around both establishments.

When taking into account the relatively lower number of reported AHS cases in and around the study area during the collection period, this could be one likely explanation for the low infection prevalence detected in field-collected midges on each establishment (Venter *et al.* 2006). This phenomenon was confirmed by the study of Durand *et al.* (2010) during the BTV epidemic in France, where an increase in cattle density resulted in lower seropositivity in the area the cattle were introduced, therefore this correlates to the study's findings, whereby the lower number of viraemic horses in the immediate area is directly proportional to the lower field infection prevalence found in those collected midges, where focus is directed at a potential dilution effect.

Furthermore, Lacono *et al.* (2013) suggested more data needs to be made available when assessing current disease frameworks, analysing how non-susceptible hosts are likely to affect the risk of AHS epidemics.

The low number of positive midges found in this study during 2014 could also be explained by the very active midge season of 2013, where the prevalence of the diseases is dependent on a build-up of virus which must first reach a critical level, thereafter spill over will occur into associated equine populations. Vector population size during the current study was also significantly lower than expected, reducing

amplification of the viruses in vector populations which coincides with current prevalence of both AHS and EE (Venter *et al.* 1997).

It is also worth noting that when comparing the midge numbers for both establishments, a significant difference was seen. *Culicoides* midge composition and population size at any collection site may vary dramatically within a relatively short period of time (Venter *et al.* 2012). Both establishments had sufficient water in various low lying terrains creating favourable oviposition sites yet midge numbers varied substantially (Figure 20).

In conclusion, it has been shown that natural infection of horses with wild-type virus will induce a broader and stronger cross-reactive immunity than that observed by attenuated vaccine serotypes (Blackburn & Swanepoel 1988). This fact which in itself lends an authority of truth, whereby continuous herd exposure to these viruses in previous season and now a greater proportion of vaccinated animals, incorporated greater herd immunity and this together with various other prophylactic measures increased the probability of protection against AHS, presenting lower disease prevalence. This was clearly demonstrated during the study whereby a more proactive approach to controlling the disease at establishment B significantly reduced the number of clinical cases and subsequently a lower mortality rate than experienced in the past. The vaccination strategies used, aimed at preventing virus amplification in equines therefore disrupting incipient AHSV outbreaks by both reducing the potential for secondary vector spill over and eliminating the threat posed by infected tissues as described by Bird *et al.* (2011) when evaluating Rift Valley fever in sheep.

Timing of vaccination plays an important role on the development of an appropriate immune response and on the overall control of the disease with the greater possibility of vaccine transmission by vectors, highlighting the need to review current vaccination strategies being practiced nationally (DAFF 2015). More studies are needed to verify the possibility of the vaccine vireamia in susceptible species and the effective competence of *Culicoides* vectors for the vaccine strains, where such data could aid in better understanding the epidemiology of AHSV circulation. Whilst moving forward in reducing the risk of orbiviral infections, improvement of our understanding of how variations of AHSV and EEV can alter their capacity to infect horses either clinically or subclinically, aswell as the influence of transmission between *Culicoides* midge vectors and equine hosts within various ecosystems, environmental conditions, including the analysis and use of the various available prophylactic measures is required.

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APPENDIX 1: RT-qPCR DATA IN ALL PARTICIPATING EQUINES

Microchip Number	Farm	Rectal Temperature	Date Collected	Sample Number	AHS C_T	AHS Status	EEV C_T	EEV Status
710098100125858	B	38.3	2013/12/20	AC00005	40	Negative	40	Negative
710098100125858	B	37.8	2014/01/03	AC00032	40	Negative	40	Negative
710098100125858	B	38.4	2014/01/25	AC00073	40	Negative	40	Negative
710098100125858	B	37.8	2014/02/05	AC00089	40	Negative	40	Negative
710098100125858	B	39.3	2014/02/14	AC00117	40	Negative	40	Negative
710098100125858	B	37.8	2014/02/28	AC00142	40	Negative	40	Negative
710098100125858	B	37.7	2014/03/14	AC00173	40	Negative	40	Negative
710098100125858	B	37.2	2014/03/28	AC00205	40	Negative	40	Negative
710098100125858	B	37.7	2014/04/11	AC00210	40	Negative	40	Negative
710098100125858	B	38	2014/04/25	AC00258	40	Negative	40	Negative
710098100125858	B	37.1	2014/05/09	AC00267	40	Negative	40	Negative
710098100125858	B	37.4	2014/05/24	AC00294	40	Negative	40	Negative
710098100126509	B	38.4	2013/12/20	AC00003	40	Negative	40	Negative
710098100126509	B	38.1	2014/01/03	AC00030	40	Negative	40	Negative
710098100126509	B	40.3	2014/01/25	AC00075	40	Negative	40	Negative
710098100126509	B	37.3	2014/02/05	AC00085	40	Negative	40	Negative
710098100126509	B	37.1	2014/02/14	AC00113	40	Negative	40	Negative
710098100126509	B	38.2	2014/02/28	AC00143	40	Negative	40	Negative
710098100126509	B	38	2014/03/14	AC00176	40	Negative	40	Negative
710098100126509	B	36	2014/03/28	AC00207	40	Negative	40	Negative
710098100126509	B	38	2014/04/11	AC00212	40	Negative	40	Negative
710098100126509	B	38.5	2014/04/25	AC00255	40	Negative	40	Negative
710098100126509	B	*	2014/05/30	AC00320	38.433086	Negative	40	Negative
710098100130556	B	38.7	2013/12/20	AC00004	40	Negative	40	Negative
710098100130556	B	38.7	2014/01/03	AC00031	40	Negative	40	Negative
710098100130556	B	38.5	2014/01/25	AC00074	**36.94164**	**Positive**	40	Negative
710098100130556	B	37.6	2014/02/05	AC00086	40	Negative	40	Negative
710098100130556	B	37.3	2014/02/14	AC00114	40	Negative	**34.987625**	**Positive**
710098100130556	B	38.1	2014/02/28	AC00141	40	Negative	**37.845127**	**Positive**
710098100130556	B	37.7	2014/03/14	AC00180	40	Negative	**38.7122**	**Positive**
710098100130556	B	36.2	2014/03/28	AC00209	40	Negative	39.44459	Negative
710098100130556	B	38	2014/04/11	AC00218	40	Negative	40	Negative
710098100130556	B	37.8	2014/04/25	AC00257	40	Negative	40	Negative
710098100130556	B	37.5	2014/05/09	AC00266	40	Negative	**36.64067**	**Positive**
710098100130556	B	37.9	2014/05/24	AC00293	40	Negative	40	Negative
710098100129464	B	36.3	2013/12/20	AC00012	40	Negative	40	Negative
710098100129464	B	37.4	2014/01/03	AC00034	40	Negative	40	Negative
710098100129464	B	37	2014/01/25	AC00077	40	Negative	40	Negative
710098100129464	B	35.1	2014/02/05	AC00096	40	Negative	40	Negative
710098100129464	B	36.1	2014/02/14	AC00124	40	Negative	40	Negative

710098100129464	B	36.7	2014/02/28	AC00151	40	Negative	40	Negative
710098100129464	B	35.7	2014/03/14	AC00172	40	Negative	40	Negative
710098100129464	B	35.4	2014/03/28	AC00197	40	Negative	40	Negative
710098100129464	B	36.2	2014/04/11	AC00219	40	Negative	40	Negative
710098100129464	B	37.1	2014/04/25	AC00265	40	Negative	40	Negative
710098100129464	B	37.1	2014/05/09	AC00276	40	Negative	40	Negative
710098100129464	B	36.2	2014/05/24	AC00303	40	Negative	40	Negative
710098100130148	B	36.1	2013/12/20	AC00002	40	Negative	40	Negative
710098100130148	B	37.2	2014/01/03	AC00033	40	Negative	40	Negative
710098100130148	B	37	2014/01/25	AC00076	40	Negative	40	Negative
710098100130148	B	36.7	2014/02/05	AC00088	40	Negative	40	Negative
710098100130148	B	38.5	2014/02/14	AC00116	40	Negative	40	Negative
710098100130148	B	37.2	2014/02/28	AC00152	40	Negative	40	Negative
710098100130148	B	34.4	2014/03/14	AC00175	40	Negative	**34.14454**	**Positive**
710098100130148	B	35.4	2014/03/28	AC00200	40	Negative	39.14527	Negative
710098100130148	B	37.5	2014/04/11	AC00217	**35.182747**	**Positive**	**38.139355**	**Positive**
710098100130148	B	37.6	2014/04/25	AC00261	38.536938	Negative	40	Negative
710098100130148	B	36.4	2014/05/09	AC00273	40	Negative	40	Negative
710098100130148	B	37.2	2014/05/24	AC00300	40	Negative	40	Negative
710098100127763	B	37.4	2013/12/20	AC00013	40	Negative	40	Negative
710098100127763	B	36.8	2014/01/03	AC00041	40	Negative	40	Negative
710098100127763	B	37.1	2014/01/25	AC00084	40	Negative	40	Negative
710098100127763	B	35.4	2014/02/05	AC00097	40	Negative	40	Negative
710098100127763	B	35.4	2014/02/14	AC00125	40	Negative	40	Negative
710098100127763	B	36.8	2014/02/28	AC00145	40	Negative	40	Negative
710098100127763	B	33.6	2014/03/14	AC00170	40	Negative	40	Negative
710098100127763	B	34.6	2014/03/28	AC00201	40	Negative	40	Negative
710098100127763	B	37.4	2014/04/11	AC00216	40	Negative	40	Negative
710098100127763	B	37.2	2014/04/25	AC00260	40	Negative	40	Negative
710098100127763	B	36.1	2014/05/09	AC00277	40	Negative	40	Negative
710098100127763	B	36.1	2014/05/24	AC00304	40	Negative	40	Negative
710098100127723	B	35.6	2013/12/20	AC00001	40	Negative	40	Negative
710098100127723	B	35	2014/01/03	AC00029	40	Negative	40	Negative
710098100127723	B	36.9	2014/01/25	AC00072	40	Negative	40	Negative
710098100127723	B	35.8	2014/02/05	AC00087	40	Negative	40	Negative
710098100127723	B	36.3	2014/02/14	AC00115	40	Negative	**36.18474**	**Positive**
710098100127723	B	37.3	2014/02/28	AC00153	40	Negative	**35.259964**	**Positive**
710098100127723	B	36.8	2014/03/14	AC00179	40	Negative	**36.511787**	**Positive**
710098100127723	B	37.1	2014/03/28	AC00199	40	Negative	**36.31506**	**Positive**
710098100127723	B	36.6	2014/04/11	AC00220	40	Negative	**37.77404**	**Positive**
710098100127723	B	37.3	2014/04/25	AC00254	40	Negative	39.561226	Negative
710098100127723	B	36.8	2014/05/09	AC00268	40	Negative	**38.363728**	**Positive**
710098100127723	B	36.3	2014/05/24	AC00295	40	Negative	40	Negative

710098100130777	B	37.3	2013/12/20	AC00009	40	Negative	40	Negative
710098100130777	B	37.9	2014/01/03	AC00036	40	Negative	40	Negative
710098100130777	B	38.8	2014/01/25	AC00080	37.32535	Negative	40	Negative
710098100130777	B	37.6	2014/02/05	AC00093	37.275482	Negative	40	Negative
710098100130777	B	36.9	2014/02/14	AC00121	40	Negative	40	Negative
710098100130777	B	37.4	2014/02/28	AC00144	40	Negative	40	Negative
710098100130777	B	37.2	2014/03/14	AC00178	40	Negative	40	Negative
710098100130777	B	35.1	2014/03/28	AC00202	40	Negative	40	Negative
710098100130777	B	37.8	2014/04/11	AC00214	40	Negative	40	Negative
710098100130777	B	38.1	2014/04/25	AC00262	40	Negative	40	Negative
710098100130777	B	36.2	2014/05/09	AC00272	40	Negative	40	Negative
710098100130777	B	37.3	2014/05/24	AC00299	**33.40537**	**Positive**	40	Negative
710098100127433	B	37.8	2013/12/20	AC00006	40	Negative	40	Negative
710098100127433	B	38	2014/01/03	AC00040	40	Negative	40	Negative
710098100127433	B	38.8	2014/01/25	AC00082	40	Negative	40	Negative
710098100127433	B	37.1	2014/02/05	AC00090	40	Negative	40	Negative
710098100127433	B	36.4	2014/02/14	AC00118	40	Negative	40	Negative
710098100127433	B	37.5	2014/02/28	AC00149	40	Negative	40	Negative
710098100127433	B	37.7	2014/03/14	AC00169	40	Negative	40	Negative
710098100127433	B	36.1	2014/03/28	AC00198	40	Negative	40	Negative
710098100127433	B	37.5	2014/04/11	AC00215	40	Negative	40	Negative
710098100127433	B	37.7	2014/04/25	AC00256	40	Negative	40	Negative
710098100127433	B	37	2014/05/09	AC00269	**37.030083**	**Positive**	40	Negative
710098100127433	B	37.5	2014/05/24	AC00296	37.833683	Negative	40	Negative
710098100130507	B	37.1	2013/12/20	AC00011	38.47143	Negative	40	Negative
710098100130507	B	37	2014/01/03	AC00035	40	Negative	40	Negative
710098100130507	B	37.7	2014/01/25	AC00079	40	Negative	40	Negative
710098100130507	B	37.2	2014/02/05	AC00095	40	Negative	40	Negative
710098100130507	B	37.1	2014/02/14	AC00123	38.272655	Negative	40	Negative
710098100130507	B	37.7	2014/02/28	AC00146	40	Negative	40	Negative
710098100130507	B	38.1	2014/03/14	AC00174	40	Negative	40	Negative
710098100130507	B	36.4	2014/03/28	AC00206	**34.44421**	**Positive**	40	Negative
710098100130507	B	37.7	2014/04/11	AC00211	37.635807	Negative	40	Negative
710098100130507	B	38	2014/04/25	AC00259	40	Negative	40	Negative
710098100130507	B	37	2014/05/09	AC00275	40	Negative	40	Negative
710098100130507	B	37	2014/05/24	AC00302	40	Negative	40	Negative
710098100129432	B	37.6	2013/12/20	AC00010	40	Negative	40	Negative
710098100129432	B	38.1	2014/01/03	AC00037	40	Negative	40	Negative
710098100129432	B	38.6	2014/01/25	AC00083	40	Negative	40	Negative
710098100129432	B	37.3	2014/02/05	AC00094	40	Negative	40	Negative
710098100129432	B	36.7	2014/02/14	AC00122	40	Negative	40	Negative
710098100129432	B	37.7	2014/02/28	AC00147	40	Negative	40	Negative
710098100129432	B	38.2	2014/03/14	AC00181	40	Negative	40	Negative
710098100129432	B	36.7	2014/03/28	AC00203	40	Negative	40	Negative

710098100129432	B	37.6	2014/04/11	AC00222	40	Negative	40	Negative
710098100129432	B	38	2014/04/25	AC00253	40	Negative	40	Negative
710098100129432	B	37.5	2014/05/09	AC00274	40	Negative	40	Negative
710098100129432	B	37	2014/05/24	AC00301	40	Negative	40	Negative
710098100130251	B	37.1	2013/12/20	AC00008	40	Negative	40	Negative
710098100130251	B	37.3	2014/01/03	AC00039	40	Negative	40	Negative
710098100130251	B	37.2	2014/01/25	AC00081	40	Negative	40	Negative
710098100130251	B	37.3	2014/02/05	AC00092	40	Negative	40	Negative
710098100130251	B	37.4	2014/02/14	AC00120	40	Negative	38.74502	Negative
710098100130251	B	38	2014/02/28	AC00148	40	Negative	39.140057	Negative
710098100130251	B	37.4	2014/03/14	AC00171	40	Negative	39.91877	Negative
710098100130251	B	36.8	2014/03/28	AC00204	40	Negative	40	Negative
710098100130251	B	37.4	2014/04/11	AC00221	40	Negative	40	Negative
710098100130251	B	37.6	2014/04/25	AC00263	40	Negative	40	Negative
710098100130251	B	36.9	2014/05/09	AC00271	40	Negative	40	Negative
710098100130251	B	37.7	2014/05/24	AC00298	40	Negative	40	Negative
710098100126782	B	37.5	2013/12/20	AC00007	39.68846	Negative	40	Negative
710098100126782	B	37.9	2014/01/03	AC00038	40	Negative	40	Negative
710098100126782	B	38.5	2014/01/25	AC00078	40	Negative	40	Negative
710098100126782	B	37.6	2014/02/05	AC00091	40	Negative	40	Negative
710098100126782	B	37.6	2014/02/14	AC00119	39.955856	Negative	40	Negative
710098100126782	B	38.2	2014/02/28	AC00150	40	Negative	40	Negative
710098100126782	B	38	2014/03/14	AC00177	40	Negative	**35.36701**	**Positive**
710098100126782	B	36.4	2014/03/28	AC00208	40	Negative	**37.5444**	**Positive**
710098100126782	B	38.1	2014/04/11	AC00213	40	Negative	38.737762	Negative
710098100126782	B	38.1	2014/04/25	AC00264	40	Negative	**38.123028**	**Positive**
710098100126782	B	38	2014/05/09	AC00270	40	Negative	40	Negative
710098100126782	B	37.4	2014/05/24	AC00297	40	Negative	**37.869915**	**Positive**

Microchip Number	Farm	Rectal Temperature	Date Collected	Sample Number	AHS C_T	AHS Status	EEV C_T	EEV Status
710098100130555	A	36.7	2013/12/27	AC00021	40	Negative	40	Negative
710098100130555	A	37.1	2014/01/10	AC00042	40	Negative	40	Negative
710098100130555	A	36.8	2014/01/25	AC00057	40	Negative	40	Negative
710098100130555	A	36.8	2014/02/07	AC00106	40	Negative	40	Negative
710098100130555	A	36.4	2014/02/24	AC00127	40	Negative	40	Negative
710098100130555	A	36.6	2014/03/07	AC00162	40	Negative	40	Negative
710098100130555	A	37	2014/03/20	AC00190	40	Negative	40	Negative
710098100130555	A	36.5	2014/04/11	AC00231	40	Negative	40	Negative
710098100130555	A	36.7	2014/04/25	AC00240	40	Negative	40	Negative
710098100130555	A	36.9	2014/05/09	AC00286	40	Negative	40	Negative
710098100130555	A	37.6	2014/05/23	AC00313	40	Negative	40	Negative
985141000622384	A	37.4	2013/12/27	AC00023	40	Negative	40	Negative

985141000622384	A	37.4	2014/01/10	AC00044	40	Negative	40	Negative
985141000622384	A	37.1	2014/01/25	AC00059	40	Negative	40	Negative
985141000622384	A	37.1	2014/02/07	AC00108	40	Negative	40	Negative
985141000622384	A	36.8	2014/02/24	AC00131	40	Negative	40	Negative
985141000622384	A	36.9	2014/03/07	AC00155	40	Negative	40	Negative
985141000622384	A	37.2	2014/03/20	AC00183	40	Negative	40	Negative
985141000622384	A	37.4	2014/04/11	AC00224	40	Negative	40	Negative
985141000622384	A	37	2014/04/25	AC00243	40	Negative	40	Negative
985141000622384	A	37.7	2014/05/09	AC00279	40	Negative	40	Negative
985141000622384	A	37.4	2014/05/23	AC00306	40	Negative	40	Negative
985141000622467	A	36.9	2013/12/27	AC00028	40	Negative	40	Negative
985141000622467	A	36.5	2014/01/10	AC00048	40	Negative	40	Negative
985141000622467	A	38.3	2014/01/25	AC00062	40	Negative	40	Negative
985141000622467	A	37.2	2014/02/07	AC00112	40	Negative	40	Negative
985141000622467	A	37.4	2014/02/24	AC00134	40	Negative	40	Negative
985141000622467	A	37.6	2014/03/07	AC00168	40	Negative	40	Negative
985141000622467	A	37	2014/03/20	AC00196	40	Negative	40	Negative
985141000622467	A	36.6	2014/04/11	AC00237	**35.515438**	**Positive**	39.085754	Negative
985141000622467	A	37	2014/04/25	AC00242	40	Negative	40	Negative
985141000622467	A	36.9	2014/05/09	AC00292	40	Negative	40	Negative
985141000622467	A	37.8	2014/05/23	AC00319	40	Negative	40	Negative
985141000621846	A	37.9	2013/12/27	AC00016	40	Negative	40	Negative
985141000621846	A	37.7	2014/01/10	AC00051	40	Negative	40	Negative
985141000621846	A	37.5	2014/01/25	AC00065	40	Negative	40	Negative
985141000621846	A	37.4	2014/02/07	AC00099	40	Negative	40	Negative
985141000621846	A	37.4	2014/02/24	AC00129	40	Negative	40	Negative
985141000621846	A	37.6	2014/03/07	AC00157	40	Negative	40	Negative
985141000621846	A	37.4	2014/03/20	AC00185	40	Negative	40	Negative
985141000621846	A	37.6	2014/04/11	AC00226	40	Negative	40	Negative
985141000621846	A	37.1	2014/04/25	AC00252	40	Negative	40	Negative
985141000621846	A	37.4	2014/05/09	AC00281	40	Negative	40	Negative
985141000621846	A	37.9	2014/05/23	AC00308	40	Negative	40	Negative
978000001359835	A	37.4	2013/12/27	AC00015	40	Negative	40	Negative
978000001359835	A	37.7	2014/01/10	AC00053	40	Negative	40	Negative
978000001359835	A	37.5	2014/01/25	AC00067	40	Negative	40	Negative
978000001359835	A	37.6	2014/02/07	AC00101	40	Negative	40	Negative
978000001359835	A	37.4	2014/02/24	AC00136	40	Negative	40	Negative
978000001359835	A	37.1	2014/03/07	AC00159	40	Negative	40	Negative
978000001359835	A	36.7	2014/03/20	AC00187	40	Negative	40	Negative
978000001359835	A	37.3	2014/04/11	AC00228	40	Negative	40	Negative
978000001359835	A	37	2014/04/25	AC00250	40	Negative	40	Negative
978000001359835	A	37.5	2014/05/09	AC00283	40	Negative	40	Negative
978000001359835	A	37.2	2014/05/23	AC00310	40	Negative	40	Negative

978000001354445	A	37.7	2013/12/27	AC00020	40	Negative	40	Negative
978000001354445	A	37.9	2014/01/10	AC00056	40	Negative	40	Negative
978000001354445	A	37.7	2014/01/25	AC00070	40	Negative	40	Negative
978000001354445	A	37.6	2014/02/07	AC00104	40	Negative	40	Negative
978000001354445	A	37.7	2014/02/24	AC00137	40	Negative	40	Negative
978000001354445	A	37.4	2014/03/07	AC00160	40	Negative	40	Negative
978000001354445	A	37.7	2014/03/20	AC00188	40	Negative	40	Negative
978000001354445	A	37.6	2014/04/11	AC00229	40	Negative	40	Negative
978000001354445	A	37.3	2014/04/25	AC00249	40	Negative	40	Negative
978000001354445	A	37.9	2014/05/09	AC00284	40	Negative	40	Negative
978000001354445	A	37.7	2014/05/23	AC00311	40	Negative	40	Negative
985141000622341	A	37.1	2013/12/27	AC00024	38.110664	Negative	40	Negative
985141000622341	A	37.5	2014/01/10	AC00045	**34.312836**	**Positive**	40	Negative
985141000622341	A	37.4	2014/01/25	AC00060	**35.408794**	**Positive**	40	Negative
985141000622341	A	37.7	2014/02/07	AC00109	**33.98109**	**Positive**	40	Negative
985141000622341	A	37.4	2014/02/24	AC00132	39.26422	Negative	40	Negative
985141000622341	A	37.4	2014/03/07	AC00165	39.259235	Negative	40	Negative
985141000622341	A	36.8	2014/03/20	AC00193	39.467644	Negative	40	Negative
985141000622341	A	37.3	2014/04/11	AC00234	40	Negative	40	Negative
985141000622341	A	37.2	2014/04/25	AC00238	39.204853	Negative	40	Negative
985141000622341	A	37.1	2014/05/09	AC00289	39.139603	Negative	40	Negative
985141000622341	A	37.8	2014/05/23	AC00316	40	Negative	**33.70489**	**Positive**
985141000622400	A	37.8	2013/12/27	AC00022	40	Negative	40	Negative
985141000622400	A	37.7	2014/01/10	AC00043	40	Negative	40	Negative
985141000622400	A	37.6	2014/01/25	AC00058	40	Negative	40	Negative
985141000622400	A	37.6	2014/02/07	AC00107	40	Negative	40	Negative
985141000622400	A	37.5	2014/02/24	AC00130	40	Negative	40	Negative
985141000622400	A	37.6	2014/03/07	AC00154	40	Negative	40	Negative
985141000622400	A	37.7	2014/03/20	AC00182	40	Negative	40	Negative
985141000622400	A	37.2	2014/04/11	AC00223	40	Negative	40	Negative
985141000622400	A	37.6	2014/04/25	AC00245	40	Negative	40	Negative
985141000622400	A	37.5	2014/05/09	AC00278	40	Negative	40	Negative
985141000622400	A	37.8	2014/05/23	AC00305	40	Negative	40	Negative
985141000622471	A	37.4	2013/12/27	AC00026	40	Negative	40	Negative
985141000622471	A	36.9	2014/01/10	AC00047	40	Negative	40	Negative
985141000622471	A	37.4	2014/01/25	AC00063	40	Negative	40	Negative
985141000622471	A	36.9	2014/02/07	AC00111	40	Negative	40	Negative
985141000622471	A	37.1	2014/02/24	AC00126	40	Negative	40	Negative
985141000622471	A	37.1	2014/03/07	AC00167	40	Negative	40	Negative
985141000622471	A	37	2014/03/20	AC00195	40	Negative	40	Negative
985141000622471	A	37	2014/04/11	AC00236	40	Negative	40	Negative
985141000622471	A	37.2	2014/04/25	AC00251	40	Negative	40	Negative
985141000622471	A	37.2	2014/05/09	AC00291	40	Negative	40	Negative
985141000622471	A	37.2	2014/05/23	AC00318	40	Negative	40	Negative

985141000622337	A	37.8	2013/12/27	AC00027	**35.51431**	**Positive**	40	Negative
985141000622337	A	37.4	2014/01/10	AC00049	37.27232	Negative	40	Negative
985141000622337	A	37.6	2014/01/25	AC00071	40	Negative	40	Negative
985141000622337	A	37.9	2014/02/07	AC00105	40	Negative	40	Negative
985141000622337	A	37.4	2014/02/24	AC00128	40	Negative	40	Negative
985141000622337	A	37.1	2014/03/07	AC00161	40	Negative	40	Negative
985141000622337	A	37.6	2014/03/20	AC00189	40	Negative	40	Negative
985141000622337	A	37.2	2014/04/11	AC00230	40	Negative	40	Negative
985141000622337	A	37.2	2014/04/25	AC00244	40	Negative	40	Negative
985141000622337	A	37.2	2014/05/09	AC00285	40	Negative	40	Negative
985141000622337	A	37.5	2014/05/23	AC00312	40	Negative	40	Negative
985141000622414	A	37.5	2013/12/27	AC00025	**33.94828**	**Positive**	40	Negative
985141000622414	A	36.9	2014/01/10	AC00046	40	Negative	40	Negative
985141000622414	A	37.4	2014/01/25	AC00061	40	Negative	40	Negative
985141000622414	A	37.1	2014/02/07	AC00110	39.263744	Negative	40	Negative
985141000622414	A	37.8	2014/02/24	AC00133	40	Negative	40	Negative
985141000622414	A	37.4	2014/03/07	AC00166	40	Negative	40	Negative
985141000622414	A	37.4	2014/03/20	AC00194	40	Negative	40	Negative
985141000622414	A	37.1	2014/04/11	AC00235	40	Negative	40	Negative
985141000622414	A	37.4	2014/04/25	AC00239	40	Negative	40	Negative
985141000622414	A	37.4	2014/05/09	AC00290	40	Negative	40	Negative
985141000622414	A	37.3	2014/05/23	AC00317	40	Negative	40	Negative
978000001363518	A	37.4	2013/12/27	AC00017	40	Negative	40	Negative
978000001363518	A	37.7	2014/01/10	AC00052	40	Negative	40	Negative
978000001363518	A	37.3	2014/01/25	AC00066	40	Negative	40	Negative
978000001363518	A	37.6	2014/02/07	AC00100	40	Negative	40	Negative
978000001363518	A	37.3	2014/02/24	AC00135	40	Negative	40	Negative
978000001363518	A	37.4	2014/03/07	AC00158	40	Negative	40	Negative
978000001363518	A	37.1	2014/03/20	AC00186	40	Negative	40	Negative
978000001363518	A	37.3	2014/04/11	AC00227	40	Negative	40	Negative
978000001363518	A	37.2	2014/04/25	AC00247	40	Negative	40	Negative
978000001363518	A	37.6	2014/05/09	AC00282	40	Negative	40	Negative
978000001363518	A	37.7	2014/05/23	AC00309	40	Negative	40	Negative
985141000622394	A	36.3	2013/12/27	AC00014	40	Negative	40	Negative
985141000622394	A	37.2	2014/01/10	AC00050	40	Negative	40	Negative
985141000622394	A	36.5	2014/01/25	AC00064	40	Negative	40	Negative
985141000622394	A	36.5	2014/02/07	AC00098	40	Negative	40	Negative
985141000622394	A	35.4	2014/02/24	AC00139	40	Negative	40	Negative
985141000622394	A	36.9	2014/03/07	AC00156	40	Negative	40	Negative
985141000622394	A	36.3	2014/03/20	AC00184	40	Negative	40	Negative
985141000622394	A	37	2014/04/11	AC00225	40	Negative	40	Negative
985141000622394	A	36.7	2014/04/25	AC00246	40	Negative	40	Negative
985141000622394	A	36.9	2014/05/09	AC00280	40	Negative	40	Negative

985141000622394	A	37	2014/05/23	AC00307	40	Negative	40	Negative
978000001357750	A	36.5	2013/12/27	AC00018	40	Negative	40	Negative
978000001357750	A	37.5	2014/01/10	AC00054	40	Negative	40	Negative
978000001357750	A	37.4	2014/01/25	AC00068	40	Negative	40	Negative
978000001357750	A	37.5	2014/02/07	AC00102	40	Negative	40	Negative
978000001357750	A	37.1	2014/02/24	AC00140	40	Negative	40	Negative
978000001357750	A	36.6	2014/03/07	AC00163	40	Negative	40	Negative
978000001357750	A	37.6	2014/03/20	AC00191	40	Negative	40	Negative
978000001357750	A	37.1	2014/04/11	AC00232	40	Negative	40	Negative
978000001357750	A	36.4	2014/04/25	AC00248	40	Negative	40	Negative
978000001357750	A	37.5	2014/05/09	AC00287	40	Negative	40	Negative
978000001357750	A	37.5	2014/05/23	AC00314	38.853348	Negative	40	Negative
041781A953	A	37.2	2013/12/27	AC00019	40	Negative	40	Negative
041781A953	A	37.5	2014/01/10	AC00055	40	Negative	40	Negative
041781A953	A	37.1	2014/01/25	AC00069	40	Negative	40	Negative
041781A953	A	37.4	2014/02/07	AC00103	40	Negative	40	Negative
041781A953	A	37	2014/02/24	AC00138	40	Negative	40	Negative
041781A953	A	37.6	2014/03/07	AC00164	40	Negative	40	Negative
041781A953	A	37.5	2014/03/20	AC00192	40	Negative	40	Negative
041781A953	A	37.1	2014/04/11	AC00233	40	Negative	40	Negative
041781A953	A	37.6	2014/04/25	AC00241	40	Negative	40	Negative
041781A953	A	37.5	2014/05/09	AC00288	40	Negative	40	Negative
041781A953	A	37.4	2014/05/23	AC00315	40	Negative	40	Negative

APPENDIX 2 CONSENT

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Equine Research Centre

Consent Form

I …….... (Owner / Manager), of ……... (Property), agree that samples can be obtained from my property for the purpose of research into the problem of African horse sickness.

The project is titled “A COMPARISON OF EQUINE ORBIVIRUS DYNAMICS ON TWO EQUINE ESTABLISHMENTS ON THE EAST RAND”.

Sampling and procedures to be included:

- Blood sampling of participating equines
- Micro-chipping of participating equines
- Basic clinical examination of participating equines
- Photographic images of the establishment

Samples will be obtained during the period of September 2013 to March 2014.

…….. Signed

…….. Date

Paraclinical Building
Private Bag X04
Onderstepoort 0110
Republic of South Africa

Email address
ant-craig@webmail.co.za
estelle.venter@up.ac.za
alan.guthrie@up.ac.za
Website:
www.up.ac.za

APPENDIX 3 ANIMAL ETHICS APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**A comparison of equine orbivirus dynamics on two equine establishments on the east rand**
PROJECT NUMBER	**V066-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. AF Craig**

STUDENT NUMBER (where applicable)	**242 824 49**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Equine**	
NUMBER OF ANIMALS	**30**	
Approval period to use animals for research/testing purposes		**November 2013-December 2014**
SUPERVISOR	**Prof. EH Venter**	

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 25 November 2013
CHAIRMAN: UP Animal Ethics Committee	Signature

APPENDIX 4 TITLE APPROVAL

UNIVERSITY OF PRETORIA	
FACULTY OF VETERINARY SCIENCE	
APPLICATION FOR APPROVAL OF TITLE OF DISSERTATION OR THESIS	
PLEASE NOTE: This form must be completed in **TYPING**. Please send the application form to: **HEAD: ACADEMIC ADMINISTRATION**.	
Name of candidate:	Mr Anthony Craig
Student number:	24282449
Degree:	MSc (Anima/Human/Ecosystem Health)
Course code:	08251008
Department:	DVTD
Name of supervisor/Leader:	Prof E H Venter
Name of co-supervisor(s)/co-leader(s)	Prof Alan Guthrie
Protocol approved : Yes **X** No ☐ **Date approved:** 16/09/2013 **Please attach a copy of protocol: V066-13**	
Title of dissertation/thesis:	
A comparison of equine orbivirus dynamics on two equine establishments on the East Rand, Gauteng Province, South Africa	
SIGNATURES OF:	
CANDIDATE:	**Date of submission:**
SIGNATURE OF SUPERVISOR/PROMOTER: EHVenter	**DATE:** 8/10/15
APPROVED BY HEAD OF DEPARTMENT:	**DATE:** 16/11/2015
APPROVED BY POST GRAD COM CHAIRMAN:	**DATE:** 01/12/15

C:\Users SM\Downloads\Application of title Anthony Craig.DOC