Seroprevalence and infection rates for equine orbivirus infections in dogs at Onderstepoort, South Africa

by

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submitted in fulfilment of the requirements for the degree

MSc (Veterinary Science Tropical Diseases)

in the Faculty of Veterinary Sciences,

University of Pretoria

November 2019

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Declaration of Originality

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Supervisor: Dr. J. E. Crafford

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Department: Veterinary Tropical Diseases

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Abstract

Recent case reports have suggested an increase in cases where dogs have been affected by African horse sickness virus (AHSV). Contrary to historic findings, several cases have been reported where dogs became infected without any evidence of contact with or consumption of infected horses or their products.

This was a prospective study to determine the prevalence of specific antibody and nucleic acid to AHSV and Equine encephalosis virus (EEV) in a high risk, isolated dog population during the high vector period in an endemic area. Dogs were kept in open kennels in close proximity to horses, sheep and cattle. Dogs in this population have historically been diagnosed with clinical AHS. Blood samples were collected on a monthly interval from February to June 2019. Antibody to AHSV was detected using an indirect ELISA while antibody to EEV was detected using a competitive ELISA. Infection rates were determined by demonstrating viral nucleic acids by RT-qPCR. All of the 37 sampled dogs remained negative for RNA to both AHSV and EEV for the duration of the study. This indicates that there were no equine

orbiviruses circulating in this dog population and is consistent with the absence of any clinical signs during the study period. This is also in line with the absence of any new AHS case reports in dogs from the area. Low levels of antibody were detected at some time points indicating transient exposure without viremia or due to cross-reactions with unknown antigens.

Acknowledgements

This study would not have been possible without the unending support and assistance of certain people. I would like to thank them for their time and expertise on both personal and professional level.

To Dr JE Crafford, my main supervisor for always putting so much time and dedication into my research. Thank you for your calmness and all your guidance during this learning process, and above all for your friendship, which has made this process much more enjoyable.

To Prof A Guthrie, my co-supervisor for your expertise in helping to shape and refine the thought process and my work.

To AgriSETA, for without their generous funding this research article would not be possible.

To the Department of Veterinary Tropical Diseases at the University of Pretoria for the equipment and materials needed for my research as well as the staff for always being a positive influence and eager to lend a helping hand during my time spent in the laboratory.

To the Onderstepoort Teaching Animal Unit for allowing me to conduct the research with the Beagle breeding population and for their willingness to assist me, and their continued support to accommodate the research in their busy schedules.

To my family and friends for their faith in me and for all their continuous, relentless support and encouragement through this process.

To my loving wife - my anchor in this mad world. Thank you for your patience, continued support, guidance, love and wisdom. I love everything you are and everything you mean to me.

Table of contents

List of tables

List of figures

List of appendixes

List of abbreviations

AHS	African horse sickness
AHSV	African horse sickness virus
ARC-OVR	Agricultural Research Centre-Onderstepoort Veterinary Research-Onderstepoort Veterinary Research
BSL	Biological safety level
BTV	Blue-tongue virus
BTV-1	Blue-tongue virus serotype 1
CFT	Complement fixation test
DIC	Disseminated intravascular coagulation
dsRNA	Double stranded ribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
EE	Equine encephalosis
EEV	Equine encephalosis virus
ERC	Equine Research Centre
IHC	Immunohistochemistry
MRU	Malelane Research Unit
NS	Non-structural protein

OIE	Office International des Épizooties
OTAU	Onderstepoort Teaching Animal Unit
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
PI	Percentage inhibition
PP	Percentage positivity
RBC	Red blood cell
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative reverse transcription polymerase chain reaction
SAVC	South African Veterinary Council
spp.	Species
VI	Virus isolation
VNT	Virus neutralisation test
VP	Viral protein

CHAPTER 1: INTRODUCTION AND SCOPE OF THE DISSERTATION

1.1 Introduction

The aim of this study was to establish whether orbiviruses, specifically African horse sickness virus (AHSV) and equine encephalosis virus (EEV) circulate in dogs (*Canis familiaris*) during the high vector season in an endemic area.

The susceptibility of dogs to African horse sickness (AHS) was first described in 1906 when Sir Arnold Theiler experimentally infected dogs with AHSV (Theiler, 1906, Theiler, 1910b). Although the validity of his findings were initially questioned by (M'Fadyean, 1910) and (Bevan, 1911), several studies have since reported on dogs being affected by AHSV (Piercy, 1951, McIntosh, 1955, Haig et al., 1956, Dardiri and Ozawa, 1969, Salama et al., 1981, van Rensburg et al., 1981). The general view amongst these authors were that dogs most probably became infected with AHSV following ingestion of contaminated horse (*Equus ferus caballus)* meat and that dogs were unlikely to play a role in the vector-borne transmission cycle of the virus.

The majority of literature pertaining to AHSV in dogs predates 1996 with the last reports on AHSV in dogs written by (van Rensburg et al., 1981) and the unlikely role of dogs as viral hosts by (Braverman and Chizov-Ginzburg, 1996). The topic of orbivirus infections in dogs resurfaced 15 years later when (Oura and El-Harrak, 2011) reported 40 out of 187 (21%) dogs in Morocco had antibody to bluetongue virus serotype 1 (BTV-1) after an outbreak of the same serotype in the ruminant population of that region. Two years later (van Sittert et al., 2013) reported a fatal

case of AHS in a dog from South Africa and a sero-prevalence of 43% in dogs sampled from the same facility. Most recently (O'Dell et al., 2018) reported retrospectively on 33 post-mortem cases of dogs which had died from AHSV over the period 2006 to 2017. Both these studies could not substantiate oral transmission of the virus as there were no evidence of dogs being exposed to AHSV contaminated meat.

All the studies on AHSV in dogs predating 1981, were either retrospective cohort studies or case reports that relied on virus neutralisation test (VNT) or the complement fixation test (CFT) for the detection of antibody to AHSV (Piercy, 1951, McIntosh, 1955, Haig et al., 1956, Dardiri and Ozawa, 1969, van Rensburg et al., 1981). Recently the more sensitive Enzyme linked immunosorbent assay (ELISA) was used for the detection of antibody to AHSV in dogs (van Sittert et al., 2013), while various highly sensitive and specific molecular techniques have been developed for the detection of nucleic acid to AHSV and EEV (Quan et al., 2010, Guthrie et al., 2013, Rathogwa et al., 2014, Weyer et al., 2015).

Here we propose a prospective study to describe the prevalence, incidence, and temporal distribution of antibody and nucleic acid specific to the two known equine orbiviruses, namely AHSV and EEV, in a high risk canine population during the high vector season. This will also be the first attempt to investigate EEV infections in dogs.

1.2 Problem Statement

New evidence in recent literature demonstrated that AHSV can indeed be transmitted to dogs other than the general traditional opinion of infection via contaminated meat.

1.3 Research Questions

1. What is the sero-prevalence of antibody to AHSV and EEV in an isolated dog population during peak vector season in an endemic area?
2. What is the infection rate of AHSV and EEV in an isolated dog population during peak vector season in an endemic area?

1.4 Aim of the Study

This study aims to determine the sero-prevalence as well as the infection rate of AHSV and EEV as measured by ELISA and Polymerase chain reaction (PCR) in an isolated canine population, in close proximity to equines in an AHS and Equine encephalosis (EE) endemic area during the high vector period.

1.5 Objectives of the Study

1. Determine baseline levels of antibody to AHSV and EEV using group specific ELISAs during the low vector period.
2. Collect blood once a month for five months during the high vector period from February to the end of June.

3. Test whole blood samples using Real time quantitative reverse transcription polymerase chain reaction (RT-qPCR) for both AHSV and EEV.
4. Test serum samples using an indirect ELISA (iELISA) for group specific antibody to AHSV.
5. Test serum samples using a competitive ELISA (cELISA) for group specific antibody to EEV.
6. Molecular typing of positive RT-qPCR samples.

1.6 Significance of the Study

By following seroconversion to the respective orbiviruses, we can estimate if and when dogs are immunologically challenged. The presence of viremia will be estimated by demonstrating viral nucleic acids by RT-qPCR in the blood. This will provide valuable data towards the role of dogs as hosts for orbiviruses. The monitoring of the whole dog colony, over a five-month period, will give information on the risks in the various age and sex sub-populations. We will also be able to correlate the viral load as represented by the levels of nucleic acid, to clinical manifestations, and attempt to determine whether dogs can harbour sub-clinical infections.

The data from this study could also serve as baseline for future studies to investigate the possibility of dogs as an experimental model for the study of the pathogenesis and the development of treatment protocols and vaccines. An extensive review of the literature concluded that there have been no reports on EEV in dogs.

CHAPTER 2: LITERATURE REVIEW

The review of the literature included the following databases: CABI: CAB Abstracts® and Global Health®, MEDLINE® (Web of Science), PubMed, ScienceDirect, Wildlife & Ecology Studies Worldwide and Zoological Record®. Search terms were initially vague and verbose and included: African AND Horse AND Sickness AND dogs. Later on, more concise search strings included for example African horse sickness AND canine OR dog AND prevalence AND epidemiology AND transmission.

2.1 Introduction

African horse sickness and Equine encephalosis are both non-contagious diseases affecting mainly equids caused by arboviruses AHSV and EEV respectively. These viruses are classified as two of 21 distinct species or distinct serogroups within the genus *Orbivirus* of the family *Reoviridae* (Coetzer and Guthrie, 2004). There are currently nine known antigenically distinct serotypes of AHSV (Coetzer and Guthrie, 2004, MacLachlan and Guthrie, 2010) and seven known antigenically distinct serotypes of EEV (Howell et al., 2004).

African horse sickness is by far not a new disease. Historically, a disease of horses resembling that of AHS was reported in Yemen in 1327. In 1569, Portuguese explorers in East Africa reported on a disease with clinical signs similar to AHS that affected horses imported from India (Coetzer and Guthrie, 2004). Horses belonging to the Dutch East India Company was described suffering from a terrible, mysterious illness causing high mortalities in 1652 after the introduction of horses from Europe (Coetzer and Guthrie, 2004). Eventually the first clinical description of AHS was

done in 1719 in the then Cape of Good Hope, South Africa where the Dutch coined the name “perreziekte” or “pardeziekte” (Theiler, 1921a, Theiler, 1921b).

Equine ephemeral fever is a disease first described by Theiler, but is no longer used in modern veterinary medicine as we know today that Theiler was actually describing EE (Theiler, 1910a).

2.2 Aetiology

The *Reoviridae* family of viruses have a genome of 10 distinct segments of linear double-stranded (ds) RNA that encode 10 proteins, four being non-structural proteins (NS1, NS2, NS3, NS3A) and seven of which are called structural viral proteins (VP1-VP7) (Gould and Hyatt, 1994, Maree and Paweska, 2005, MacLachlan and Guthrie, 2010). These linear dsRNA viruses pose difficulties when it comes to both detection and vaccination strategies due to their marked genetic and phenotypic diversity as seen in the large number of serotypes that occur with each virus (MacLachlan and Guthrie, 2010).

When studying the structure of orbiviruses it becomes apparent how scientists use unique structural proteins to identify between them and to distinguish between certain serogroups and serotypes. Examples of unique structural proteins that contain immune-dominant, serogroup-specific epitopes and are reserved in AHSV are NS 1 and NS 2 and VP 1, 3, 4, 6, 7.

NS 1 form long tubular structures within infected cell cytoplasm and NS 2 form part of viral inclusion bodies. (Mellor and Mertens, 2008).

VP's are present in the core particle (Maree and Paweska, 2005) and VP 7 is highly conserved among the nine AHSV serotypes as demonstrated by Maree and Paweska (2005) who confirmed analytical specificity as a group-specific antigen using recombinant VP 7 antigen. The location of these proteins is illustrated in Figure 1.

Cross-immunity reactions between these epitopes and different AHSV serotypes is the basis to distinguish for example AHSV from other orbivirus species like BTV and EEV during serological assays (Mellor and Mertens, 2008), although antigenic variability phenomena has been described to occur due to serial passages in susceptible equine hosts (Rodriguez et al., 1992).

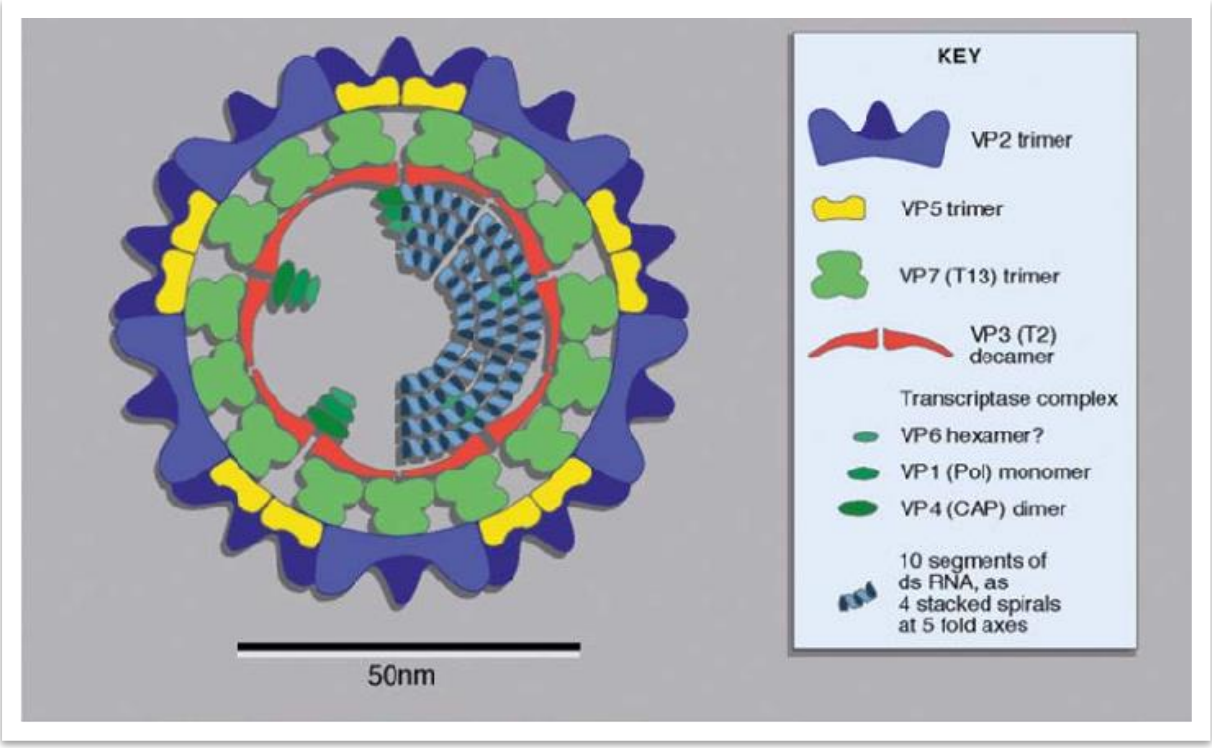

Figure 1 Diagrammatic illustration of the structure of the African horse sickness virus particle with special interest in the structural location of VP 7 trimers making up the inner layer of the viral capsid. Courtesy of P.P.C. Mertens and S. Archibald – reproduced from Mertens PPC, Maan S, Samuel A, and Attoui H (2005) Orbivirus, Reoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, and Ball LA (eds.) Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, pp. 466–483. San Diego, CA: Elsevier Academic Press, with permission from Elsevier.

When AHSV is studied under an electron microscope, as seen in Figure 2, the characteristic icosahedral lattice or shape comprising of a triple inner capsid layer composed of five- and six-membered rings of VP 7 trimers can be appreciated (Maree and Paweska, 2005, Mellor and Mertens, 2008).

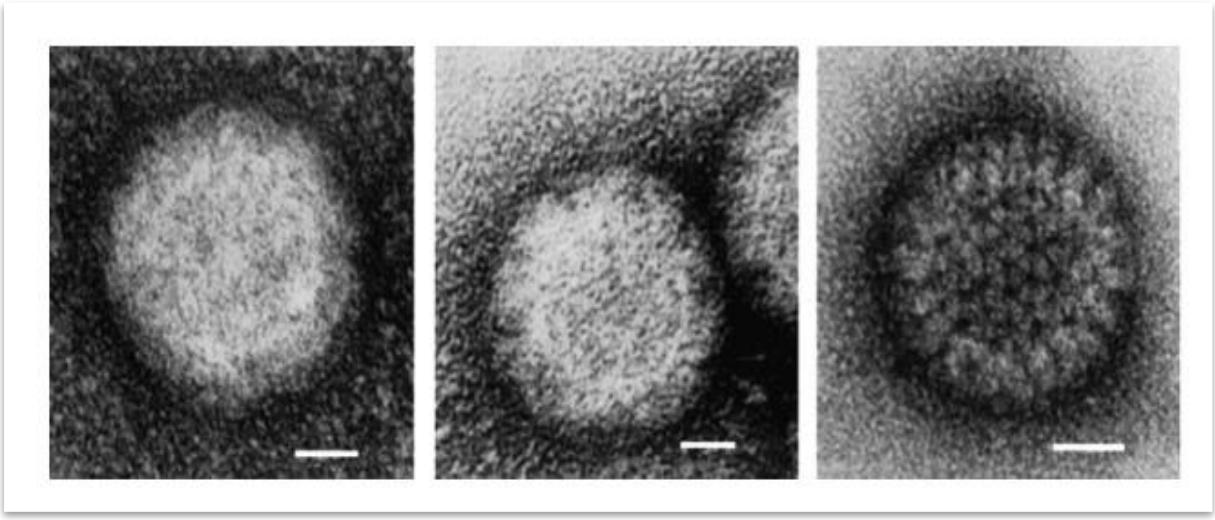

Figure 2 Electron micrographs of African horse sickness virus (AHSV) serotype 9. The image on the right shows the core-surface layer of a 20-faced polyhedron ring structure made up of VP7 trimers. Reproduced from Mertens PPC, Maan S, Samuel A, and Attoui H (2005) Orbivirus, Reoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, and Ball LA (eds.) Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, pp. 466–483. San Diego, CA: Elsevier Academic Press, with permission from Elsevier.

The ability to form and purify large morphological VP 7 crystals in situ is exploited and used in diagnostics (Maree and Paweska, 2005). For example, an iELISA for the detection of AHSV has been developed by expressing and purifying recombinant AHSV-3 VP 7 (Maree and Paweska, 2005).

2.3 Distribution range

African horse sickness and Equine encephalosis are both endemic to Africa with AHSV being endemic in the central tropical regions in a band from Senegal and

Gambia to Ethiopia and Somalia. Some sources report that AHSV might also be endemic in Yemen (Mellor and Mertens, 2008). From there it is known to seasonally spread north and north-west with only AHSV serotype 2, 7 and 9 found in western and northern Africa whereas all nine serotypes of AHSV have been reported in eastern and southern Africa (Mellor and Mertens, 2008). Outbreaks of AHSV have occurred in horses outside the normal distribution range such as countries of the Middle East (1959–1963), Spain (1966, 1987–1990), Portugal (1989), Yemen (1997) and the Cape Verde Islands (1999) (Mellor and Mertens, 2008). Figure 3 illustrates both the endemic distribution range of AHS as well as some of the outbreaks documented outside the endemic areas (Coetzer and Guthrie, 2004). Table 1 summarises all the known AHS outbreaks worldwide for the period 1928-2010 with source articles referenced.

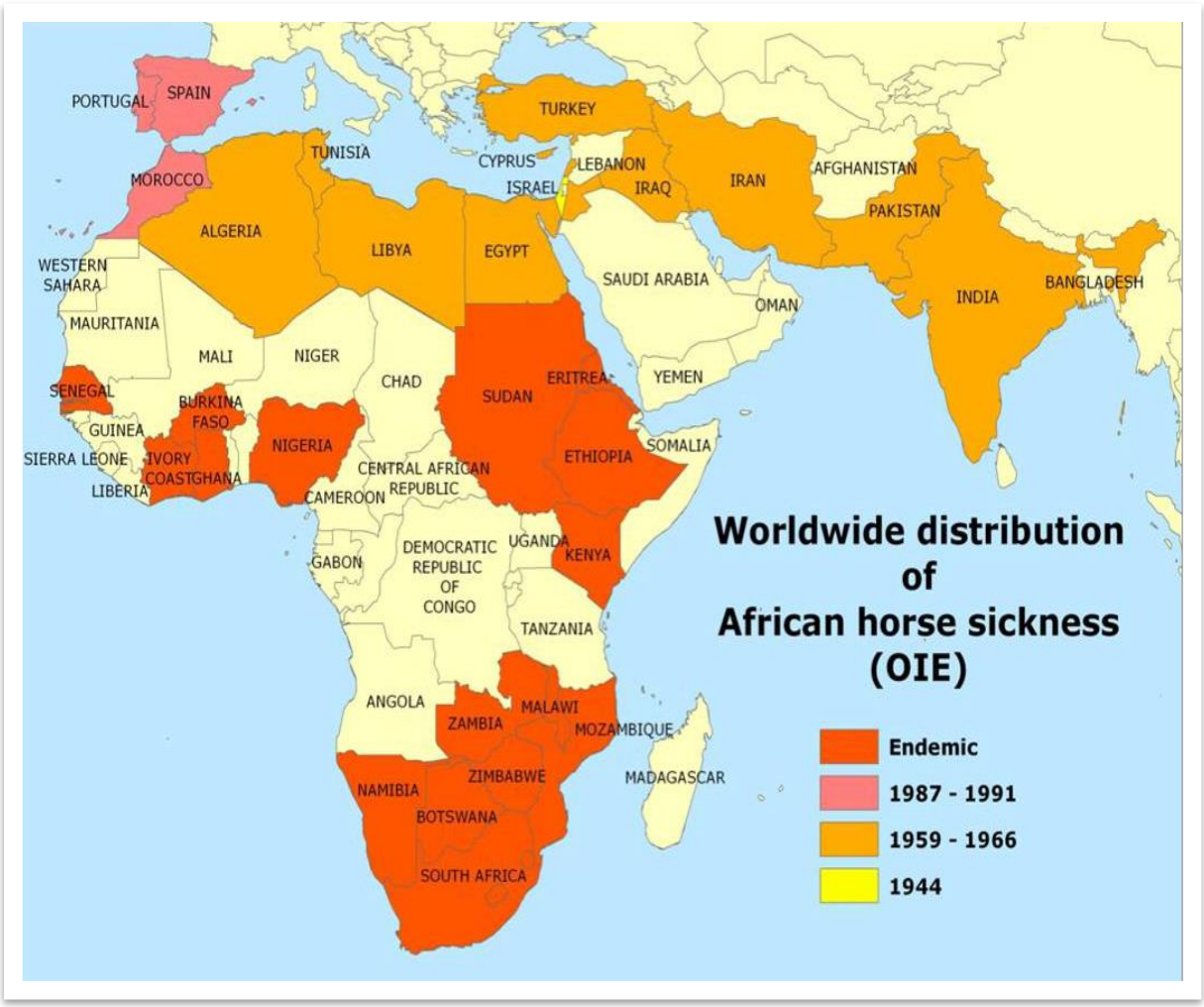

Figure 3 A map that illustrates the endemic distribution range of AHS as well as some of the outbreaks documented between 1944 and 1991 (Coetzer and Guthrie, 2004).

Table 1 Summary of AHS outbreaks reported for the period 1928 - 2010 in horses outside the endemic area in chronological order.

Country	Year	AHSV Serotype	Diagnostic test	Reference(s)
Egypt	1928, 1943, 1953, 1958	*	Virus isolation and VNT	(MacLachlan and Guthrie, 2010)
Yemen	1930	*	Virus isolation and VNT	(MacLachlan and Guthrie, 2010)
Middle East (Syria, Lebanon, Jordan, Palestine)	1944	*	Virus isolation and VNT	(Alexander, 1948, MacLachlan and Guthrie, 2010)
Middle East (Iran, Iraq, Afghanistan Pakistan)	1959-1964	9	Virus isolation and VNT	(Howell, 1960, Howell, 1962, Wilson et al., 2009, MacLachlan and Guthrie, 2010)
India	1960	9	Virus isolation and VNT	(Howell, 1960, Gohre et al., 1964–1965, Wilson et al., 2009)
Eastern Mediterranean (Turkey, Cyprus, Syria, Lebanon and Jordan	1960	9	Virus isolation and VNT	(Rodriguez et al., 1992, MacLachlan and Guthrie, 2010)
India	1964	6	Virus isolation and VNT	(Keerti, 1964)
North Africa (Libya, Morocco Algeria and Tunisia)	1965-1966	9	Virus isolation and VNT	(Rodriguez et al., 1992, Wilson et al., 2009, Pedgley and Tucker, 1977)
Portugal	1966	9	Virus isolation and VNT	(Rodriguez et al., 1992)
Spain	1966	9	Virus isolation and VNT	(Rodriguez et al., 1992)
Spain, Portugal	1987-1990	4	Virus isolation and VNT	(Rodriguez et al., 1992, Wilson et al., 2009)
Saudi-Arabia	1989-1990	9	Virus isolation and VNT	(Anderson et al., 1989, Rodriguez et al., 1992)
Morocco	1990	4	Virus isolation and VNT	(Rodriguez et al., 1992)
Yemen, Saudi Arabia	1997	*	Virus isolation and VNT	(MacLachlan and Guthrie, 2010)
Cape Verde Islands	1999	*	Virus isolation and VNT	(MacLachlan and Guthrie, 2010)

Ethiopia	2003	6, 9	Virus isolation and VNT	(Zeleke et al., 2005, MacLachlan and Guthrie, 2010)
Kenya	2007	4	*	(Wilson et al., 2009)
Senegal	2007	2, 7, 9	*	(Wilson et al., 2009, MacLachlan and Guthrie, 2010)
West Africa (Ghana, Mali, Nigeria and Mauritania)	2007	7	*	(Wilson et al., 2009, MacLachlan and Guthrie, 2010)
Ethiopia	2007-2010	2, 4, 6, 8, 9	RT-PCR	(MacLachlan and Guthrie, 2010, Aklilu et al., 2014)

* Information not available

Based on African serological surveys EEV is currently known to be endemic to southern Africa (South Africa, Botswana, Namibia, Zimbabwe) and Kenya (Barnard, 1997). However, after the EE outbreak in Israel (2009), studies conducted on the prevalence of EEV in other countries reported antibody to EEV from Ethiopia in East Africa, as well as from Gambia and Ghana in West Africa (Oura et al., 2012, Tirosh-Levy et al., 2017).

2.4 Seasonal occurrence

The occurrence of AHS and EE in equid populations are regarded as seasonal. Clinical cases usually occur from the end of summer and the incidence starts to wane after the first winter frost. The incidence and prevalence for both AHS and EE are directly related to the number of vectors present with insect numbers rising during early spring and peaking during the warm, wet summer months (Rodriguez et al., 1992, Gordon et al., 2017). This corroborates with recent literature of clinical AHS cases in dogs documented between January and May (van Sittert et al., 2013, O'Dell et al., 2018).

2.5 Mode of transmission

It is commonly accepted that the main mode of transmission of AHSV and EEV in equids is through biting midges - haematophagous artrhopods of the genus *Culicoides*, order Diptera, family Ceratopogonidae (Meiswinkel and Paweska, 2003, Mellor and Mertens, 2008).

Culicoides spp. in general have quite a large vertebrate host range, ranging from equids and ruminants to lagomorphs, bats, primates and birds (Wilson et al., 2009), but seems to have preferences for larger mammals (Venter et al., 2014).

Prior to the work done by Meiswinkel and Paweska (2003), *C. imicola* was regarded as the sole field vector for AHSV for well over 50 years (Braverman and Chizov-Ginzburg, 1996). During an outbreak in the Clarens valley of the eastern Free State, South Africa between February and May of 1998, in which approximately 100 horses died of AHS, *C. bolitinos* accounted for most (65%) of all the *Culicoides* spp. collected (Meiswinkel and Paweska, 2003). *C. bolitinos* was also the only midge species that occurred on each of the AHS affected farms and was the only species from which AHSV was isolated (Meiswinkel and Paweska, 2003). Therefore both *C. imicola* and *C. bolitinos* are accepted vectors for AHSV and EEV (Meiswinkel and Paweska, 2003, Coetzer and Guthrie, 2004, Mellor and Mertens, 2008, MacLachlan and Guthrie, 2010).

Potential vectors outside the normal distribution range of AHSV, are summarized in Table 2. The role of these midge species as potential vectors become significant

when their ability to feed on multiple hosts increases the risk of further spread of AHSV past its normal distribution range into non endemic areas.

Table 2 Midge species that may act as vectors and country where these species are of relevance

Midge species	Comment	Reference(s)
C. sonorensis	Shown as a likely *in vitro* vector in North America.	(Mellor and Mertens, 2008).
C. pulicaris	Wild-caught *culicoides* spp. during the 1988 Spanish outbreak. Their significance was however underestimated.	(Mellor, 1994).
C. obsoletus		
C. subfascipennis	Speculated to be able to feed on horses in the Mediterranean and so hypothetically be able to transmit AHSV.	(Braverman and Chizov-Ginzburg, 1996).
C. punctatus		
C. chiopterus	These midges are said to be able to feed on dogs in Siberia. Also found in the Mediterranean.	(Braverman and Chizov-Ginzburg, 1996, van Sittert et al., 2013).
C. pulicaris		
C. fascipennis		
C. schultzei	These midges are known to feed on different species of mammals and could also be a potential equine vector.	(Nevill and Anderson, 1972, Braverman and Chizov-Ginzburg, 1996).

The fact that *C. bolitinos,* as an alternative vector for AHSV, is known to breed in manure of domestic and wild ruminants like cattle (*Bos indicus*), buffalo (*Syncerus caffer*) and wildebeest (*Connochaetes* spp), increases the risk of disease transmission between horses kept with or close to livestock (Meiswinkel, 1989). No literature explicitly states unusual midge species that might be implicated in the spread of EEV.

So far only horizontal transmission of orbiviruses between insects has been demonstrated (Wilson et al., 2009). The reason for the lack of vertical transmission

stems from the membrane surrounding the culicoides egg. This membrane has been shown to be impermeable to intact virus particles as demonstrated for bluetongue virus and *C. variipennis* (Nunamaker et al., 1990).

The pre-patent period, the period where the virus incubates within the vector, is known to be eight to ten days at 25 ºC. During this time infectious virus particles reach the salivary glands and have replicated by a factor of up to 10^3 and are able to re-infect the next animal (Mellor and Mertens, 2008). This is also called extrinsic incubation (Wilson et al., 2009).

When reading the literature on AHS outbreaks outside the endemic area, it became apparent that certain transmission factors were involved, and two themes seem to unfold. Firstly, mechanical dispersion of either the vector or the host and secondly the increased vector distribution range largely attributed to climate change. Regarding the first theme, the 1966 Spanish outbreak was attributed to the dispersal of arthropod vectors from the outbreak in Morocco either by wind across the Strait of Gibraltar or by boat (Pedgley and Tucker, 1977). Similarly was the Spanish outbreak of 1987 blamed on the importation of infected Zebra (*Equus quagga*) from Namibia (Rodriguez et al., 1992). The epizootic in the Middle East and south-west Asia was also largely attributed to mechanical dispersal of the host (horses) by means of travelling nomads and Gypsies. This epizootic was exacerbated when infected horses migrated to large river basins and the infection spread due to the large number of insect vectors that were present (Howell, 1960). The Spanish outbreak of 1988 could not be explained by conventional theories, but mules and donkeys (*Equus asinus*) have been proposed to be blamed as sources of AHS

during the interim periods between the Spanish AHS outbreaks of 1987, 1988, 1989 and 1990 by allowing the persistence of AHSV among equine population at low levels without being diagnosed (Rodriguez et al., 1992). The possibility of other host species, such as dogs, as sources of the virus was not considered by (Rodriguez et al., 1992). MacLachlan and Guthrie (2010) was of the opinion that the spread of BTV-8 throughout Europe could have involved unknown mechanical dispersion of either vector or host.

The second theme centres on the worldwide increase in vector distribution ranges seen due to the effects of climate change. This theme is more difficult to explain, but increased vector distribution ranges have been seen to be a problem worldwide and so too can the natural endemic area of AHS and EE expand due to more favourable geographical distribution ranges of the vectors. An example of increased global distribution ranges of vectors due to climate change can be found in the BTV outbreak in Europe where BTV invaded non-endemic geographical regions like the Mediterranean from adjacent endemic regions in North Africa or the Middle East (MacLachlan and Guthrie, 2010) .

There are some reports that propose other blood sucking insects like mosquitos or ticks to be involved in the transmission of orbiviruses. These might be less significant but still require further investigation (Wilson et al., 2009, Alexander et al., 1995, Braverman and Chizov-Ginzburg, 1996, Mellor and Mertens, 2008). In an isolated case AHSV was isolated from the Camel tick, *Hyalomma dromadarii,* in Egypt (Awad et al., 1981, Wilson et al., 2009). The role of ticks in the epidemiology of AHS is probably negligible but remains uncertain.

2.6 Host range

African horsesickness virus and EEV share the same vertebrate host range which are solidungulate species with horses being unequivocally the most clinically affected and mules and donkeys being less affected (Mellor and Mertens, 2008).

Zebra, indigenous equids to Africa, are thought to be part of the natural cycle of AHSV and are mostly sub-clinically infected (Barnard, 1993, Alexander et al., 1995, Mellor and Mertens, 2008). A study by Barnard (1993) in the Kruger National Park, South Africa found that from blood collected from various age groups of zebra, foals start losing passive maternal immunity at around six months and that AHSV infections increase from 31% at around seven months to almost a 100% just prior to 12 months. Some authors propose that zebra may be natural reservoirs for AHSV and maintain the virus throughout the year i.e. zebra might be over-wintering hosts for these viruses (Binepal et al., 1992, Barnard, 1993). It would be of interest to learn the viral load and duration of viraemia in sub-clinical infected zebra which certainly warrants further investigation.

Studies have presented compelling evidence that wild carnivores can be infected with AHSV by the oral route after ingesting AHSV-infected meat (Alexander et al., 1995, Mellor and Mertens, 2008). A comprehensive survey on AHSV in African carnivores was conducted by Alexander et al. (1995). In this survey article, antibodies to AHSV were found to be widespread among African carnivores with AHSV serotype 4 and 7 being most prevalent. Table 3 show data demonstrating the wide range of sero-positive AHSV carnivore species. The author concluded that these animals where likely infected via the oral route based on two aspects of the

data. Firstly, the higher antibody prevalence in larger carnivores which would either prey or scavenge on zebra and horses and secondly that a more even distribution would have been expected among all species if vector-borne transmission were at play.

Table 3 Wide range of seropositive AHSV animals, other than dogs and horses, extracted from the literature tabulated according to species, country of origin, prevalence and AHSV serotype (Alexander et al., 1995).

Animal species	**Country**	**AHSV prevalence**	**AHSV serotype**
Lion (*Panthera leo)*	Tanzania	19/24 (48%)	4, 7
Cheetah (*Acinonyx jubatus*)	Tanzania	3/28 (11%)	7
African wild dog (*Lycaon pictus*)	Tanzania	5/18 (28%)	4, 7
African wild dog (*Lycaon pictus*)	Botswana	13/24 (54%)	4, 7, 9
African wild dog (*Lycaon pictus*)	Zimbabwe	3/6 (50%)	*
African wild dog (*Lycaon pictus*)	South Africa	4/11 (36%)	4
Lion (*Panthera leo)*	South Africa	18/29 (62%)	4, 7
Jackal (*specific spp. not mentioned*)	Kenya	5/39 (13%)	*
Domestic cat (*Felis catus*)	Kenya	0/29 (0%)	*
Civet (*Civettictis civetta*)	Kenya	0/6 (0%)	*
Genet (*Genetta* spp.)	Kenya	2/13 (15%)	*
White-tailed mongoose (*Ichneumia albicauda*)	Kenya	0/14 (0%)	*
Marsh mongoose (*Atilax paludinosus*)	Kenya	0/3 (0%)	*
Hyena (*specific spp. not mentioned*)	Kenya	35/65 (54%)	1, 4, 6, 7
African wild dog (*Lycaon pictus*)	Kenya	2/15 (13%)	4, 7

* Information not available

Even though the author concluded that oral infection rather than vector-borne infection was the rout of infection in carnivore species, some data did support the latter. For example, in Kenya, the most common serotype found in carnivores, such as African wild dogs, hyena, jackals and leopard was serotype seven, (Davies et al.,

1993, Alexander et al., 1995) but serotype seven has not been reported in horses in Kenya after 1952. Serotype two, five and nine were the most dominant serotypes isolated in zebra in Tanzania (Hamblin et al., 1990a) but that they were not able to isolate serotype two or five from carnivores from the same country and that serotype nine was only found in wild dogs in Botswana. Smaller scavenger species were either antibody negative or had very low antibody titres. The domestic dog can be considered part of this group, as they would not actively hunt large prey in rural circumstances.

Since the first reports by Sir Arnold Theiler in 1906 (Theiler, 1906, Theiler, 1910b) the role of dogs as host for vector-born AHSV infection was questioned (M'Fadyean, 1910). Theiler wanted to show that AHSV can be transferred from horse to dog, dog to dog as well as from dog to horse. He conducted experiments where he directly infected subjects intravenously with 2, 5 and 10 ml of AHSV infected blood and described the clinical manifestations that would follow. However, M'Fadyean strongly disagreed with Theiler's methodology, did his own infection studies and highlighted the following inconsistencies form Theiler's work:

1. The route of infection i.e. intravenous, was unnatural.
2. The possibility might have existed that by using unfiltered blood, contaminants in the blood could have caused a reaction mistakenly seen as disease.
3. The quantity of blood used to infect subjects were also unnatural and led to the quick temperature responses described in Theiler's article.

4. The passage of virus from his infected dogs to horses could be attributed to actual viral persistence in the blood and not due to viral multiplication.

5. The rise in temperature was always mild and Theiler never described a pyrexic dog. This M'Fadyean concluded could suggest a possible phase of incubation, since he himself could not transmit infection from these dogs to others during the mild febrile period.

M'Fadyean concluded that dogs are indeed susceptible to AHS, but was the first to hypothesize the oral route of infection over that of natural vector-borne transmission.

Theiler responded to his peers' critique by repeating his experiments to specifically address the above mentioned points:

1. On point one and two, he infected and re-infected dogs and horses with filtered blood via the subcutaneous route demonstrating that AHS can indeed be transferred between these two species.

2. On point three, he reduced his inoculation volumes to 1ml and still documented positive results.

3. On point four, he remarked on the viral load based on percentage blood drawn calculated on the animals' live weight. He argued that viral persistence in blood is without merit if the same conclusion is made where horses were infected with canine blood with much lower viral particles calculated on live weight. Theiler went further stating that after successful initial inoculation of a dog from an infected horse,

he reproduced canine and equine fatalities up to the 30th dog passage from the same blood.

4. On point number five, Theiler re-iterates the fact that “horse-sickness in dogs has a very rapid course, both in incubation and temperature reaction.” He goes on by questioning that horses in M’Fadyean’s experiments were only injected from dogs 14 days after being inoculated but why not after four or five days, when dogs showed a temperature reaction. From this he draws attention to the fact that AHS recovered animals are no longer infective.

Point number three in Theiler’s response is an important concept to grasp. His scientific approach was quite remarkable even though the method is very crude by today’s standards. Modern infection studies now use titres median mouse intracerebral lethal doses ($MICLD_{50}$).

McIntosh (1955) detected specific AHSV antibodies in one out of 13 tested dog serums (7.7% prevalence) and concluded that the dog “is not an important host in insect-borne African horse sickness”. Critique on this study is obviously the small sample size used which could lead to biased estimates due to increased sampling variances. The low seroprevalence of antibody to AHSV in domestic dogs is often mentioned in the literature (McIntosh, 1955, Baba et al., 1992, Alexander et al., 1995). Table 4 presents a comprehensive summary of all AHSV cases documented in the domestic dog (Piercy, 1951, McIntosh, 1955, Haig et al., 1956, Keerti, 1964, Dardiri and Ozawa, 1969, van Rensburg et al., 1981, Salama et al., 1981, Baba et

al., 1992, Alexander et al., 1995). In spite of the small sample size in these studies, some authors argue that it supports the notion that dogs do not play a significant role in the overwintering of the virus (M'Fadyean, 1910, Dardiri and Ozawa, 1969, Salama et al., 1981). Passive surveillance by Salama et al. (1981) reported a 5% (6 out of 111) prevalence rate in rural dogs from the Aswan Province in Egypt. This area experienced sporadic outbreaks of AHS in their equine populations in spite of regular vaccinations (Salama et al., 1981). AHSV serotype 9 was identified in all positive canine cases. The author conceded that these infections were most probably due to oral ingestion of contaminated meat. Interestingly enough, the frequency of BTV titres in dogs was significantly higher when compared to AHSV (Alexander et al., 1995). They argue that this might be related to the higher probability of dogs being fed contaminated production animal meat (cattle, sheep, goats) than being fed contaminated horse, donkey or zebra meat (Alexander et al., 1995). Even domestic cats (*Felis catus*) tested positive for antibody to BTV (21% seroprevalence) but not AHSV.

Table 4 Summary of the literature on African horse sickness in dogs stating the year of publication, country where the sample(s) were taken, seroprevalence, serotype, diagnostic test used and the reference.

Year of study	Country	Sero-prevalence	Sero-type	Diagnostic test	Reference
1951	Kenya	*	*	*	(Piercy, 1951)
1955	In vitro study	1/13 (8%)	3	VNT	(McIntosh, 1955)
1956	South Africa	19/30 (63%)	*	Virus isolation and VNT	(Haig et al., 1956)
1964	India	*	*	VNT	(Keerti, 1964)
1969	In vitro study	9/18 (50%)	9	CFT, VNT	(Dardiri and Ozawa, 1969)
1981	South Africa	13/17 (76%)	6	*	(van Rensburg et al., 1981)
1981	Egypt	6/111 (5%)	9	CFT	(Salama et al., 1981)
1989	Kenya	1/46 (2%)	1, 4	cELISA, VNT	(Alexander et al., 1995)
1990	Kenya	6/87 (7%)	Could not be typed		
1991	Kenya	0/51 (0%)	N/A		
1992	Kenya	2/62 (3%)	Could not be typed		
1992	Botswana	6/53 (11%)	4, 7		
1992	Botswana	1/47 (2%)	Could not be typed		
1992	Nigeria	* (9%)	*	CFT, VNT	(Baba et al., 1992)
1993	Nigeria	28/80 (35%)		HI	(Baba et al., 1993)
2013	South Africa	24/56 (43%)	Could not be typed	iELISA, RT-qPCR	(van Sittert et al., 2013)
2006-2017	South Africa	*	6	IHC, RT-qPCR	(O'Dell et al., 2018)
2018	South Africa	*	6	IHC, iELISA, RT-qPCR	(Whitehead et al., 2018)

* Information not available

From Alexander et al. (1995) we see that AHSV serotypes four and seven were identified using virus neutralisation. Serotype one and four were prevalent in the

domestic dog population sampled in Kenya and only serotype seven found in Botswana. Serotype one was only found in dogs and hyenas and only in Kenya. In their article van Sittert et al. (2013) gives different percentages to studies done in Kenya, Botswana, Nigeria and South Africa. In their article they mention AHSV seroprevalences of 4%, 8%, 9% and 1% respectively. An infection rate of 9% in dogs sampled was found in a study conducted in Nigeria by using CFT to determine positive cases, unfortunately no reference was made to the serotype isolated (Baba et al., 1992).

Other vertebrate hosts, other than canids, that are known to have tested positive for antibody to AHSV include camelids, bovids and African elephants (*Loxodonta africana*) (Binepal et al., 1992, Alexander et al., 1995). Although there have been reports of humans contracting AHS, these were from accidental exposure in a vaccine plant. The disease is still considered not to be a zoonosis (Mellor and Mertens, 2008).

The importance of these positive serological cases in unconventional hosts are unclear, but most authors do attribute their infections as probably spill over or dead end hosts and that infection is uncommon and not significant (Binepal et al., 1992).

That said, the study conducted by van Sittert et al. (2013) at the Malelane Research Unit (MRU) re-opened the chapter of natural canine AHSV vector-borne infection. It was the first published case of AHS in a dog to substantiate natural vector-borne transmission (van Sittert et al., 2013) and was the highest seroprevalence (43%

prevalence rate) recorded in a naturally infected population. The article describes that dogs at this unit was strictly fed a commercial pelleted diet, with absolutely no way for the introduction of horse and or horse meat products. This sentence bears more weight than one might think as discounting this fact would imply that these commercialised foods deliberately or unknowingly include horse and or horse meat products into their brand. Even if these brands did include horse meat products it would also be highly unlikely that the virus would be able to withstand the kibbling process which entails high temperatures and desiccation of the product.

A well written argument in favour of dogs being naturally infected via an insect vector is where they investigated suspect clinical cases or retrospectively reviewed old records of cases where dogs died of respiratory failure between the year 2006 and 2017 at the Faculty of Veterinary Science, Onderstepoort (O'Dell et al., 2018). They identified 33 canines which tested positive either by Immunohistochemistry (IHC) or RT-qPCR or both similar to van Sittert et al. (2013). Included in their data was epidemiological records such as signalment and clinical presentation, however, they specifically noted that none of the cases had a history where horse meat or horse meat products had been given as part of their diets.

Recently a case study report was published about two supposedly naturally infected canine AHS cases presented at the Onderstepoort Veterinary Academic Hospital (OVAH) (Whitehead et al., 2018). The report gives post mortem, immunohistochemical, serological and PCR findings, but unfortunately lacks in elaborating about RT-qPCR cyclic threshold and typing results as well as ELISA percentage positivity (PP) values. That said, the report does make a detailed

documentation on the clinical presentation, haematological-, biochemical-, thoracic radiological-, arterial blood gas-, trans-tracheal aspirate cytology and treatment protocols of these two unrelated respiratory compromised patients as well as the road to uncovering a surprise diagnosis of AHS. This report also involves a Beagle from the Onderstepoort Teaching Animal Unit (OTAU), which upon investigation revealed to have tested positive for AHSV-6 (Crafford, J. E., (2018) Personal communication, 26 September 2019).

Studies on other orbiviruses in dogs revealed 21% seroprevalence (40 out of 187) for BTV (Oura and El-Harrak, 2011). Six dogs that were experimentally infected with BTV-1 seroconverted within 14 days but with no increase in viral RNA using RT-PCR nor was it possible to isolate virus from the blood of the infected dogs (Oura et al., 2014). There is currently no literature on EEV in dogs.

To summarise, the role that species, other than equids play in the epidemiology of AHS is still not fully understood but older studies and reports maintain that dogs play little or no part and are merely incidental dead-end or spill over hosts.

2.7 Pathophysiology

Pathogenesis of orbiviruses are very similar and follow much the same pattern (MacLachlan and Dubovi, 2017). Upon entering the bloodstream of a susceptible vertebrate host, the virus spreads to the regional lymph nodes and starts to replicate. This is followed by the virus spreading via the blood throughout the body. This is called primary viraemia or primary dissemination. The virus then infects the

lungs, spleen, other lymphoid tissues and certain endothelial cells throughout the hosts' body. It appears that the primary lesions are associated with damage to the endothelial cells. The exact physiology behind vascular injury is not clearly understood but activation of monocyte-macrophages, leakage, haemorrhage, inflammation and disseminated intravascular coagulation are all implicated (Clift et al., 2009, MacLachlan and Dubovi, 2017). After the virus multiplies in these tissues, the virus is again released into the bloodstream. This stage is called secondary viremia and is marked with a fever.

In horses the incubation period – from infection until the secondary viraemia - occur within nine days (experiments show a variation of between two and 21 days) and usually lasts for four to eight days and has never been shown to extend beyond 21 days. In zebra, viraemia occasionally extends for as long as 40 days. The duration of viraemia as well as viral titres in donkeys ($10^{2.5\text{-}5.0}$ $TCID_{50}$ ml^{-1}) is intermediate to that of horses ($10^{5.0}$ $TCID_{50}$ ml^{-1}) and zebra (Mellor and Mertens, 2008). Mellor and Mertens (2008) reports that dogs' titres have been found to be very low, viraemia often of short duration with low to none seroprevalences recorded. This was also discussed by Dardiri and Ozawa (1969) who showed low titres in dogs experimentally infected with AHSV with transitory viraemia's and all dogs used remaining clinically healthy.

It has been stated that in experimentally infected horses, exhibiting the per-acute form of the disease, viral antigen is detected primarily in the large mononuclear cells of the cardiovascular and lymphatic systems with the primary locations of antigens found being endothelial cells, red pulp of the spleen and surrounding lymphoid

follicles (Mellor and Mertens, 2008). This suggests that they are a primary target for the virus as illustrated in Figure 4. However, since orbiviruses are closely associated with the cellular compartment of blood (both red and white blood cells) and there is very little virus present in the plasma, the virus is therefore protected from antibody and it is common to find virus in the presence of antibody in the blood. This has been reported in zebra but not in horses (Coetzer and Guthrie, 2004).

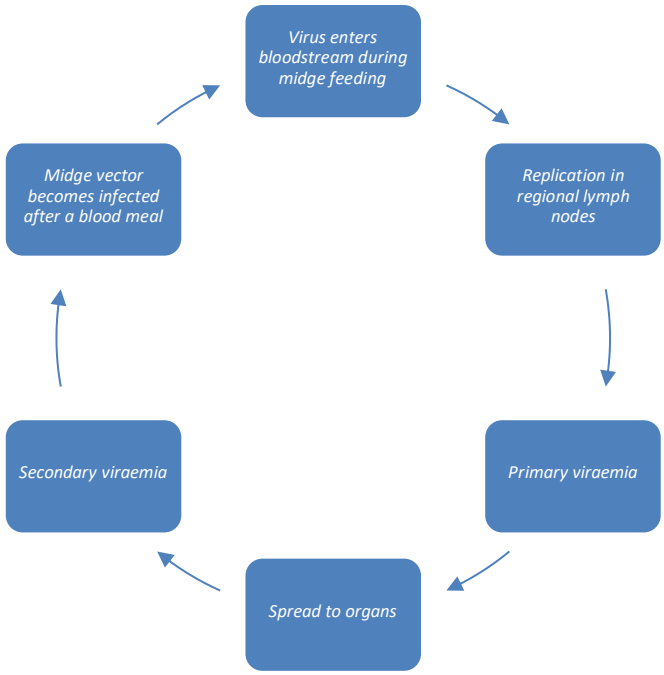

Figure 4 Flowchart demonstrating the cyclic nature of AHSV in its epidemiology as well as illustrating the primary target of virus after introduction into its host.

2.8 Clinical signs

Typically AHS is characterised as a disease that affects the cardio-pulmonary system causing effusions and haemorrhages in the organs and can lead to failure of these systems (O'Dell et al., 2018). Ante-mortal findings in equids were first described by Theiler and are grouped into different syndromes still being used today (Theiler, 1921a, Theiler, 1921b, Coetzer and Guthrie, 2004). The three syndromes

are named “Dikkop” a sub-acute cardiac from. “Dunkop”, a per-acute pulmonary form, and a “mixed” form. They will be discussed here in ascending order of severity.

“Dikkop” meaning thick head in Afrikaans can be attributed to the characteristic trait of oedema of the supraorbital fossae. The mortality rate is about 50%. Horses suffering from “Dunkop”, meaning thin head, have conjunctival petechiae and sublingual ecchymosis, may or may not show signs of oedema of the supraorbital fossae and surrounding ocular tissues that can extend to areas of the head, neck, and chest. Dunkop has a high mortality rate up to 90%. The third and most severe form described in horses is the “mixed” form which can share clinical and pathological signs of both of the two previously mentioned syndromes and can have a mortality rate higher than 90%.

It is suggested that a fourth mild, non-fatal form of AHS exists, named Horse sickness fever where animals could be infected with a less virulent strain or when some degree of immunity exists within the host (Theiler, 1921a, Theiler, 1921b, Coetzer and Guthrie, 2004, Mellor and Mertens, 2008). This may be an alternate explanation when it comes to zebra and donkeys as to why they rarely show disease other than just being resistant to infection. Horses with AHS may also show non-specific clinical signs such as colic, pyrexia (39-41$_{o}$C), excessive perspiration, dyspnoea with or without coughing, periods of recumbency and ataxia (Coetzer and Guthrie, 2004, Mellor and Mertens, 2008).

Equine encephalosis is a mild febrile or even subclinical disease, however extreme cases of supraorbital fossa oedema, respiratory distress, ataxia, seizures, abortions and acute deaths have been attributed to the virus (Howell et al., 2004). In practice these two viruses are often tested concurrently as EE can be an important differential diagnosis for the mild form of AHS.

Except for equids the domestic dog is the only other species confirmed to be clinically affected by AHSV. Clinical manifestations of AHSV infection in dogs was described in relative detail by Theiler during his early experiments with AHSV and dogs (Theiler, 1906, Theiler, 1910b). Subsequent authors described similar clinical manifestations to Theiler. Clinical signs were the same or similar to those found in horses, with a rapid incubation period and pyrexia. Morbidity rates ranged from 33% to 76% and mortality rates ranged from 20% to 78% (Bevan, 1911, Piercy, 1951, Haig et al., 1956, van Sittert et al., 2013). In their article the author states that dogs that were treated at OVAH for acute respiratory distress syndrome, typical of what is seen in dogs affected with AHS, the mortality rate was >95% (O'Dell et al., 2018). The exact number of clinical patients presented was not stated. Syndromes from dogs recorded suffering from AHSV after being infected via the oral route has been described as being per-acute. The incubation period in dogs is mostly 1-2 weeks (Theiler, 1906, Theiler, 1910b, Bevan, 1911) with clinical symptoms ranging from mild transient fevers (van Rensburg et al., 1981) to respiratory distress, diarrhoea and convulsions or per acute death (Haig et al., 1956, van Rensburg et al., 1981).

The case report from the MRU describes a dog that had died of a "chronic" form of AHS (van Sittert et al., 2013). The dog worsened progressively over the course of a

week and died in spite of intervention. The progression of clinical signs in the dog was similar to that of the pulmonary form in horses. It started with progressive inappetence, depressed habitus and a low-grade pyrexia or even sometimes a normal rectal temperature. The clinical signs worsened and eventually lead to hyperpnoea with dyspnoea, tachycardia and congested mucous membranes with petechial haemorrhages. This was similar to recently described clinical presentation of confirmed canine AHS cases presented at The Faculty of Veterinary Science, Onderstepoort (O'Dell et al., 2018, Whitehead et al., 2018). It is this author's opinion that the term "chronic" infection is not applicable to canine AHS infections and should rather be substituted for sub-acute since chronic infections imply an indefinite duration with no change in clinical presentation. Thus, in the sub-acute canine forms, acute anorexia and lethargy was noted which progressed rapidly to respiratory failure and death. Acute deaths or per-acute cases of respiratory distress syndrome were said to be easily and often confused by owners with malicious poisoning. These dogs were presented with severe dyspnoea, excessive salivation, tachypnoea, crackles on pulmonary auscultation with or without copious serous oro-nasal discharge leading to respiratory failure and inevitably death (O'Dell et al., 2018). Morbidity rates in dogs are difficult to evaluate as mildly affected dogs i.e. mildly elevated temperatures, transient inappetence, mildly depressed habitus etc. can easily be overlooked and not recorded.

2.9 Pathology

Post mortem findings of both equid and canid species are very specific. The most noticeable post mortem findings of the pulmonary form found in both these species

include pulmonary oedema and oedema of the interlobular septa of the lungs, hydrothorax, froth and serofibrinous fluid in the trachea, occasionally ascites, hydrothorax and hyperaemia and oedema of the stomach mucosa (Mellor and Mertens, 2008, O'Dell et al., 2018, Whitehead et al., 2018). A diagnosis of the cardiac form, arise from the lack of pulmonary pathology and usually include intermuscular connective tissue oedema, hydropericardium, petechial haemorrhages of the left ventricle of the heart, and congestion and petechiation of the mucosa of the gastrointestinal tract (Mellor and Mertens, 2008)

2.10 Histopathology

Mellor and Mertens (2008) describes the histopathological changes seen in horses as: "a result of increased permeability of the capillary walls and consequent impairment in circulation". The lungs exhibit serous infiltration of the interlobular tissues with distension of the alveoli and capillary congestion. The central veins of the liver may be distended, with interstitial tissue containing erythrocytes and blood pigments while the parenchymous cells show fatty degeneration. Cellular infiltration can be seen in the cortex of the kidneys while the spleen is heavily congested. Congestion may also be seen in the intestinal and gastric mucosae, and cloudy swelling in the myocardial and skeletal muscles (Mellor and Mertens, 2008).

Lesions seen on histopathological evaluation of dogs are: acute serofibrinous pneumonia or interstitial pneumonia with marked protein-rich oedema, cerebral and intestinal oedema and congestion, leucocytic (monocytic and lymphocytic) infiltration of the alveoli and myocardium, alveolar haemorrhage, centrilobular

hepatic congestion, multifocal sub-endocardial hyaline degeneration and necrosis and small lymphoid follicles in the spleen and lymph nodes (van Rensburg et al., 1981, van Sittert et al., 2013, O'Dell et al., 2018, Whitehead et al., 2018).

2.11 Diagnosis

The OIE give relevant, short, precise information in the form of technical disease cards as part of their database of infectious diseases (OIE, 2018). Therein describes appropriate samples and diagnostic methods as currently being prescribed to make a diagnosis of AHS in any country and will then be accepted by the international community. Therein virus isolation, PCR and serology; blocking ELISA's, indirect ELISA's and CFT, are mentioned as prescribed tests to diagnose AHS. Table 5 is taken from the OIE Terrestrial manual (OIE, 2018). From this table the diagnostic modality is linked to the purpose of testing.

Table 5 Test methods currently utilised for the diagnosis of African horsesickness and their various epidemiological purposes. Adapted from (OIE, 2018)

Method	**Purpose**			
	Population free from infection	Individual free from infection	Confirmation of clinical cases	Prevalence of infection
Agent identification				
Real time RT-PCR	+	+++	+++	++
Virus isolation	-	++	+++	-
Detection of immune response				
ELISA (serogroup specific based on VP 7)	+++	++	+++	++
CFT	+	+	+	+
VN	+	+	+	+

(+++) = recommended method, (++) = suitable method, (+) = may be used in some situations, (-) = not appropriate for this purpose

Mellor and Mertens (2008) also gives short notes on how to confirm AHS in suspected equid cases whereby the virus is either identified directly by means of virus isolation or PCR and then the serotype is established by means of serotype-specific virus neutralisation using plaque reduction assays. The author elaborates on how the identification of virus strains and the analysis of their nucleic acid sequences is an important aspect of viral epidemiology (Mellor and Mertens, 2008) and in South Africa RT-qPCR is being used for the screening of clinical cases as part of movement control (Quan et al., 2010, Guthrie et al., 2013, Rathogwa et al., 2014, Weyer et al., 2015).

2.11.1 Virus Isolation

Some authors still mention Virus isolation (VI) to be a reference test for detection of AHSV (Guthrie et al., 2013). Investigating Table 5 the role VI plays in diagnosing AHS becomes evident. The test is indicated as one of just two recommended methods, the other being RT-PCR, for confirming infectious agents during surveillance studies.

Traditionally virus identification has been accomplished by culture and isolation of causative agents by virus isolation and identification procedures. However there is some critique regarding this test particularly that it is very time-consuming and present many technical difficulties that may lead to long delays in diagnosing viral infections (Maree and Paweska, 2005). The OIE states that particularly during outbreak studies initial tests should be ELISA's or PCR which are quicker to perform (OIE, 2018). Other critique stems from its apparent insensitivity in detecting low

number of viral particles. An example of its inferior sensitivity was shown in the development of newer RT-qPCR which showed a 10-fold increase in sensitivity than that of VI (Quan et al., 2010). Other authors state that the sensitivity of VI was shown to be 44.2% compared to 97.8% of the RT-qPCR of (Guthrie et al., 2013). It is also stated that demonstrating circulating viral nucleic acid is much more possible than the virus identification itself as nucleic acid continues in circulation within the host for much longer making direct demonstration of circulating nucleic acid more accurate as a positive diagnosis than the infectious agent (Quan et al., 2010).

2.11.2 Agar gel immunodiffusion

Traditionally, CFT and agar gel immunodiffusion tests have been used for the detection of group-specific antibody to orbiviruses. These tests have fallen out of favour and is not even listed by the OIE anymore for either AHS or EE (OIE, 2018).

2.11.3 Complement Fixation Tests

Complement fixation tests (CFT) are of the older tests to diagnose viral infections and AHSV retrospective cohort studies and case reports done before 1990 relied heavily on CFT to detect antibodies (Piercy, 1951, McIntosh, 1955, Haig et al., 1956, Dardiri and Ozawa, 1969, van Rensburg et al., 1981). Historically CFT was known as the “gold standard” in the demonstration of group-specific antibodies such as Immunoglobulin (Ig) M to AHSV. Although often described as lacking in sensitivity due to the subjective interpretation of data (Rathogwa et al., 2014, Souf, 2016, OIE, 2018), CFT is still listed as a prescribed test in the OIE Terrestrial manual (Oie.int., 2018) for international trade but is largely being replaced by ELISA’s which have

higher sensitivity and larger degree of standardisation. Anti-complementary effects that occur with zebra and donkey sera which can seriously affect the reliability and efficiency of the test has not been fully addressed when dog serum is used (OIE, 2018).

CFT demonstration and titration of group-specific IgM antibodies are done on samples collected either ante mortem, preferably during the febrile phase, or post mortem of which the spleen is a prerequisite. After positive samples are identified concurrent or subsequent typing is then done using micro-titre VNT (Meiswinkel and Paweska, 2003).

2.11.4 Virus Neutralisation Tests

Virus neutralization tests (VNT) were traditionally used to type new virus isolates; however, they are laborious and need validated reference viruses and serotype-specific antisera (Alexander et al., 1995, Maree and Paweska, 2005). Consequently, only a few highly specialized reference laboratories still perform VNT's.

These tests are not as sensitive when compared to some ELISA's for the detection of antibody as (Alexander et al., 1995) reported that 50% of positive carnivore serums using cELISA did not have neutralising activity. Similarly, Maree and Paweska (2005) found the iELISA was much more sensitive in the detection of declining levels of passive immunity in foals when compared to VNT's. However, it must be mentioned that even during development of new ELISA's, VNT's still play a crucial role as a reference standard and is still regarded as a gold standard for

detection of antibodies to AHSV (Maree and Paweska, 2005) as well as serotyping AHSV isolates (OIE, 2018). Seroprevalence studies conducted on EEV outside of South Africa used VNT (Tirosh-Levy et al., 2017), cELISA and RT-qPCR (Oura et al., 2012).

2.11.5 Immunohistochemistry

Immunohistochemistry (IHC) has been shown to be quite useful in preserved tissues with some immunoperoxidase staining reports to have as high as a 100% sensitivity and specificity for AHSV present in equine heart and lung tissue (Clift et al., 2009). However, in general the short comings of IHC can be summarised as an amalgamation of inadequate diagnostic performance, lack of standardisation and unsuitability when screening large numbers of samples (Maree and Paweska, 2005, Clift et al., 2009).

Novel AHSV immunohistochemical staining of dog tissues were done by van Sittert et al. (2013) and recorded feint AHSV-positive staining within the myocardium only. This drew the attention of the author to comment on the need for future investigation on the sensitivity and specificity of AHS IHC in dog tissues. Indeed this was further investigated by O'Dell et al. (2018) who recorded that positive immunohistochemical staining using AHSV specific NS 4 antibody were ascertained within microvascular endothelial cells, monocytes and macrophages. In contrast to van Sittert et al. (2013), nuclear and cytoplasmic AHSV specific labelling was most apparent in pulmonary tissue and occasionally splenic tissue.

2.11.6 Enzyme Linked Immunosorbent Assays

Polyclonal and monoclonal antibodies have been used for the detection of AHSV with ELISA (Hamblin et al., 1991, van Wyngaardt et al., 1992, Maree and Paweska, 2005). ELISA's also rely on purified virus or recombinant antigen as a source of antigen (Hamblin et al., 1990b).

Various types of assays have been described and used successfully for the detection of antibody to various orbiviruses such as BTV (Thevasagayam et al., 1996, Vandenbussche et al., 2008) epizootic haemorrhagic disease of deer (Thevasagayam et al., 1996), AHSV (Hamblin et al., 1990b, Hamblin et al., 1991, Rubio et al., 1998, Gordon et al., 2017), EEV (Gordon et al., 2017) and specifically in dogs for detection of antibody to BTV (Oura and El-Harrak, 2011) as well as AHSV (Alexander et al., 1995, van Sittert et al., 2013).

Viral protein seven or whole virus has been used as a group specific antigen in ELISA (Maree and Paweska, 2005). The use of species specific conjugates could limit the use of ELISA to specific species, but could be mitigated by using more inclusive conjugates like protein G or recombinant protein A-G.

Competitive or blocking ELISAs have the advantage that they are not limited by the ability of the conjugate to detect antibody to different species such as for example the competitive ELISA (cELISA) for the detection of antibody to EEV (Crafford et al., 2011). Here an EEV specific guinea pig antiserum was used to compete with the test serum for a pre-titrated EEV antigen. This assay can therefore be used to test

any species except guinea pig for the antibody to EEV and is serogroup specific and able to identify all seven known EEV serotypes. The specificity of the assay is evident in the complete absence of any significant cross reactions with 47 other orbivirus reference antisera (Crafford et al., 2011).

2.11.7 Real Time Quantitative Reverse Transcription Polymerase Chain Reaction

Various RT-PCR assays have been developed, some assays which target AHSV genome segment one, two and three which code VP 1, VP 2 and VP 3 proteins respectively (Bachanek-Bankowska et al., 2014) others target genome segment 7 which code for VP 7 and NS 2 proteins (Quan et al., 2010, Guthrie et al., 2013). PCR's have also been developed and used for other orbiviruses such as BTV (Hoffmann et al., 2009) and EEV (Rathogwa et al., 2014).

The PCR described and recently being used with great success in the diagnosis of AHSV and EEV cases in South African equine populations is distinctive due to its optimisation as a RT-qPCR (Quan et al., 2010, Guthrie et al., 2013). This optimisation of a RT-qPCR assay as currently used by the Equine Research Centre (ERC), Veterinary Genetics Laboratory, Faculty of Veterinary Sciences, Onderstepoort resulted in a high diagnostic sensitivity (97.8%) and specificity (99.9%) as similar results regarding analytical sensitivity and specificity was found in the original assay (Guthrie et al., 2013). This very same RT-qPCR was used to retrospectively detect AHSV infections in frozen tissue samples as well as whole

blood from dogs in previously mentioned recent studies (van Sittert et al., 2013, O'Dell et al., 2018).

2.12 Differential diagnosis

These two equine orbiviruses namely AHSV and EEV share a very similar epidemiology, however where AHSV can cause high mortalities EEV usually manifests as a mild or even subclinical infection (MacLachlan and Guthrie, 2010). More often than not AHSV and EEV can infect the same vertebrate host simultaneously (Mellor and Mertens, 2008).

It is not uncommon in private practice to test for EEV, equine theileriosis (*Theileria equi*) and equine babesiosis (*Babesia caballi*) simultaneously with AHSV as these diseases often present with similar clinical signs, especially early on. Haemorrhages and oedema of AHSV horses are similar to those encountered in cases of purpura haemorrhagica and equine viral arteritis with clinical signs including pyrexia, lethargy and inappetence (Mellor and Mertens, 2008).

2.13 Treatment

There is no specific treatment for neither AHSV nor EEV apart from supportive therapy (Mellor and Mertens, 2008). O'Dell et al. (2018) noted that symptomatic treatment of dogs presented with AHS had a mortality rate of over 95% (O'Dell et al., 2018).

2.14 Prevention

South Africa has a unique procedure in ensuring its protected zone stays free. Equines moved from infected zones to virus-free zones are restricted for lengthy periods of time. Before importation, as well as if permitted, animals are quarantined for 60 days in insect-proof accommodation (Mellor and Mertens, 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

A prospective cohort study design was used. Blood was collected from a closed canine population over a five-month period and screened for antibody and viral nucleic acid to AHSV and EEV. Blood was collected in November 2018 at the beginning of the study, during the low vector season, where baseline levels for antibody and nucleic acid were determined. Serial blood collections were performed during the high vector season (February 2019 to June 2019) as indicated in Figure 5.

For ethical and welfare reasons dogs were familiarised to the blood collection procedures before the onset of the study to reduce stress. This conditioning included walking the dogs to the area of collection, lifting each individual onto the table while applying mild-to-moderate physical restraint during the preparation of the area over the cephalic vein. To further reduce stress an anaesthetic cream was applied on the day of venepuncture and positive reinforcement was used in the form of high quality commercial dog treats. Dogs that were difficult to handle due to aggression or aversion to having their paws handled were identified.

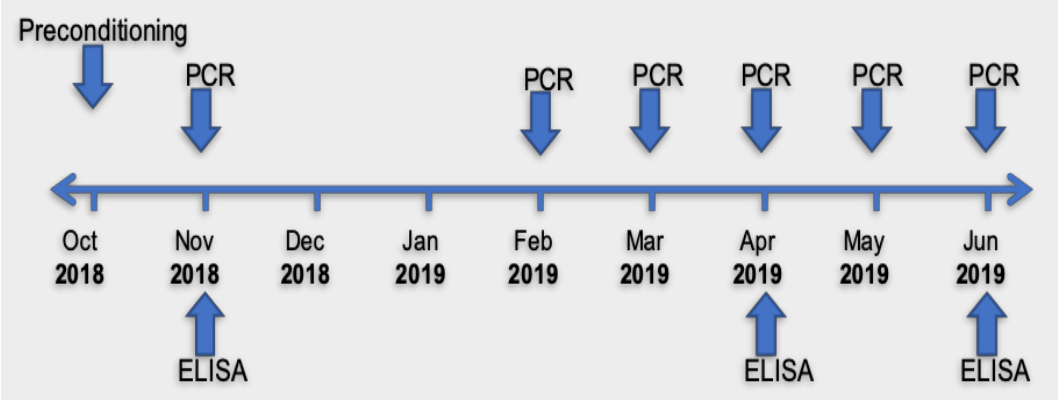

Figure 5 Timeline showing the study design, emphasising temporal distribution of blood collection and indicating the months where ELISA and PCR were performed.

3.2 Study population

The study population consisted of a high risk canine population. The Beagle breeding colony at OTAU was chosen as it was located within the AHS infected zone of South Africa. The facility is situated in proximity to horses, cattle, sheep and goats as illustrated in Figure 6. From this satellite image it can be seen that OTAU is situated within a 300m radius of stables, kraals and irrigated grazing paddocks with horses, cattle, sheep and goats. There is also an open concrete water reservoir, labelled with a blue circle, 20 meters from the kennels which is used to supply water for the irrigation systems.

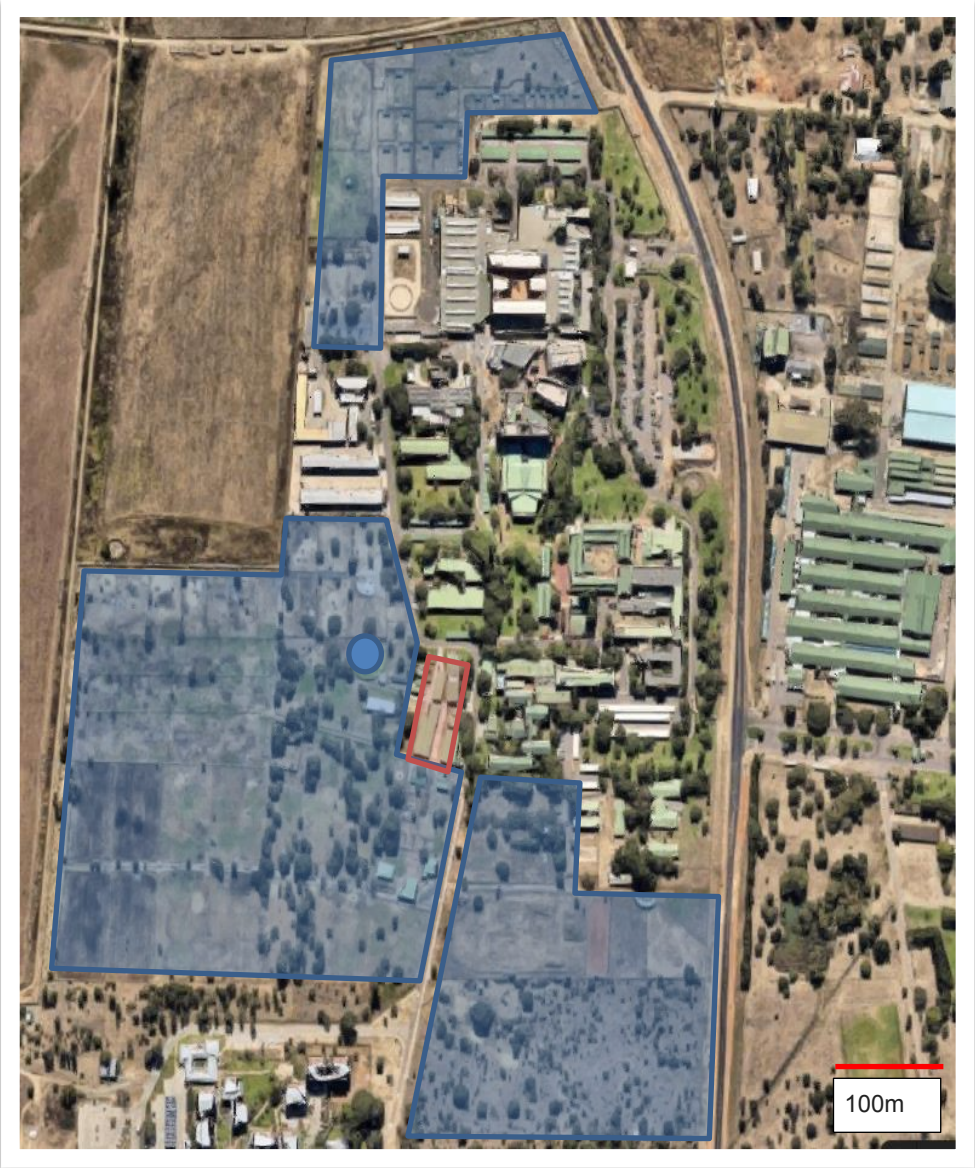

Figure 6 Satellite imaging of the Faculty of Onderstepoort illustrating the Onderstepoort Teaching Animal Unit (RED) and its proximity to paddocks where horses, cattle and sheep are kept (BLUE) (Maps, 2019).

The OTAU facility itself consists of open kennels enclosed with steel mesh fencing and concrete floors. All dogs received a high end pelleted commercial diet with routine care and husbandry performed by dedicated OTAU personnel and veterinary care was independently provided by the OVAH. No new animals were introduced during the study.

3.3 Sample size

The sample size was derived using a probability formula for surveys to substantiate freedom from disease in Survey Toolbox for Livestock Diseases (Cameron, 1999). Calculations were performed using the Epitools Web application (Sergeant, ESG, 2018. EpiTools Epidemiological Calculators. Ausvet. Available at: (http://epitools.ausvet.com.au.)

The following parameters were used:

1. Test sensitivity = 0.8
2. Test specificity = 0.9
3. Design prevalence = 0.3
4. Analysis method = Modified hypergeometric exact
5. Target Type I error = 0.05

This gave a maximum sample size of 36 participants and since this is a small isolated population with an unknown sero-prevalence and infection rate all the dogs (n = 37) were sampled. Dogs were allocated a number from 1-37 with their respective individual microchip number and signalment given in Appendix 1.

3.4 Sample collection

Blood samples were collected from the cephalic or jugular vein using a 21 x G needle and 3 ml syringe. One ml of blood was deposited into an EDTA (BD Vacutainer; K2E (EDTA) 7.2 mg; LOT 8199547; Becton, Dickinson and Company 1 Becton Drive Franklin Lakes, NJ 07417-1880) tube and 2 ml into a Serum

Separator Tube (SST) (BD Vacutainer; SST II Advance; LOT 8227719; Becton, Dickinson and Company 1 Becton Drive Franklin Lakes, NJ 07417-1880). After clotting the serum tubes were centrifuged at 1 300 x g for 15 minutes. Serum was harvested and stored at -20 ºC until tested. Whole blood samples were stored at 4 ˚C until tested for viral nucleic acid.

3.5 Detection of AHSV and EEV nucleic acid

Whole blood samples were tested according to the standard operating procedures of the ERC, a South African National Accreditation System accredited laboratory. A validated group specific RT-qPCR assay as described by Guthrie et al. (2013) was used to detect nucleic acid to AHSV. This assay has a diagnostic sensitivity and specificity of 97.8% and 99.9%, respectively. Nucleic acid to EEV was detected using the real-time RT-qPCR assay as described by Rathogwa et al. (2014). This assay is very specific and has an efficiency and sensitivity of 81% and 95% respectively.

Viral nucleic acid extraction was performed according to the manufacturer's recommendations with slight modifications. Nucleic acid extraction was performed on a Kingfisher 96 magnetic particle processor (Thermo Fisher Scientific Inc.; Thermo Fisher Scientific, 168 Third Avenue Waltham, MA USA 02451) using the MagMAX™ pathogen RNA/DNA kit (Applied Biosystems part number 4462359, Thermo Fisher Scientific Inc.; Thermo Fisher Scientific, 168 Third Avenue Waltham, MA USA 02451). Thereafter the RT-qPCR was performed on a StepOnePlus™ Real-

Time PCR System (Applied Biosystems; Thermo Fisher Scientific Inc.; Thermo Fisher Scientific, 168 Third Avenue Waltham, MA USA 02451).

3.6 Detection of AHSV antibody

Serum samples were tested for antibody to AHSV at the Agricultural Research Centre-Onderstepoort Veterinary Research (ARC-OVR) using an iELISA as described by Maree and Paweska (2005). This iELISA detects IgG antibodies to VP 7 using recombinant protein G conjugated with horseradish peroxidase. The results were expressed as a percentage of the positive control and were calculated according to the following formula:

$$PP = \left(\frac{meanA_{492}\ test\ sample - meanA_{492}\ neg\ control}{meanA_{492}\ pos\ control - meanA_{492}\ neg\ control}\right) \times 100$$

The diagnostic specificity and sensitivity of this assay in horses, are 100% and 99.4% respectively and has been used in the detection of AHSV in dogs before (van Sittert et al., 2013, Whitehead et al., 2018).

3.7 Detection of EEV antibody

Serum samples were tested for antibody to EEV at the Serology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria using an indirect cELISA as described by Crafford et al. (2011). Samples were labelled using the same numerical order used for the iELISA.

Crude sucrose density purified EEV type 1 was used as antigen in the ELISA. Briefly, 50µL EEV antigen diluted to 1:500 in carbonate/bicarbonate buffer (0.05M carbonate–bicarbonate buffer, pH 9.6 (Sigma), was passively adsorbed on to a 96 well Maxizorb Immuno plate (Nunc). The plate was incubated at 37 ˚C for an hour on an orbital shaker. Plates were washed three times with phosphate buffered saline (PBS, pH= 7.2) and blotted dry. Test sera was diluted 1:5 in blocking buffer (PBS, pH= 7.2) supplemented with 0.05% Tween 20 (Sigma), 5.0% skimmed milk powder (Sigma) and 1% bovine serum albumin (Sigma,) and 50 µL were added to duplicate wells of columns 1–10. A positive equine control serum was titrated across eight wells of column 11 from 1:5 to 1:640. Wells 12 A and 12 B each received 100µL of blocking buffer and were used as the blank control wells. Wells C 12 and D 12 received a 50µL of a 1:5 dilution of negative EEV serum and blocking buffer and were used as the negative control wells. Wells 12 E–H received 50 µL/well of blocking buffer and were used as the 100% absorbance value control wells. 50 µL canine serum diluted to 1:5 with blocking buffer was added in duplicate wells and 50 µL of guinea pig anti-EEV-infectious sub-viral particle serum, diluted to 1:150 with blocking buffer was also added to all the wells except 12 A and 12 B (total volume in each well =100µL). After incubation (37 ˚C for 1h) on an orbital shaker and washing, 50µLrabbit anti-guinea pig immunoglobulin (DAKO, Ely, UK) conjugated to horseradish peroxidase optimally diluted 1/1 000 in blocking buffer was added to all the wells. Plates were incubated for a third time and washed as before. Freshly prepared substrate indicator system ortho-phenylenediamine (Sigma) at a concentration of 0.04 mg/mL and containing 0.05% H_2O_2 (30% v/v) was then added to each well and incubated in the dark at room temperature for 10

minutes. The reaction was stopped after 10 minutes by addition of 1.0 N H_2SO_4 to all the wells. Figure 7 is a representation of a typical ELISA plate showing duplicate samples as well as positive (CPOS), negative controls (CNEG), 100% control wells (C100) as well as blank control wells (BL). The plates were read spectrophotometrically at a wavelength of 492 nm.

The percentage inhibition (PI) of the 100% colour reaction determines the level of competition observed in the test wells and was used to differentiate between positive and negative samples. The PI for each sample was calculated using the formula below and a cut-off value of 30% was used to distinguish between positive and negative sera.

$$PI = 100 - \left(\frac{meanA_{492}oftestsample}{medianA_{492}ofguineapigantiserumcontrol}\right) \times 100$$

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	3	3	4	4	5	5	CPOS	BL
B	6	6	7	7	8	8	9	9	10	10	CPOS	BL
C	11	11	12	12	13	13	14	14	15	15	CPOS	CNEG
D	16	16	17	17	18	18	19	19	20	20	CPOS	CNEG
E	21	21	22	22	23	23	24	24	25	25	CPOS	C100
F	26	26	27	27	28	28	29	29	30	30	CPOS	C100
G	31	31	32	32	33	33	34	34	35	35	CPOS	C100
H	36	36	37	37	38	38	39	39	40	40	CPOS	C100

Figure 7 A typical ELISA plate illustrating the layout of the plate which can run 40 samples simultaneously as well as showing the position of positive controls (CPOS), negative controls (CNEG)100% control (C100) and blank control wells (BL).

3.8 Statistical analysis

Statistical analysis was performed using IBM© SPSS© Statistics version 26.0.0.0 and Microsoft Excel® (Microsoft). Data was tested for normality using the Shapiro-Wilk test. Data for the three different time points were compared using the non-parametric related samples Friedman's Two-Way Analysis of Variance by Ranks at a significance level of 0.05. Pairwise comparisons significance values have been adjusted by the Bonferroni correction for multiple tests.

3.9 Ethical considerations

Approval to use animal tissue in research in terms of Section 20 of the Animal Diseases Act (Act 35 of 1984) was obtained before the project commenced (Reference number 12/11/1/1/2 (903) as well as approval by the Animal Ethics

Committee and the OTAU ethics panel (Certificate V076-18) as well as the Faculty of Veterinary Science Research Ethics Committee (Certificate REC071-18), see Appendix 2.

In order to maintain the wellbeing of these dogs, as well as to ensure their continuous use as educational tools for future students and research purposes it was important that the dogs not become aggressive towards neither the handling nor sampling process. To ensure this wellbeing certain prerequisites were imparted unto the researcher by the OTAU ethics panel, these included:

1. Sampling must take place in a novel location in case they form a negative association with the location. The sampling should therefore not take place in the OTAU facility or any place where the dogs are used during practical sessions on a regular basis.
2. The use of muzzles is noted and if used, it is the responsibility of the researcher to ensure that they are first habituated to them.
3. Dogs with high stress levels should ideally be sedated prior to subsequent procedures. Highly distressed animals that do not respond well to sedation should be withdrawn.

CHAPTER 4: RESULTS

4.1 Detection of AHSV and EEV nucleic acid

No viral RNA from AHSV or EEV could be detected in any of the blood samples collected during each of the time points. Examples of the original RT-qPCR results for November 2018, April 2019 and June 2019 are attached under Appendix 3. Raw data for the entire study is presented in Appendix 5.

4.2 Detection of AHSV and EEV antibody

4.2.1 Indirect ELISA for antibody to AHSV

Serum samples were tested for antibody to AHSV during November 2018 as baseline, April 2019 as reference interval for the middle of the study and June 2019 as the end reference of the study. The raw data from the iELISA performed by the ARC-OVR are attached under Appendix 4. The descriptive statistics for the AHS iELISA results on these three different sampling points are presented in Table 6 and Figure 8. The cut-off as defined for horse sera is a PP of 11.9% (Maree and Paweska, 2005). The highest PP obtained from all the sampling points was -1,21%, implicating that all the sera tested during the study period was classified as negative. The median PP value obtained for the November 2018 time point was -3,95% and the data was skewed to the right with the minimum PP at -4.74% and the maximum at -1.97%. Pairwise comparison indicated that there were no significant differences in the PP values between November 2018 and April 2019 ($p = 0.192$).

Table 6 Descriptive statistics for the AHSV iELISA Percentage Positivity (PP) values during the different sampling points

	November 2018	April 2019	June 2019
Median (Mean#)	-3.95	-3.72 (-3.68#)	-2.00
Standard Deviation	-	0.09	-
Minimum	-4.74	-4.54	-4.39
Maximum	-1.97	-2.27	-1.21
Shapiro-Wilk*	0.004	0.083*	0.000

* If the value of the Shapiro-Wilk Test is greater than 0.05, the data is normal

mean is reported only for parametric data.

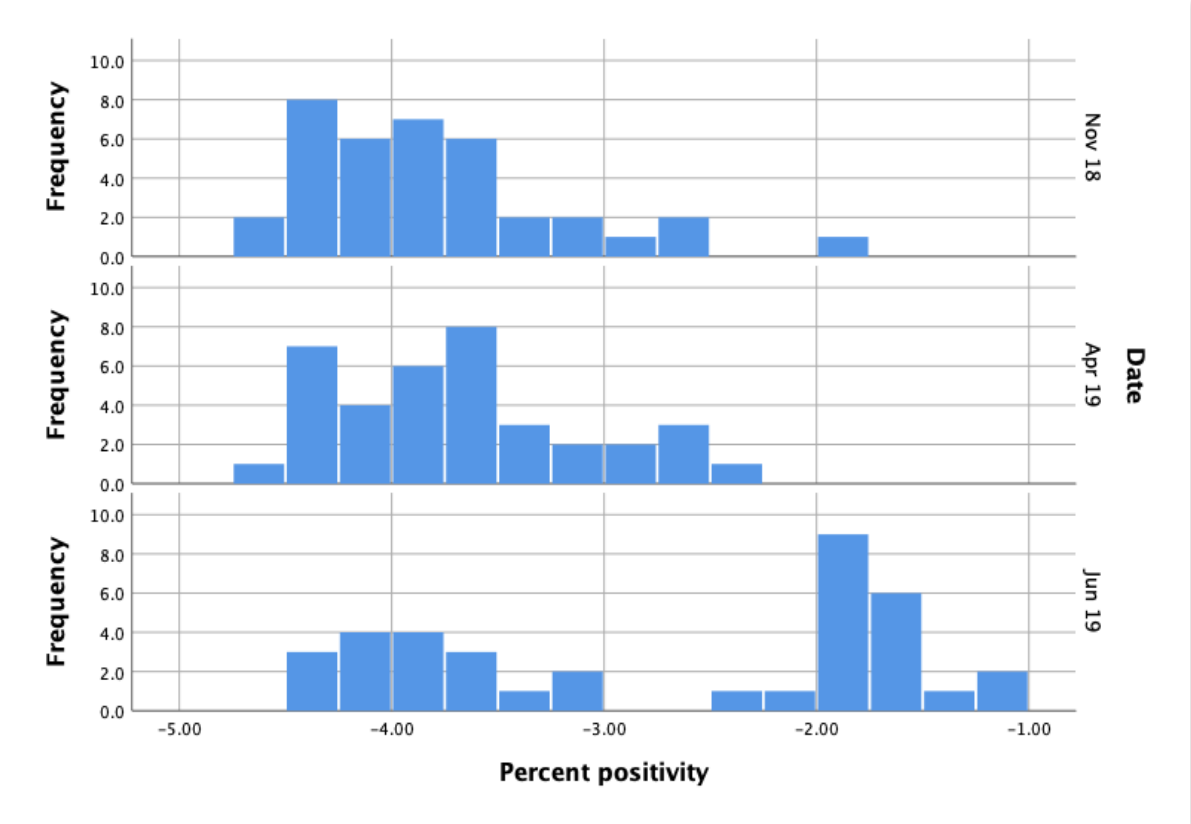

Figure 8 Distribution of the Percent Positivity (PP) values obtained in the AHSV iELISA for the different time points. November 18 represents the baseline at the start of the study while April 19 and June 19 represents the middle and end of the study period respectively.

Evident from Figure 9, there was a significant difference between the distributions for November 2018 and June 2019 (p = 0.006). For the June 2018 sampling point there were 19 dogs with PP values >= -2.0% while 17 dogs had PP <= -3.0%. One dog had a PP of 2.4%. There was not a significant difference in the mean PP value between November 2018 and April 2019 (p = 0.603).

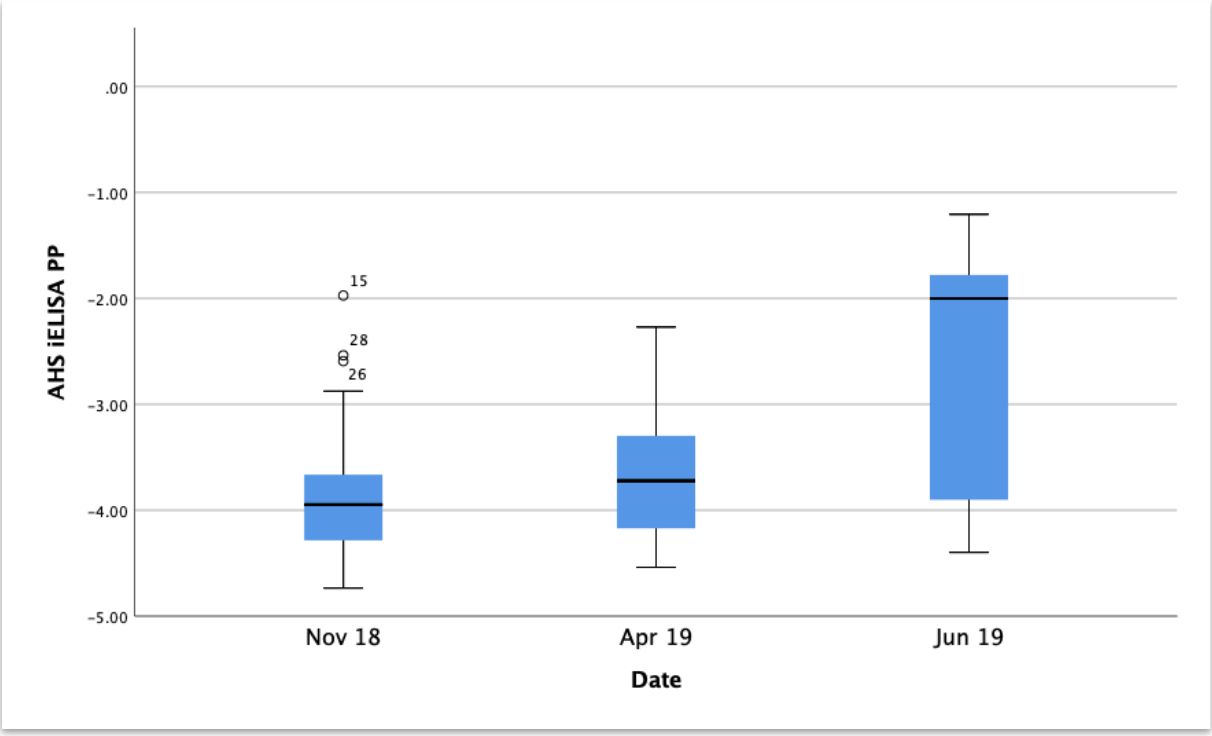

Figure 9 Box and whisker plots representing the Percent Positivity (PP) of the AHSV iELISA at the different time points. The box represents the median, 25th and 75th percentiles while the whiskers represents the minimum and maximum values. Outliers are indicated as circles. There was a significant difference between November 2018 and June 2019 (p = 0.006).

4.2.2 Competitive ELISA for antibody to EEV

Serum samples were tested for antibody to EEV at the same time points as for AHSV above. The descriptive statistics for the EEV cELISA are presented in Table 7 and Figure 10. The raw data from the cELISA are attached under Appendix 5. The cut-off as defined for horse sera is a PI of 29.5% (Crafford et al., 2011). The highest PI obtained from all the sampling points was 35.9%, implicating that some of the

sera tested as low positive. The median PI value obtained for the November 2018 time point was -4.50% and the data was normally distributed with the minimum PI at -42.0% and the maximum at 20.2%.

Table 7 Descriptive statistics for the EEV cELISA Percentage Inhibition (PI) values during the different sampling points

	November 2018	**April 2019**	**June 2019**
Median (Mean#)	-4.50 (-6,59#)	21.70 (20,63#)	-0.20
Standard Deviation	2.05	1.27	-
Minimum	-42.0	4,1	-46.2
Maximum	20.2	35,9	23.4
Shapiro-Wilk*	0.296*	0.942*	0.000

* If the value of the Shapiro-Wilk Test is greater than 0.05, the data is normally distributed.
# mean is reported only for parametric data.

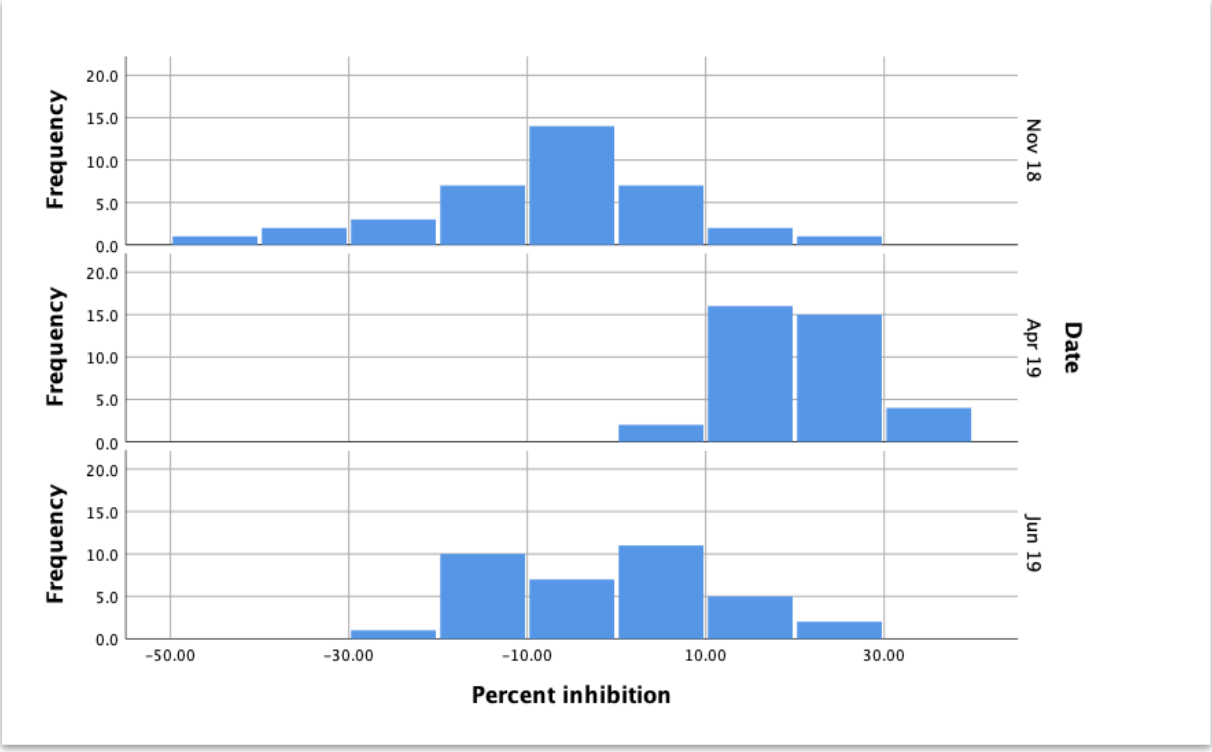

Figure 10 Distribution of the Percent Inhibition (PI) values obtained in the EEV cELISA for the different time points. November 18 represents the baseline at the start of the study while April 19 and June 19 represents the middle and end of the study period respectively.

There were significant differences in the distribution of PI values between November 2018 and April 2019 ($p < 0.0005$) and between April 2019 and June 2019 ($p < 0.0005$) as demonstrated in Figure 11. For the April 2019 sampling point there were six dogs with PI values >= 29.5% while 31 dogs had PI values between 4.1% and 28.7%. The PI values for dogs in June 2019 varied between -46,2 and 23.4 with a median of -0.20%. There was not a significant difference between November 2018 and June 2019 ($p = 1.000$).

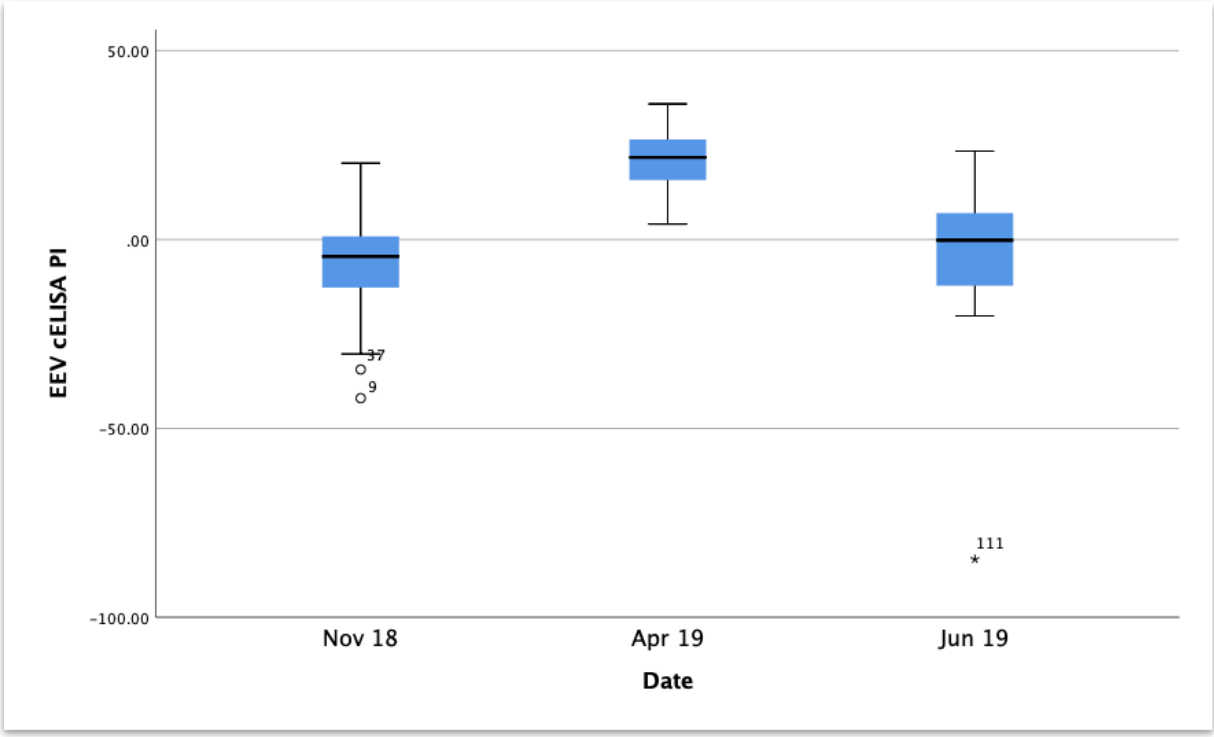

Figure 11 Box and whisker plots representing the Percent Inhibition (PI) of the EEV cELISA at the different time points. The box represents the median, 25th and 75th percentiles while the whiskers represents the minimum and maximum values. Outliers are indicated as circles. There was a significant difference in the mean PI value between November 2018 and April 2019 ($p < 0.0005$). There were no significant differences between November 2018 and June 2019 ($p = 1.000$).

CHAPTER 5: DISCUSSION

Recent literature has indicated an increase in cases where dogs have been affected by AHS. Contrary to historic findings, several of these cases have been reported where dogs became infected without any evidence of contact with or consumption of infected horses or their products. Here we report on a prospective study to determine the prevalence of specific antibody and nucleic acid to two equine orbiviruses namely AHSV and EEV in a high risk, isolated dog population during the high vector period in an endemic area. This was also the first attempt to investigate prevalence of EEV in dogs.

An attempt was made to detect antibody to AHSV using an indirect ELISA while a competitive ELISA was used to detect antibody to EEV. Infection rates were determined by demonstrating viral nucleic acids by RT-qPCR. All of the 37 sampled dogs remained negative for RNA to both AHSV and EEV as well as antibody to AHSV for the duration of the study. Six dogs (prevalence = 16%) had low positive PI values for antibody to EEV in April 2019.

Dogs were kept in open kennels in close proximity to horses, sheep and cattle. Horses on the premises are vaccinated annually against AHS with a registered live vaccine (OBP; Onderstepoort Biological Products, African Horse Sickness vaccine for horses, donkeys and mules, Reg. No. G 0116 (Act 36/1947); Onderstepoort, Pretoria, 0110). In spite of annual vaccination, a 16% infection rate has previously been reported in these horses (Weyer et al., 2013). Dogs in this population have

also historically been diagnosed with clinical AHS with one confirmed mortality during 2016 (Whitehead et al., 2018).

Our sampling strategy was based on historic data of the temporal distribution of AHS cases as indicated in Figure 12. Baseline data was collected in November before the onset of the anticipated AHS season. Blood samples were collected on a monthly interval from February to June 2019 to coincide with the expected peak season.

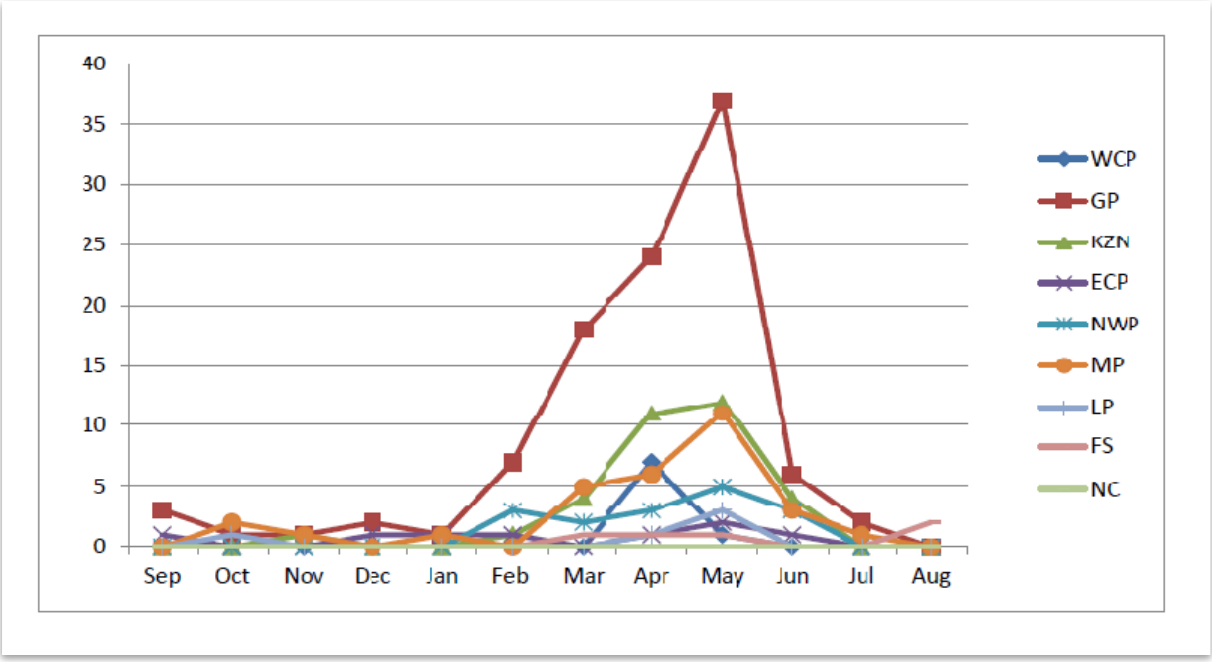

Figure 12 Temporal distribution of reported AHS outbreaks in each province of South Africa from September 2015 – August 2016.-(Directorate Animal Health, 2016)

We hypothesised that the risk of exposure of these dogs to the culicoides vectors of equine orbiviruses were high due to the proximity to horses and irrigated pastures shared with cattle and sheep. *C. imicola* have a predilection to horses while *C. bolitinos* have a predilection for ruminants (Meiswinkel and Paweska, 2003). Both these species are able to transmit AHSV and occur in and around the study area.

Furthermore, an above average rainfall of 642.4 mm was recorded during the study period (November 2018-July 2019) which could have supported increased vector numbers (Figure 13). The prevalence of the vector and its temporal distribution are currently being determined in a separate study and could shed more light on the host preference and feeding habits of the vector.

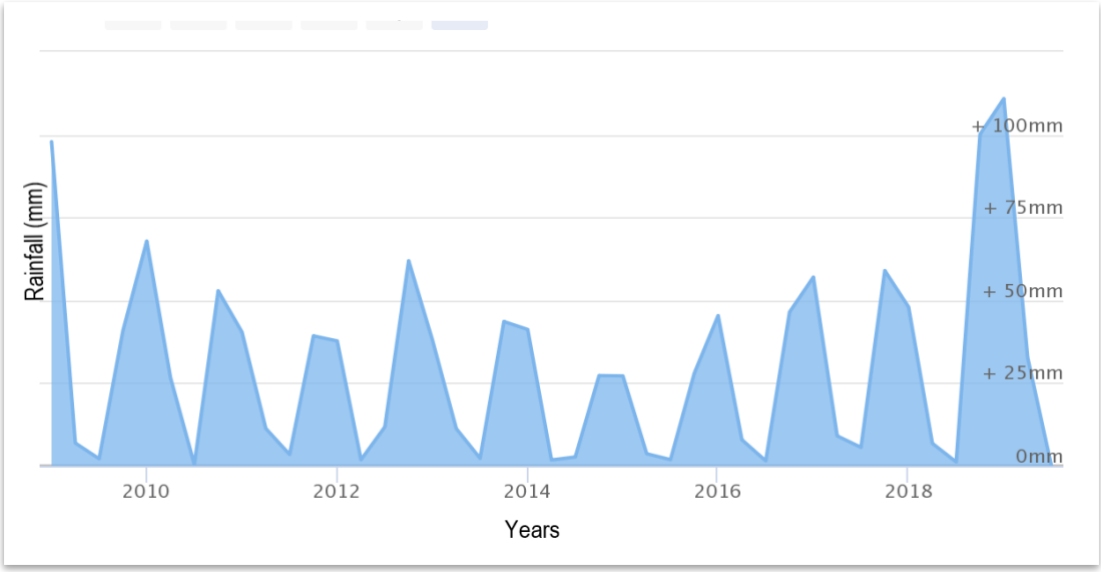

Figure 13 Rainfall (mm) between 2010-2019 for the Onderstepoort area, Pretoria where the study was based. (Adapted from 'https://www.worldweatheronline.com/' title='Historical average weather' Data provided by WorldWeatherOnline.com, 2019, 6 September 2019, (https://www.worldweatheronline.com/onderstepoort-weather-averages/gauteng/za.aspx)

Our data suggests that there were no equine orbiviruses circulating in this dog population during the study period and this is consistent with the absence of any clinical signs reported in the same population during the same time period. Interestingly, it is also important to note that there have not been any reports of clinical canine AHS in dogs from the area during the specific time period.

The RT-qPCR that was used is very sensitive and specific and has been effectively used in the detection of AHSV and EEV in horses (Quan et al., 2010, Guthrie et al.,

2013, Rathogwa et al., 2014, Weyer et al., 2015, Weyer et al., 2013). The primers used in these assays were developed to recognize all the AHSV and EEV strains that are currently circulating in Southern Africa. Although it has not been validated for use in dogs, there are currently no accredited diagnostic tests validated for the detection of AHSV or EEV in dogs. However, canine AHSV has previously been identified using this PCR (Whitehead et al., 2018, O'Dell et al., 2018).

Both the AHS iELISA and the EE cELISA used in this study are not validated for detecting antibody in dogs. However, both the AHS iELISA (Maree and Paweska, 2005) and the EE cELISA (Crafford et al., 2011) have been extensively validated for the detection of antibody in horses.

The EE cELISA uses polyclonal EEV specific guinea pig serum as the competitor and should be able to detect antibody to EEV in any species except guinea pigs. This was the first attempt to use this assay in the canine species. The 16% prevalence of low positive PI values for antibody to EEV in April 2019 could suggest that some of the dogs were exposed to EEV without becoming viraemic or that these low positives could be from cross-reactions with another unrelated antigen or orbivirus, as low levels of cross-reactivity has been seen for some bluetongue antisera in this EE cELISA (Crafford et al., 2011).

The AHS iELISA has been successfully used before to positively identify seroconversion in dogs (van Sittert et al., 2013, Whitehead et al., 2018). It must be mentioned that the protein-G horseradish peroxidase conjugate used in the assay

only has moderate affinity for binding to dog immunoglobulin and will impact on the sensitivity of the test. (Thermo Fisher Scientific Inc., 2013, Binding characteristics of antibody-binding proteins: Protein A, Protein G, Protein A/G and Protein L, viewed 15 August 2019. https://www.thermofisher.com/order/catalog/product/21193#/21193) The cut-off values for dogs should be re-evaluated using a known panel of positive and negative sera.

The significant right shift in the AHS iELISA PP values for June 2019 could be interpreted as due to cross-reactions with other orbiviruses circulating in the population or that the assay in its current format is not sensitive enough in dogs. The probability that we missed possible viraemic individuals was unlikely since it has been demonstrated that experimentally infected AHSV viraemic horses can be detected seven days post-inoculation and that AHSV viral nucleic acid could still be identified in blood for up to 97 days post-inoculation (Quan et al., 2010). From the literature it is suggested that viremia in dogs are of shorter duration (Dardiri and Ozawa, 1969, Mellor and Mertens, 2008). However, it is known that BTV binds with high affinity to red blood cells (RBCs) (Coetzer and Guthrie, 2004, Mellor and Mertens, 2008, MacLachlan and Dubovi, 2017), and the lifespan of RBCs is approximately 104 days in the average dog (Abrams-Ogg, 2010). If we assume that other orbiviruses such as AHSV and EEV behave similarly to that of BTV we should be able to detect viral nucleic acid for roughly the same time as the lifespan of a canine RBC.

Although we have not evaluated breed predilection, it has been discussed by some authors (van Sittert et al., 2013, O'Dell et al., 2018). Having short hair, as Beagles

do, could influence vector contact by allowing easier access for vectors to a blood meal (Schneider and Higgs, 2008, Wilson et al., 2009). This theory needs further investigation.

Rectal temperatures were taken for all dogs during blood collections with only two dogs exhibiting pyrexia, i.e. a rectal temperature exceeding 39.5 °C (see Appendix 5). During their study van Sittert et al. (2013) documented short periods of pyrexia which they said could lead to sick animals easily being missed. A short transient rise in temperature was also documented by Theiler who admitted that a rise in temperature was always mild in canine AHSV infections (Theiler, 1906, Theiler, 1910b). Clinical cases presented by Whitehead et al. (2018) either presented with hypothermia (37.0 °C) or was not recorded. Since the two dogs which exhibited pyrexia in this study did not test positive for viral nucleic acid by PCR no correlation or interpretation towards viremia and pyrexia can be made.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusions

The following conclusions can be drawn from this study:

- Consecutively negative PCR results throughout the study indicate that no AHSV or EEV viraemia could be demonstrated.
- A significant right shift in the negative AHS iELISA values for antibody could be due to low sensitivity of the assay in dogs or cross-reaction with other orbiviruses like bluetongue virus, however this should be interpreted with caution due to a lack of proper validation of the assay in dogs.
- There is serological evidence for a transient right shift of EE cELISA values indicating either low level EEV exposure without viremia or cross-reaction with other orbiviruses like bluetongue virus.
- No evidence of subclinical infection could be demonstrated in the study population

6.2 Strengths of this study

The following strengths were identified in this study:

- The Beagle population at OTAU was ideal for this study as dogs were kept outside in close proximity to horses, cattle and sheep in an orbivirus endemic area.

- Serial data collection occurred during a time period that was highly favourable towards vector midges, based on rainfall parameters.
- Sensitive assays were used for the detection of viral nucleic acid.

6.3 Limitations of this study

The following limitations were identified in this study:

- Concurrent vector collection and blood meal analysis did not form part of this study and could have given more of a definitive answer towards natural vector-borne transmission of equine orbiviruses to dogs.
- No positive control sera from dogs with known antibody levels to EEV or AHSV were available.

6.4 Recommendations for Future Studies

Concurrent vector collection and blood meal analysis in the vicinity of the study population will shed more light on the prevalence and host preferences of the vectors. It will be valuable to repeat this study in the future when positive cases are confirmed in the area, since no new cases have been reported since 2017. Further investigation into the correlation of AHS cases in horses compared to dogs during the presence or absence of the disease in dogs or the use of insecticides could also shed more light. It is evident that there could be other factors that play a role in the epidemiology in spite of high rainfall.

Further validation of the ELISA assays will be needed to accurately investigate antibody levels to orbiviruses in dogs.

Future prospective studies where dogs are experimentally infected with orbiviruses in a controlled environment either by needle or oral infection are unlikely to occur purely based on ethical reasons. These studies will however be valuable to study the pathogenesis of AHS in dogs and dogs could potentially act as a model for AHS.

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APPENDIXES

Appendix 1: Canine identification, microchip numbers and signalment

Animal name	Case number	Microchip number	Age	Sex
Raven	1	953010001847343	2 years	Female
Faye	2	953010001847814	2 years	Female
Aleandro	3	953010002160842	2 years	Male
Watson	4	900008800546509	6 years	Male
Sarabi	5	953010001847812	2 years	Female
Connor	6	953010000907755	4 years	Male
Kiara	7	953010001847818	2 years	Female
Fletcher	8	900008800715532	6 years	Male
Thea	9	953010001815130	3 years	Female
Mozart	10	953010001815242	3 years	Male
Eevee	11	953010001815181	3 years	Female
Bonaparte	12	900008800546506	6 years	Male
Sherlock	13	953010001234051	3 years	Male
Dawn	14	953010001815234	3 years	Female
Luna	15	953010001815282	3 years	Female
Leo	16	953010001847676	2 years	Male
Raffa	17	953010001234043	9 years	Male
Trientjie	18	953010001222193	3 years	Female
Annemieke	19	953010000903107	3 years	Female
Dippie	20	945000001611303	4 years	Female
Danica	21	900008800324998	7 years	Female

Elena	22	900008800324186	7 years	Female
Nala	23	953010000432371	5 years	Female
Dixie	24	945000001611256	4 years	Female
Kira	25	953010000430244	5 years	Female
Smiler	26	953010000430375	5 years	Female
Miame	27	900008800396155	8 years	Female
Lallie	28	900008800612228	6 years	Female
Neo	29	953010000506938	4 years	Male
Lindile	30	900008800544045	6 years	Female
Tammy-lee	31	4C3F326223	6 years	Female
Mieke	32	953010000144151	5 years	Female
Carien	33	945000001520295	5 years	Female
Puppy 3	34 (P3)	953010002481973	4 months	Female
Puppy 6	35 (P6)	953010002478634	4 months	Male
Puppy 7	36 (P7)	953010002481965	4 months	Male
Puppy 11	37 (P11)	953010002478643	4 months	Male

Appendix 2: Animal ethics committee, Research ethics committee and Section 20 approval letters

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Response to the Animal Ethics Committee (AEC) regarding topic: Seroprevalence and infection rates of African horse sickness virus and equine encephalosis virus in dogs at the Faculty of Veterinary Sciences, Onderstepoort (NH Jacobs)**
PROJECT NUMBER	**V076-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. NH Jacobs**

STUDENT NUMBER (where applicable)	**U_26222028**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Canine**
NUMBER OF ANIMALS	**33**
Approval period to use animals for research/testing purposes	**August 2018-August 2019**
SUPERVISOR	**Dr. JE Crafford**

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 10 September 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

Approve only ethically obtained stored samples

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Sero prevalence and infection rates of African horse sickness virus and equine encephalosis virus in dogs at the Faculty of Veterinary Sciences, Onderstepoort.**
PROJECT NUMBER	**REC071-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**NH Jacobs**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**JE Crafford**

APPROVED	Date **9 October 2018**
CHAIRMAN: UP Research Ethics Committee	Signature

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Golcio • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/12

Dr Nicholaas Hendrik Jacobs
Pierre Van Ryneveld Veterinary Clinic
Cell: 074 893 5467
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RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Dr Jacobs

Your application dated 29 June 2018 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form dated 29 June 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HenryG@daff.gov.za;
3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HenryG@daff.gov.za;
4. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;
5. Samples for this research project may only be collected from the Onderstepoort Teaching Animal Unit (OTAU) Beagle dog colony;

6. Samples from the Beagles may be sent to the following laboratories:

   6.1. Equine Research Centre/Veterinary Genetics Laboratory for AHS and EEV PCR and typing;

   6.2. ARC-OVR Virology Laboratory for AHS Elisa

   6.3. Department of Veterinary Tropical Diseases - Serology Laboratory for EEV Elisa;

7. As the Equine Research Centre is not SANAS accredited or DAFF approved to perform AHS PCR as a diagnostic test and as AHS is a controlled animal disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), the results of this test may not be distributed to the owners of the animals or to other persons;

8. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the state veterinarian of the area;

9. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project by incineration.

10. Only a registered waste disposal company may be used for the removal of all potentially infectious waste from the research project;

11. Records must be kept for five years for auditing purposes;

12. A dispensation for the storage of serum and whole blood from the OTAU Beagle dog colony is attached.

Title of research/study: Seroprevalence and infection rates of African horse sickness virus and Equine encephalosis virus in dogs at the Faculty of Veterinary Science, Onderstepoort
Researcher: Dr Nicholaas Hendrik Jacobs
Institution: Equine Research Centre/Veterinary Genetics Laboratory, Faculty of Veterinary Science, Onderstepoort; ARC-OVR Virology Laboratory; Serology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort.
Permit Expiry date: 31 December 2019
Our ref Number: 12/11/1/1/2 (903)
Your ref: v076-18

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 28

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

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Reference: 12/11/1/12

Dr Nicholaas Hendrik Jacobs
Pierre Van Ryneveld Veterinary Clinic
Cell: 074 893 5467
E-mail: dmicholl@pierrevanreynelveldvet.com, u02554763@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "SEROPREVALENCE AND INFECTION RATES OF AFRICAN HORSE SICKNESS VIRUS AND EQUINE ENCEPHALOSIS VIRUS IN DOGS AT THE FACULTY OF VETERINARY SCIENCE, ONDERSTEPOORT"

A dispensation is hereby granted on Point 9 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Serum and whole blood collected from the OTAU Beagle dog colony must be stored under access control at the ERC and DVTD Serology Laboratory;

ii) Serum and whole blood collected from the OTAU Beagle dog colony must not be outsourced or used for further research without prior written approval from the Director: Animal Health.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -09- 28

Appendix 3: RT-qPCR test reports from the ERC

Test Report: E180975

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science
Veterinary Genetics Laboratory
Molecular Diagnostics

Date opened: 23 November 2018
Date closed: 27 November 2018

Date of report: 27 November 2018
Report reference number: E180975
Report type: Final

Purpose of Testing: Passive Surveillance

Pierre van Ryneveld Veterinary Clinic
PO Box 60394
Pierre van Ryneveld
0157

Tel: (012) 662 2502
Fax:
E-mail: mariska@pierrevanryneveldvet.com; lab

Dear Dr. N. H. Jacobs

Results from submitted samples

Owner surname: Otau
Animal details: 'Raven', no age details, no sex details, no breed details
Your reference: No details — Animal Reference: E180975_1

Sample ID: EP16945 — Date collected: No details
Sample type: Blood (EDTA) — Date received: 26 November 2018
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 26 November 2018
Result: AHSV-VP7 Result Not Reported — Date completed: 26 November 2018

Test: EEV Real-Time Testing Protocol (4) — Date started: 26 November 2018
Result: EEV-VP7 negative — Date completed: 26 November 2018

Owner surname: Otau
Animal details: 'Faye', no age details, no sex details, no breed details
Your reference: No details — Animal Reference: E180975_2

Sample ID: EP16946 — Date collected: No details
Sample type: Blood (EDTA) — Date received: 26 November 2018
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 26 November 2018
Result: AHSV-VP7 Result Not Reported — Date completed: 26 November 2018

Test: EEV Real-Time Testing Protocol (4) — Date started: 26 November 2018
Result: EEV-VP7 negative — Date completed: 26 November 2018

Owner surname: Otau
Animal details: 'Alice', no age details, no sex details, no breed details
Your reference: No details — Animal Reference: E180975_3

Private Bag X04
ONDERSTEPOORT
0110

Tel: (012) 529 8068
Fax: (012) 529 8301
E-mail: erc@up.ac.za

Tel: (012) 529 8068
Fax: (012) 529 8301
E-mail: alan.guthrie@up.ac.za

November 2018

Test Report: E191091

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Veterinary Genetics Laboratory
Molecular Diagnostics

Date opened: 15 May 2019
Date closed: 17 May 2019

Date of report: 17 May 2019
Report reference number: E191091
Report type: Final

Purpose of Testing: Passive Surveillance

Pierre van Ryneveld Veterinary Clinic
PO Box 60394
Pierre van Ryneveld
0157

Tel: (012) 662 2502
Fax:
E-mail: mariska@pierrevanryneveldvet.com; lab

Dear Dr. N. H. Jacobs

Results from submitted samples

Owner surname: Otau
Animal details: 'Raven', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191091_1

Sample ID: EP19299 — Date collected: 26 May 2019
Sample type: Blood (EDTA) — Date received: 15 May 2019
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 15 May 2019
Result: AHSV-VP7 negative — Date completed: 16 May 2019

Test: EEV Real-Time Testing Protocol (4) — Date started: 15 May 2019
Result: EEV-VP7 negative — Date completed: 16 May 2019

Owner surname: Otau
Animal details: 'Faye', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191091_2

Sample ID: EP19300 — Date collected: 26 May 2019
Sample type: Blood (EDTA) — Date received: 15 May 2019
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 15 May 2019
Result: AHSV-VP7 negative — Date completed: 16 May 2019

Test: EEV Real-Time Testing Protocol (4) — Date started: 15 May 2019
Result: EEV-VP7 negative — Date completed: 16 May 2019

Owner surname: Otau
Animal details: 'Allie', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191091_3

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Fax: (012) 529 8301
E-mail: erc@up.ac.za

Tel: (012) 529 8068
Fax: (012) 529 8301
E-mail: alan.guthrie@up.ac.za

Test Report: E191345

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Veterinary Genetics Laboratory
Molecular Diagnostics

Date opened: 04 July 2019
Date closed: 15 July 2019

Date of report: 15 July 2019
Report reference number: E191345
Report type: Final

Purpose of Testing: Passive Surveillance

Pierre van Ryneveld Veterinary Clinic
PO Box 60394
Pierre van Ryneveld
0157
Tel: (012) 662 2502
Fax:
E-mail: mariska@pierrevanryneveldvet.com; lab

Dear Dr. N. H. Jacobs

Results from submitted samples

Owner surname: OTAU
Animal details: 'Raven', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191345_1

Sample ID: EP19887 — Date collected: 21 June 2019
Sample type: Blood (EDTA) — Date received: 08 July 2019
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 08 July 2019
Result: AHSV-VP7 negative — Date completed: 12 July 2019

Test: EEV Real-Time Testing Protocol (4) — Date started: 08 July 2019
Result: EEV-VP7 negative — Date completed: 12 July 2019

Owner surname: OTAU
Animal details: 'Faye', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191345_2

Sample ID: EP19888 — Date collected: 21 June 2019
Sample type: Blood (EDTA) — Date received: 08 July 2019
Condition of sample upon receipt: Acceptable

Test: AHSV/Xeno Real-Time Testing Protocol (3) — Date started: 08 July 2019
Result: AHSV-VP7 negative — Date completed: 12 July 2019

Test: EEV Real-Time Testing Protocol (4) — Date started: 08 July 2019
Result: EEV-VP7 negative — Date completed: 12 July 2019

Owner surname: OTAU
Animal details: 'Allie', no age details, no sex details, Dog
Your reference: No details — Animal Reference: E191345_3

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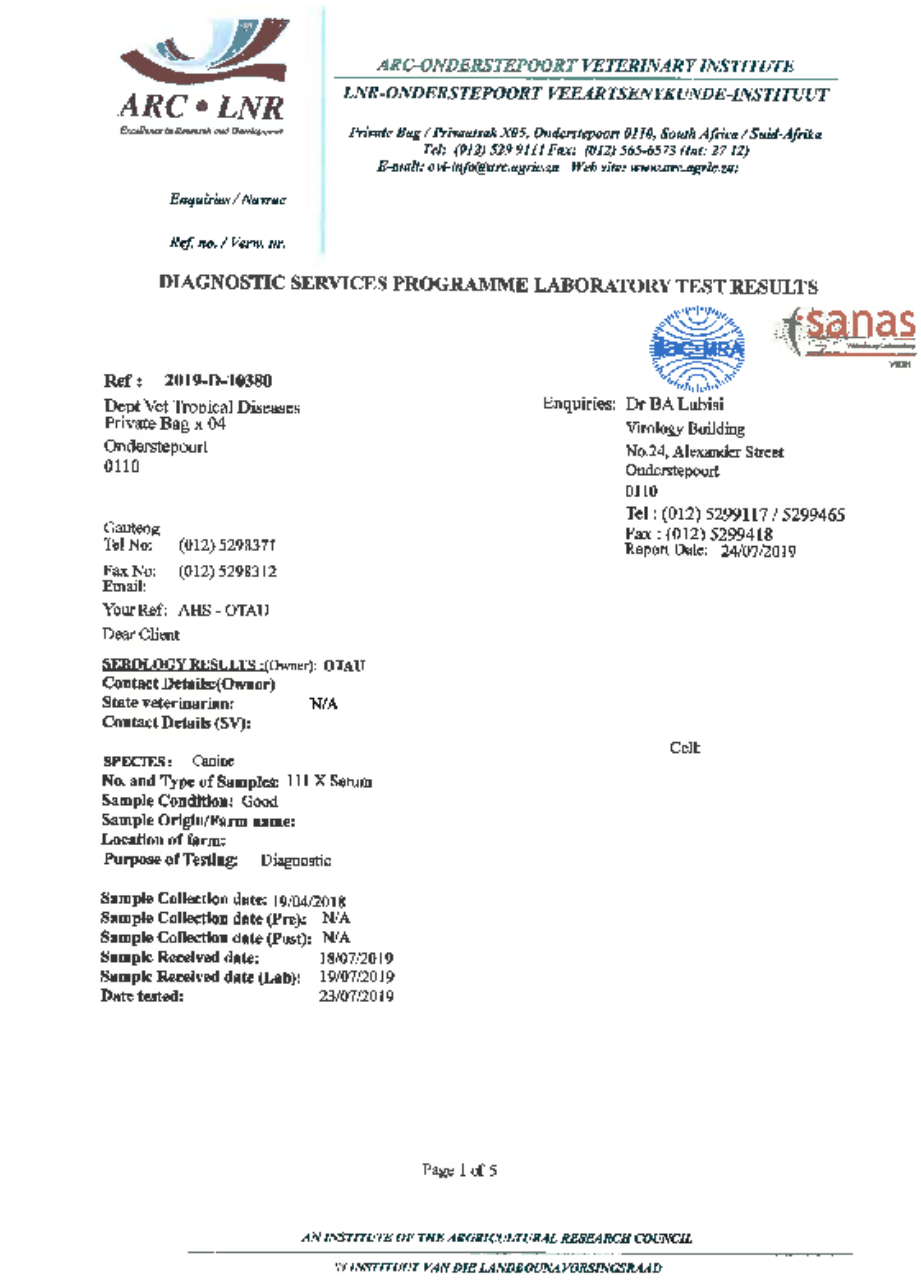
E-mail: alan.guthrie@up.ac.za

Appendix 4: iELISA results



ARC • LNR

ARC-ONDERSTEPOORT VETERINARY INSTITUTE
LNR-ONDERSTEPOORT VEEARTSENYKUNDE-INSTITUUT

Private Bag / Privaatsak X05, Onderstepoort 0110, South Africa / Suid-Afrika
Tel: (012) 529 9111 Fax: (012) 565-6573 (Int: 27 12)
E-mail: ovi-info@arc.agric.za Web site: www.arc.agric.za

Enquiries / Navrae

Ref. no. / Verw. nr.

DIAGNOSTIC SERVICES PROGRAMME LABORATORY TEST RESULTS

Ref : 2019-D-10380

Dept Vet Tropical Diseases
Private Bag x 04
Onderstepoort
0110

Enquiries: Dr BA Lubisi
Virology Building
No.24, Alexander Street
Onderstepoort
0110
Tel : (012) 5299117 / 5299465
Fax : (012) 5299418
Report Date: 24/07/2019

Gauteng
Tel No: (012) 5298371
Fax No: (012) 5298312
Email:

Your Ref: AHS - OTAU

Dear Client

SEROLOGY RESULTS :(Owner): OTAU
Contact Details:(Owner)
State veterinarian: N/A
Contact Details (SV):

Cell:

SPECIES: Canine
No. and Type of Samples: 111 X Serum
Sample Condition: Good
Sample Origin/Farm name:
Location of farm:
Purpose of Testing: Diagnostic

Sample Collection date: 19/04/2018
Sample Collection date (Pre): N/A
Sample Collection date (Post): N/A
Sample Received date: 18/07/2019
Sample Received date (Lab): 19/07/2019
Date tested: 23/07/2019

Page 1 of 5

AN INSTITUTE OF THE AGRICULTURAL RESEARCH COUNCIL
'N INSTITUUT VAN DIE LANDBOUNAVORSINGSRAAD

Ref : **2019-D-10380**

VIRUS SEROLOGY RESULTS : (Owner): OTAU

NO	SAMPLE REFERENCE	AHS
		I-ELISA
	METHOD NO	VR_ME_010
1	1	Neg
2	2	Neg
3	3	Neg
4	4	Neg
5	5	Neg
6	6	Neg
7	7	Neg
8	8	Neg
9	9	Neg
10	10	Neg
11	11	Neg
12	12	Neg
13	13	Neg
14	14	Neg
15	15	Neg
16	16	Neg
17	17	Neg
18	18	Neg
19	19	Neg
20	20	Neg
21	21	Neg
22	22	Neg
23	23	Neg
24	24	Neg
25	25	Neg
26	26	Neg
27	27	Neg
28	28	Neg
29	29	Neg
30	30	Neg
31	31	Neg
32	32	Neg
33	33	Neg

Ref : 2019-D-10380

VIRUS SEROLOGY RESULTS : (Owner): OTAU

NO	SAMPLE REFERENCE	AHS
		I-ELISA
	METHOD NO	VR_ME_010
34	P3-34	Neg
35	PC-35	Neg
36	P7-36	Neg
37	P11-37	Neg
38	38	Neg
39	39	Neg
40	40	Neg
41	41	Neg
42	42	Neg
43	43	Neg
44	44	Neg
45	45	Neg
46	46	Neg
47	47	Neg
48	48	Neg
49	49	Neg
50	50	Neg
51	51	Neg
52	52	Neg
53	53	Neg
54	54	Neg
55	55	Neg
56	56	Neg
57	57	Neg
58	58	Neg
59	59	Neg
60	60	Neg
61	61	Neg
62	62	Neg
63	63	Neg
64	64	Neg
65	65	Neg
66	66	Neg

Ref : **2019-D-10380**

VIRUS SEROLOGY RESULTS : (Owner): OTAU

NO	SAMPLE REFERENCE	AHS
		I-ELISA
	METHOD NO	VR_ME_010
67	67	Neg
68	68	Neg
69	69	Neg
70	70	Neg
71	71	Neg
72	72	Neg
73	73	Neg
74	74	Neg
75	75	Neg
76	76	Neg
77	77	Neg
78	78	Neg
79	79	Neg
80	80	Neg
81	81	Neg
82	82	Neg
83	83	Neg
84	84	Neg
85	85	Neg
86	86	Neg
87	87	Neg
88	88	Neg
89	89	Neg
90	90	Neg
91	91	Neg
92	92	Neg
93	93	Neg
94	94	Neg
95	95	Neg
96	96	Neg
97	97	Neg
98	98	Neg
99	99	Neg

Ref : **2019-D-10380**

VIRUS SEROLOGY RESULTS : (Owner): OTAU

NO	SAMPLE REFERENCE	AHS
		I-ELISA
	METHOD NO	VR_ME_010
100	100	Neg
101	101	Neg
102	102	Neg
103	103	Neg
104	104	Neg
105	105	Neg
106	106	Neg
107	107	Neg
108	108	Neg
109	109	Neg
110	110	Neg
111	111	Neg

Interpretation and Comment:

Disclaimer

1. The report relates only to the samples supplied to the ARC-OVI Virology laboratory.
2. This report shall not be reproduced, except in full, without the written approval of the Head of Laboratory or the Quality Manager.
3. The opinions and interpretations that may be available in this report, fall outside the scope of accreditation.

Sincerely,

Dr BA Lubisi
Technical Manager: Virology

Appendix 5:

A summary of the laboratory results for the entire study. PCR results are given as qualitative values as reported from the ERC. PP and PI values are given in brackets underneath the iELISA and cELISA results respectively. Blank spaces indicates where tests were not conducted.

Collection date: 21 November 2018				
Animal number	AHSV PCR result	EEV PCR result	AHSV iELISA (PP)	EEV cELISA (PI)
1	N	N	N (-3,777)	N (-30,3)
2	N	N	N (-4,228)	N (-20,7)
3	N	N	N (-3,890)	N (-23,3)
4	N	N	N (-4,453)	N (-11,9)
5	N	N	N (-4,510)	N (-15,0)
6	N	N	N (-3,044)	N (-2,7)
7	N	N	N (-4,284)	N (1,6)
8	N	N	N (-3,720)	N (3,1)
9	N	N	N (-4,340)	N (-42,0)
10	N	N	N (-3,495)	N (-13,7)
11	N	N	N (-2,875)	N (-6,0)
12	N	N	N (-3,439)	N (-3,2)
13	N	N	N (-4,397)	N (0,8)
14	N	N	N (-4,228)	N (-4,0)
15	N	N	N (-1,973)	N (-4,7)
16	N	N	N	N

			(-3,551)	(-2,5)
17	N	N	N (-3,664)	N (-0,1)
18	N	N	N (-3,720)	N (-12,7)
19	N	N	N (-4,228)	N (-5,9)
20	N	N	N (-4,115)	N (-8,8)
21	N	N	N (-3,946)	N (-8,6)
22	N	N	N (-3,946)	N (-0,6)
23	N	N	N (-3,777)	N (14,4)
24	N	N	N (-4,340)	N (1,4)
25	N	N	N (-3,664)	N (-15,5)
26	N	N	N (-2,593)	N (-4,5)
27	N	N	N (-3,720)	N (-0,3)
28	N	N	N (-2,537)	N (-23,8)
29	N	N	N (-4,735)	N (18,1)
30	N	N	N (-4,397)	N (8,1)
31	N	N	N (-4,284)	N (20,2)
32	N	N	N (-3,890)	N (5,8)
33	N	N	N (-4,115)	N (-10,8)
34	N	N	N (-3,946)	N (-10,3)
35	N	N	N (-3,213)	N (-3,9)
36	N	N	N (-4,002)	N (2,7)
37	N	N	N (-4,284)	N (1.1)
Collection date: 15 February 2019				
Animal number	AHS iELISA result	EEV PCR result	AHSV iELISA	EEV cELISA (PI)

1	N	N		
2	N	N		
3	N	N		
4	N	N		
5	N	N		
6	N	N		
7	N	N		
8	N	N		
9	N	N		
10	N	N		
11	N	N		
12	N	N		
13	N	N		
14	N	N		
15	N	N		
16	N	N		
17	N	N		
18	N	N		
19	N	N		
20	N	N		
21	N	N		
22	N	N		
23	N	N		
24	N	N		
25	N	N		
26	N	N		
27	N	N		
28	N	N		
29	N	N		
30	N	N		
31	N	N		

32	N	N		
33	N	N		
34	N	N		
35	N	N		
36	N	N		
37	N	N		
Collection date: 27 March 2019				
Animal number	AHS iELISA result	EEV PCR result	AHSV iELISA (PP)	EEV cELISA (PI)
1	N	N		
2	N	N		
3	N	N		
4	N	N		
5	N	N		
6	N	N		
7	N	N		
8	N	N		
9	N	N		
10	N	N		
11	N	N		
12	N	N		
13	N	N		
14	N	N		
15	N	N		
16	N	N		
17	N	N		
18	N	N		
19	N	N		
20	N	N		
21	N	N		
22	N	N		
23	N	N		

24	N	N		
25	N	N		
26	N	N		
27	N	N		
28	N	N		
29	N	N		
30	N	N		
31	N	N		
32	N	N		
33	N	N		
34	N	N		
35	N	N		
36	N	N		
37	N	N		
Collection date: 26 April 2019				
Animal number	AHS iELISA result	EEV PCR result	AHSV iELISA (PP)	EEV cELISA (PI)
1	N	N	N (-3,269)	N (18,3)
2	N	N	N (-3,777)	N (24,4)
3	N	N	N (-3,684)	N (16,8)
4	N	N	N (-4,115)	N (13,4)
5	N	N	N (-3,664)	N (18,3)
6	N	N	N (-4,397)	N (19,5)
7	N	N	N (-4,171)	N (22,6)
8	N	N	N (-3,777)	N (22,5)
9	N	N	N (-4,171)	N (14,5)
10	N	N	N (-3,901)	N (27,5)
11	N	N	N (-3,865)	N (16,8)

12	N	N	N (-2,27)	N (24,6)
13	N	N	N (-3,546)	N (15,8)
14	N	N	N (-2,943)	N (22,1)
15	N	N	N (-3,050)	LP (35,9)
16	N	N	N (-2,660)	N (24,2)
17	N	N	N (-2,730)	N (21,7)
18	N	N	N (-3,830)	N (16,1)
19	N	N	N (-3,014)	N (12,2)
20	N	N	N (-4,007)	N (26,5)
21	N	N	N (-3,972)	N (22,1)
22	N	N	N (-3,723)	LP (29,8)
23	N	N	N (-3,511)	N (28,7)
24	N	N	N (-4,397)	LP (32,3)
25	N	N	N (-4,397)	N (12,7)
26	N	N	N (-4,433)	N (10,2)
27	N	N	N (-4,362)	N (15,7)
28	N	N	N (-3,582)	N (12,8)
29	N	N	N (-4,397)	LP (30,6)
30	N	N	N (-4,397)	LP (29,8)
31	N	N	N (-4,539)	N (28,1)
32	N	N	N (-3,404)	LP (33,1)
33	N	N	N (-3,298)	N (6,1)
34	N	N	N (-3,63)	N (10,8)

35	N	N	N (-3,688)	N (4,1)
36	N	N	N (-2,979)	N (23,1)
37	N	N	N (-2,672)	N (19,5)
Collection date: 23 May 2019				
Animal number	AHS iELISA result	EEV PCR result	AHSV iELISA	EEV cELISA (PI)
1	N	N		
2	N	N		
3	N	N		
4	N	N		
5	N	N		
6	N	N		
7	N	N		
8	N	N		
9	N	N		
10	N	N		
11	N	N		
12	N	N		
13	N	N		
14	N	N		
15	N	N		
16	N	N		
17	N	N		
18	N	N		
19	N	N		
20	N	N		
21	N	N		
22	N	N		
23	N	N		
24	N	N		
25	N	N		

26	N	N		
27	N	N		
28	N	N		
29	N	N		
30	N	N		
31	N	N		
32	N	N		
33	N	N		
34	N	N		
35	N	N		
36	N	N		
37	N	N		
Collection date: 21 June 2019				
Animal number	AHS iELISA result	EEV PCR result	AHSV iELISA (PP)	EEV cELISA (PI)
1	N	N	N (-3,475)	N (-1,4)
2	N	N	N (-3,759)	N (3,6)
3	N	N	N (-3,085)	N (-12,2)
4	N	N	N (-3,05)	N (-12,8)
5	N	N	N (-2,447)	N (-13,3)
6	N	N	N (-3,936)	N (-9,4)
7	N	N	N (-4,397)	N (-13,1)
8	N	N	N (-4,291)	N (-10,2)
9	N	N	N (-4,184)	N (-20,2)
10	N	N	N (-4,22)	N (-13,4)
11	N	N	N (-4,362)	N (-8,8)
12	N	N	N (-3,546)	N (-8,4)
13	N	N	N	N

			(-3,901)	(-14,0)
14	N	N	N (-4,078)	N (-16,6)
15	N	N	N (-3,723)	N (1,7)
16	N	N	N (-3,652)	N (-15,6)
17	N	N	N (-3,972)	N (-8,5)
18	N	N	N (-4,184)	N (7,0)
19	N	N	N (-1,207)	N (-12,0)
20	N	N	N (-1,588)	N (5,8)
21	N	N	N (-1,747)	N (7,2)
22	N	N	N (-1,811)	N (10,0)
23	N	N	N (-1,747)	N (2,1)
24	N	N	N (-2,001)	N (4,7)
25	N	N	N (-1,207)	N (22,5)
26	N	N	N (-1,779)	N (19,9)
27	N	N	N (-1,938)	N (8,3)
28	N	N	N (-1,429)	N (11,4)
29	N	N	N (-1,906)	N (13,6)
30	N	N	N (-1,715)	N (-0,2)
31	N	N	N (-1,811)	N (-6,6)
32	N	N	N (-1,811)	N (4,8)
33	N	N	N (-1,874)	N (13,0)
34	N	N	N (-1,906)	N (23,4)
35	N	N	N (-1,906)	N (3,0)
36	N	N	N (-1,715)	N (3,8)

37	N	N	N (-1,684)	N (-46,2)

(N) = Negative, (LP) = Low positive.

During each sample collection rectal temperatures were recorded with only two dogs during the entire study exhibiting pyrexia (Temperature > 39.5 ˚C). These were Animal numbers 10 (39.5 ˚C) and 14 (40.0 ˚C) during 29 April 2019.

In addition, during each sample collection period clinically relevant remarks were recorded such as multiple mild cranial bite wounds with a supra-orbital draining tract from Animal number 10 and focal ringworm from Animal number 36 during 29 April 2019.