Correlating vaccination status of confirmed African horse sickness cases with disease outcome in South Africa

by

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List of abbreviations

Ab	Antibody/antibodies
AGID	Agar gel immunodiffusion
AHS	African horse sickness
AHS1	African horse sickness vaccine combination 1
AHS2	African horse sickness vaccine combination 2
AHSV	African horse sickness virus
AHSV-x	African horse sickness virus serotype (x)
BHK	Baby hamster kidney fibroblasts
BTV	Bluetongue virus
CA	Controlled area
CFR	Case fatality rate
CFT	Complement fixation test
Cq	*Quantification cycle*
Ct	Cycle threshold
DAFF	Department of Agriculture, Forestry and Fisheries
DEET	N, N-Diethyl-meta-toluamide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
E	Case reference number
EC	Eastern Cape
EC	*External control*
EDTA	Ethylenediaminetetraacetic acid
EEV	Equine encephalosis virus
EIA	Equine infectious anaemia
ELISA	Enzyme-linked immunosorbent assay

ENSO	El Niño–Southern Oscillation
EP	Individual sample reference number
ERC	Equine Research Centre
EVA	Equine viral arteritis
FDPs	*F*ibrin degradation products
FS	Free State
FZ	Free zone
GP	Gauteng Province
GPS	Global Positioning System
GS	*Group-specific*
Hgb	Haemoglobin
Ht	Haematocrit
IFA	Immunofluorescent assay
IFA	Indirect immunofluorescent antibody
IL-1	Interleukin-1
IZ	Infected zone
KZN	KwaZulu-Natal
LAV	Live attenuated vaccine
LP	Limpopo
Lymph	Lymphocyte
Mab	Maternal antibody
MP	Mpumalanga
NC	Northern Cape
Neut	Neutrophil
NS	Virus non-structural proteins
NW	North West

OBP	Onderstepoort Biological Products
OIE	World Organization for Animal Health/ Office International des Epizooties
PCR	Polymerase chain reaction
PZ	Protection zone
QGIS	Quantum geographical information system
RCC	Red cell count
RNA	Ribonucleic acid
RSA	Republic of South Africa
RT- qPCR	Reverse transcriptase quantitative polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SANAS	South African National Accreditation System
SNT	Serum neutralization test
ST	Serotype
SZ	Surveillance zone
TCID	Tissue culture infectious dose
Temp	Temperature
TNF- α	Tumour necrosis factor alpha
TS	*Type-specific*
UP	University of Pretoria
VGL	Veterinary Genetics Laboratory
VI	Virus isolation
VNT	Virus neutralization test
VP	Virus structural proteins
WC	Western Cape
WNV	West Nile Virus

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Abstract

African horse sickness (AHS) is one of the economically most important equid diseases in Southern Africa, contributing significantly to equine morbidity and mortality. This makes the incidence, distribution and control of AHS a matter of importance to affected and at-risk countries. AHS is caused by African horse sickness virus (AHSV), of the genus *Orbivirus,* family *Reoviridae*. Disease occurrence and spread are mainly determined by the spread of its insect vector, midges of the *Culicoides* spp.

Annual vaccination with the Onderstepoort Biological Products polyvalent live attenuated AHS vaccine is currently the mainstay of prevention and protection in South Africa. The vaccine consists of two components given at least 3 weeks apart. Component one (AHS1) consists of types 1,3 and 4; component two (AHS2) consists of types 2, 6, 7 and 8. Types 6 and 8 provide cross-protection to types 9 and 5, respectively. Most horses only develop neutralizing antibodies against all types after multiple vaccination courses.

Disease diagnosis is performed with real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) which is World Organization for Animal Health (OIE) approved and used in South Africa for disease outbreak and surveillance purposes.

This study retrospectively reviewed the history of suspected AHS cases from South Africa submitted to the Veterinary Genetics Laboratory, University of Pretoria,

that were confirmed positive by real-time RT-qPCR during the period 1 September 2017 - 30 June 2019 to assess the effect of AHS vaccination on case outcome. For this period 233 AHSV RT-qPCR positive samples were obtained. The study confirmed that increased case fatality was not due to vaccine failure, but rather due to multiple variables, with an increased population of unvaccinated horses being one of these. Of the 115 cases that died 90 were unvaccinated or had a questionable vaccination history. With the case fatality rate for unvaccinated cases (0.80) being increased when compared to vaccinated cases (0.57). A significant association was shown between vaccination and case outcome (p=0.001), with unvaccinated animals being more likely to die. Other variables that were shown to influence disease outcome included the number of days since last AHS vaccination, age and sex.

Keywords: AHS, AHS vaccination, vaccination status, case outcome.

Chapter 1 General Introduction

1.1 Introduction

Over the centuries African horse sickness virus (AHSV) has been one of the most devastating diseases to the equine population in sub-Saharan Africa (Zientara, Weyer and Lecollinet, 2015). Researchers postulate that due to climate change geographical areas historically free of African horse sickness (AHS) have become favourable for its insect vector (*Culicoides* midges), as shown by the dramatic spread of bluetongue virus to Europe (MacLachlan and Guthrie, 2010; Purse *et al.*, 2006; Mellor and Hamblin, 2004). In 2011, 744 cases of AHS were reported in South Africa by the Department of Agriculture, Forestry and Fisheries (DAFF) for the season (1 September 2010 - 31 August 2011), with a maximum number of 320 cases reported in one month (Health, 2016).

1.2 Problem statement

A previous study in Zimbabwe indicated that repeated immunization will result in more encompassing antibody response to the various AHS serotypes and higher individual titres (N. K. Blackburn and Swanepoel, 1988; N.K. Blackburn and Swanepoel, 1988 b). However, the authors noted that some horses did not produce a satisfactory response to one or more of the AHS serotypes. Blackburn and Swanepoel (1988a) did however not indicate the number of immunizations required to produce an adequate immune response that will be protective against a virulent field challenge. It is important to note that this study was conducted using the old mouse-brain attenuated vaccine containing only 7 AHS types which were all included in a single bottle. Therefore, the results are not comparable to the current study. It is however notable that this has been the only previous study comparing vaccination status with disease outcome.

There have also been associations between the polyvalent AHS live attenuated vaccine (LAV) produced by Onderstepoort Biological Products (OBP) Ltd., South Africa, and some safety concerns and alleged cases of vaccine failure or vaccine-induced disease (von Teichman and Smit, 2008). However, no clinical symptoms typical of AHS were observed in vaccinated horses by Von Teichman (2008) and all horses showed a good immune response. The AHS-LAV is

administered as two separate components given at least 3 weeks apart. Each component consists of a combination of different serotypes of AHS: combination one (AHS1) contains serotypes 1, 3 and 4 and combination two (AHS2) contains serotypes 2, 6, 7 and 8. These AHS serotype combinations were selected during vaccine development based on cross-neutralization results and to ensure minimal interference between serotypes. Until recently AHS serotype 9 rarely occurred in southern Africa and was therefore not included in the vaccine, together with the cross-protection afforded by AHS serotype 6. Originally AHS serotype 5 was included in the polyvalent vaccine however, it caused severe reactions and deaths after vaccination, which resulted in its removal from the LAV. Follow-up immunization studies in susceptible horses showed that the combination of AHS serotypes in AHS1 was the cause of vaccine-associated disease reported in previous studies (von Teichman and Smit, 2008).

The claims of vaccine-associated disease were explained by Von Teichman and Smit (2008). However, no study has looked at the claims of vaccine failure and whether vaccinated horses are just as likely to die of AHSV as unvaccinated horses.

This study aimed to address the claims of AHS-LAV failure by evaluating the likelihood of death in vaccinated horses.

1.3 Hypothesis

$H_0$1: There is no significant relationship between AHS vaccination status and disease outcome.

H_A1: There is a significant relationship between AHS vaccination status and disease outcome.

$H_0$2: The disease outcome is independent of the number of AHSV types the sample tested positive for.

H_A2: The disease outcome is related to the number of AHSV types the sample tested positive for.

$H_0$3: The mean cycle threshold (Ct) value of the real-time RT-qPCR for horses that died due to AHSV and those that recovered from AHSV are equal.

H_A3: The mean Ct value of the real-time RT-qPCR for horses that died due to AHSV and those that recovered from AHSV are unequal.

$H_0 4$: Type of clinical signs observed does not influence disease outcome.

H_A4: There is a significant relationship between the type of clinical signs observed and disease outcome.

$H_0 5$: There is no correlation between the order in which the two AHS vaccine components are given and disease outcome.

H_A5: There is a correlation between the order in which the two AHS vaccine components are given and disease outcome.

$H_0 6$: There is no significant difference between the case fatality rates (CFR) for the years observed during the study period.

H_A6: There is a significant difference between the CFR of the years observed during the study period.

$H_0 7$: Disease outcome is independent of individual equine age.

H_A7: Disease outcome is related to the individual equine age.

$H_0 8$: There is no difference in disease outcome between the different sexes.

H_A8: There is a significant difference in disease outcome between the different sexes.

1.4 Aim and objectives

The primary aim of this study was to correlate the AHS vaccination status of horses that tested positive for AHSV with real-time RT-qPCR with the disease outcome (death or recovery). The secondary aims were to evaluate the vaccination frequency, disease-causing AHSV type prevalence, geographical distribution and outbreak dynamics, disease seasonality, clinical symptoms related to disease outcome and lab results related to disease outcome. Tertiary aims included age group in which disease was most prevalent, sex-related to disease outcome and order in which vaccine components were administered.

Objectives:

a) To determine if there was a significant relationship between vaccination status and disease outcome.
b) To evaluate whether infection with more than one AHSV type leads to a greater likelihood of death.
c) To describe the difference in the mean cycle threshold (Ct) values for the real-time RT-qPCR between cases that died and recovered.
d) To evaluate whether the type of clinical symptoms observed influenced disease outcome.
e) To determine whether the order in which AHS vaccine components were administered (AHS1 then AHS2 versus AHS2 then AHS1) affects disease outcome.
f) To compare the case fatality rates (CFR) for AHS cases between the years observed.
g) To evaluate the relationship between age and disease outcome.
h) To evaluate the relationship between sex and disease outcome.

1.5 Application of results

This project could impact the vaccination protocols currently used in South Africa, with the intention to make the vaccination protocols more effective for disease prevention. The study aimed to identify the equine age group with the highest risk of disease. Because AHS is a listed disease by the World Organization for Animal Health (Office International des Epizooties (OIE)), research into the disease and vaccination protocols would be of international interest. This could also affect the South African equine import and export industry.

Chapter 2 Literature review

2.1 Introduction

African horse sickness (AHS) was first recognized as a disease during the 1719 epidemics in South Africa (Mornet and Gilbert, 1968), after the introduction of European horses during the exploration of the continent (M'Fadyean, 1900). AHSV was likely circulating in the wildlife of the area prior to this (Mornet and Gilbert, 1968). Since then AHSV has been one of the most devastating diseases to the equine population in sub-Saharan Africa and Mediterranean countries (Zientara, Weyer and Lecollinet, 2015). AHSV caused epidemics at intervals of approximately 20 to 30 years, prior to 1953. With major losses in 1780, 1801, 1839, 1855, 1862, 1891, 1914, 1918, 1923, 1946 and 1953. Of these, the 1855 epidemic is still considered the worst recorded, with up to 40 per cent (70 000 horses) of the equine population of the Cape of Good Hope succumbing to the disease (Bayley, 1856). AHS is endemic to the tropical and subtropical sub-Saharan areas of Africa, spreading west as far as Senegal, east to Ethiopia and as far south as South Africa (MacLachlan and Guthrie, 2010; Guthrie and Weyer, 2015). Spread out of this area has occurred with severe epidemics occurring in newly infected populations (Middle East, North Africa and Spain) (Lubroth, 1988, 1992; Zientara, Weyer and Lecollinet, 2015). North America and Australia also have competent vectors and are therefore at risk of an epidemic in the case of vector exposure to the AHSV (Baylis *et al.*, 1999). Figure 2-1 illustrates the current OIE members' official AHS status as updated in May 2019. Note that the AHS free status has been suspended for Myanmar and Kyrgyzstan due to failure to comply with the OIE Terrestrial Code provisions for the maintenance of an AHS free status (*Suspension/reinstatement of status: OIE - World Organisation for Animal Health*, no date).

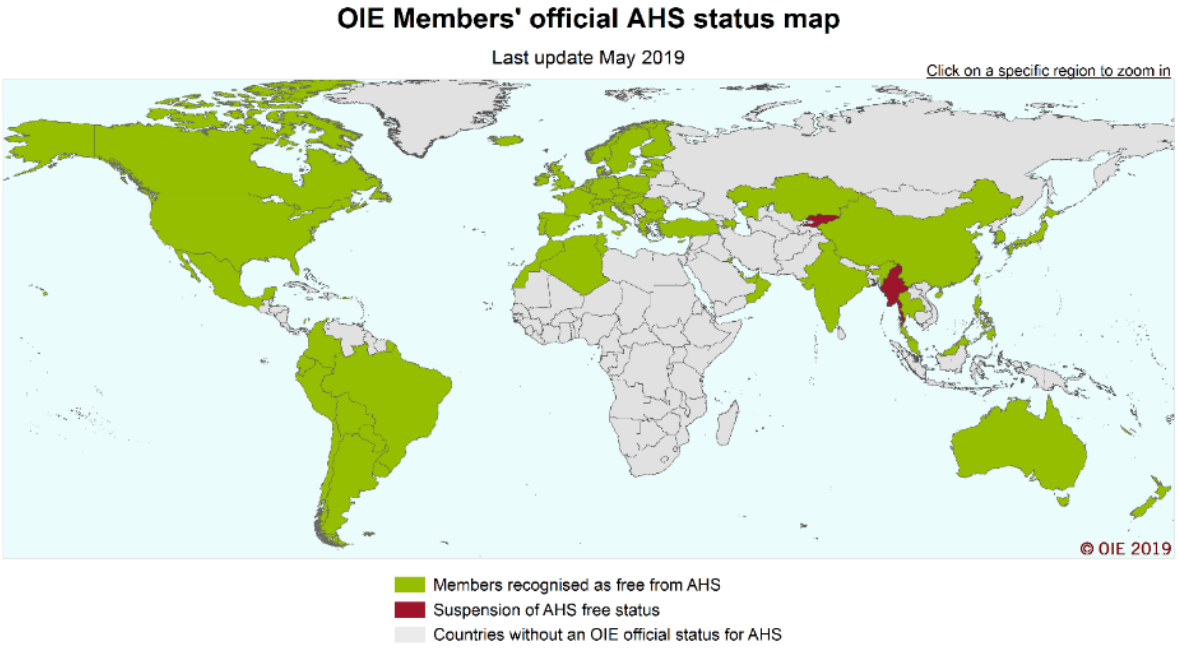

Figure 2-1: A map compiled by the OIE to indicate the AHS status of its members (OIE - World Organisation for Animal Health, 2019).

2.2 Aetiology

AHS is an infectious, non-contagious, arthropod-borne disease caused by AHSV, an *Orbivirus* in the family *Reoviridae* (Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004). Nine AHSV types (numbered 1 to 9) have been reported (Howell, 1962). Orbiviruses cause major animal diseases such as Bluetongue virus and AHS. The members of this genus share morphological, physicochemical and genomic characteristics (Murphy *et al.*, 1971). These include being non-enveloped viruses, consisting of an icosahedral core surrounded by an outer capsid; with a segmented double-stranded (ds) ribonucleic acid (RNA) genome enclosed by these. The genus *Orbivirus* is divided into groups, which is further divided into serotypes based on virus neutralization tests. AHSV particles contain seven structural viral proteins (VP) (VP1 – VP7), as illustrated in Figure 2-2, and five non-structural proteins (NS) (NS1, NS2, NS3, NS3A and NS4). The genome consists of ten dsRNA segments of different lengths and has been entirely sequenced (Roy, P.C. Mertens and Casal, 1994). The main proteins used for AHSV diagnosis and typing is VP2, VP3 and VP7. The VP2 protein, encoded by segment 2, is the principal component of the outer capsid and is the main type-specific antigen (Vreede and Huismans, 1994). The

VP3 and VP7 proteins, encoded by segments 3 and 7 respectively, contain group-specific antigenic determinants (Chuma *et al.*, 1992; Roy, P.C. Mertens and Casal, 1994). The VP7 protein is used for the serological diagnosis of AHSV-serogroup-infected animals.

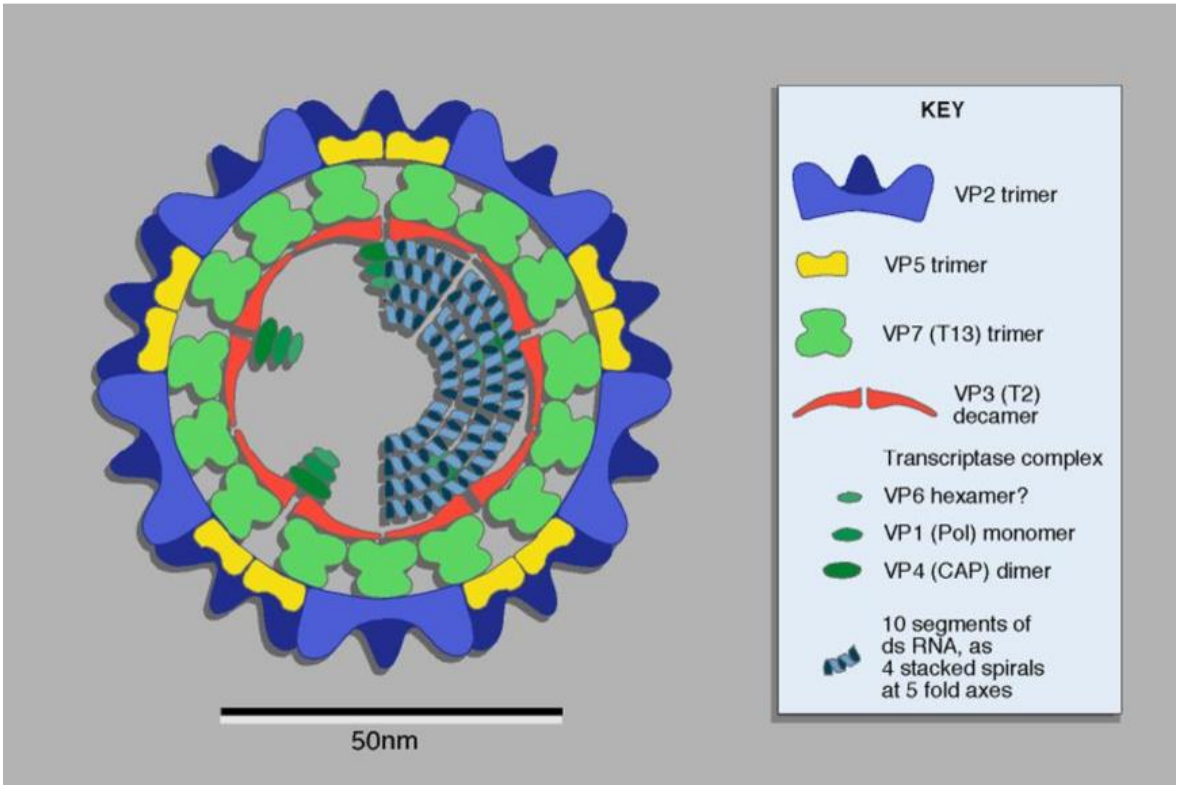

Figure 2-2: A diagrammatic representation of the structure of the African horse sickness viral particle, showing the positioning of the seven structural viral proteins (VP 1-7) and the segmented RNA genome (Wilson *et al.*, 2009).

2.3 Epidemiology

AHSV is indirectly transmitted to equids by hematophagous arthropods. Multiple vectors have been found infected with AHSV (Lord *et al.*, 2002). However, the main biological vectors are biting midges of the family *Culicoides*, *C. imicola* and *C. bolitinos* in South Africa (Du Toit, 1944; Venter, Graham and Hamblin, 2000; Meiswinkel and Paweska, 2003). A high viraemia in the host is required for vector contamination, as the minimum dose needed to experimentally infect *C. imicola* is 10_4 – $10_{4.5}$ $TCID_{50}$/0.02ml blood (Zientara, Weyer and Lecollinet, 2015). Disease distribution is largely controlled by the abundance, prevalence and seasonal incidence of its insect vectors. It has been postulated that due to climate change

geographical areas historically free of AHS have become favourable for its insect vector (*Culicoides* midges), as shown by the dramatic spread of Bluetongue virus into Europe (MacLachlan and Guthrie, 2010; Purse *et al.*, 2006; Mellor and Hamblin, 2004). Apart from this during the 1987 – 1990 outbreaks in Spain AHSV was isolated from a mixed pool of *Culicoides* (especially *C. obsoletus* and *C. pulicaris*), which could be possible disease vectors in Europe (Lubroth, 1988). The disease spread due to climate change is also demonstrated by looking at the interaction of the disease epidemics with the El Niño–Southern Oscillation (ENSO) phenomenon (Baylis *et al.*, 1999). Baylis et al. (1999) showed that there is a strong association between the timing of the major epidemics of AHSV in South Africa occurring every 10 to 15 years and the warm phase (El Niña) of the ENSO phenomenon. This suggests that outbreaks are related to the combination of rainfall and drought, most likely when drought in the early season is followed by heavy rainfalls later in the season (Paton, 1863). The factors necessary for high vector activity are highlighted by these findings, which include a warm and humid climate. Infection rates, virogenesis and transmission increase with temperature increases. However, midge survival decreased with temperature increase (Mellor *et al.*, 1998). It is important to note that AHSV does not seem to replicate below 15 °C (Wellby *et al.*, 1996). Midges can survive for up to 90 days at these lower temperatures, with 'latent' virus persisting in some individuals. It has been shown that when these midges are exposed to the temperature range in which AHSV can replicate this 'latent' virus is able to replicate and rapidly reach sufficiently high titers for transmission to occur (Wellby *et al.*, 1996). This has been hypothesized to be a virus overwintering mechanism in the absence of infected vertebrates. From this data on AHSV vectors and viral replication characteristics it is evident why AHS has generally been described as a seasonal disease in southern Africa, with cases starting to appear shortly after the first rainfall in summer rainfall areas (mainly from November or December), the majority of cases clustered in March and April, and the disease slowing to even disappearing after the first frost (generally in June) (Theiler, 1915; Coetzer and Guthrie, 2004). These observations were taken into account when DAFF made the official restrictions and recommendations to AHS vaccination periods (DAFF, 2015).

Horses are the most susceptible equids, with infection often leading to high mortalities of up to 90% (Zientara, Weyer and Lecollinet, 2015). Donkeys and mules are also susceptible, but generally only develop a mild form of the disease (Fassi-Fihri, el Harrak and Fassi-Fehri, 1998; Hamblin *et al.*, 1998; Hasnaoui *et al.*, 1998). Zebra are highly resistant to the disease (Erasmus *et al.*, 1978; Barnard, 1998; Coetzer and Guthrie, 2004). The occasional occurrence of multiple cases of disease over a short time period suggests the existence of a virus reservoir with a long-lasting, reactivatable viraemia or a continuous circulation of the virus within a reservoir herd. The second scenario is the more likely one. The herd acting as a reservoir needs to be big enough and have non-seasonal breeding to allow for naïve young animals to be perpetually exposed. Zebra appears to play a role as a natural vertebrate reservoir of AHSV, with herds in the Kruger National Park having the capacity (Barnard, 1998; Porphyre and Grewar, 2019). However, the disease is also endemic in countries where there is no zebra population, highlighting the need to investigate other reservoirs as well (Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004). Dogs are the only other species that have been shown to be highly susceptible to AHSV, developing fatal pulmonary forms (Lubroth, 1988). They are however regarded as epidemiological bottlenecks as their contribution to the transmission cycle is minor, if any.

Due to the severity of AHS in endemic and naïve populations; and its ability to rapidly spread to new areas from historically endemic areas without apparent warning signs African horse sickness has been listed by the World Organization for Animal Health as a notifiable disease relating to the international trade and movement of horses (Mellor and Hamblin, 2004; OIE, 2015) and is a controlled disease in South Africa (South African Government Gazette, 2018). Spread occurs mainly due to the movement of infected animals, however the possible spread due to movement of insect vectors over large distances by winds have not been excluded (Mellor and Hamblin, 2004). In South Africa outbreaks usually occur in late summer in the summer rainfall areas (Coetzer and Guthrie, 2004).

2.4 Pathogenesis

After infection and primary viraemia, with initial multiplication in the regional lymph nodes, the virus is haematogenously spread to the endothelial cells of the target organs (lungs, heart, spleen and lymphoid tissue) (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar, Laegreid, *et al.*, 1995; Mellor and Hamblin, 2004). Virus replication in these target organs causes a second viraemia, damaging endothelial cells by most likely causing ultra-structural changes in, and separation of, the endothelial cells (Laegreid *et al.*, 1992; Gómez-Villamandos *et al.*, 1999). Endothelial damage results in effusions into body cavities, oedema and serosal and visceral haemorrhage (Guthrie and Quan, 2009). The secondary viraemia also causes macrophage activation, resulting in the release of inflammatory cytokines (such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF- α)). The virus is closely associated with erythrocytes in the blood (Theiler, 1921) and high concentrations are found in lymphoid tissue, possibly explaining the lymphopenia noticed in experimental cases of AHS (Erasmus, 1973; Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995).

2.5 Clinical signs

The OIE defines AHS as an infection of equids with AHSV based on diagnostic techniques used (OIE, 2014). “An infection with AHSV can be defined as a) AHSV isolated and identified from an equid or product derived from that equid; or b) viral antigen or viral RNA specific to AHSV identified in samples from an equid showing clinical signs consistent with AHS, or epidemiologically linked to a suspected or confirmed case; or c) serological evidence of active infection with AHSV by detection of seroconversion with production of antibodies against structural or non-structural proteins of AHSV, that is not a consequence of vaccination, have been identified in an equid that either shows clinical signs consistent with AHS or is epidemiologically linked to a suspected or confirmed case” (OIE, 2014).

The clinical symptoms exhibited in equids with AHS and the range of signs are well documented (Theiler, 1921; Coetzer and Guthrie, 2004). Clinical signs are due to endothelial injury caused by the virus, with resultant oedema of the lungs,

pleura and subcutaneous tissues, and petechiae and haemorrhages observed macroscopically with concurrent pyrexia (Kazeem *et al.*, 2008). The severity of these signs is determined by virus-dependent factors (strain virulence, infectious dose, etc.) and host-dependent factors (breeding conditions, immune status, etc.). The AHSV incubation period can vary from three to fifteen days; with five to seven days being the average in experimental cases (Coetzer and Guthrie, 2004). This depends on the virus strain and the sensitivity of the equid host. The AHSVs infective period is 21 days, but considered 40 days by the OIE (OIE, 2014).

The onset of pyrexia corresponds with the viraemic period (Laegreid *et al.*, 1992), which can persist for two to fourteen days (House, House and Mebus, 1992), as shown by viral isolation (Erasmus, 1973; Sailleau *et al.*, 1997). There are no exact figures on how long the viraemia extend after this due to viral levels often being lower than those detectable by virus isolation (Sailleau *et al.*, 1997). Quan et al. (2010) showed that AHSV dsRNA, detected with RT-qPCR, peaks at approximately 15 days post-challenge and remained present for up to 97 days after initial exposure to the virus (Quan *et al.*, 2010). Whether this is related to red blood cell life span in blood (145 days) and a non-infectious state or horses are truly infectious to midges the entire period that viral RNA can be detected with RT-qPCR have not been clarified and requires further study (Theiler, 1921; Latimer, Mahaffey and Prasse, 2003).

AHS generally manifests in one of four forms; which has been described as the pulmonary (peracute), cardiac (acute), mixed (acute/subacute) and horse sickness fever forms historically (Erasmus, 1974). The pulmonary form, colloquially known as ‘dunkop’, oftentimes develops so fast that animals will die without previous signs of disease. It generally causes a marked depression and fever (39 – 41^{O}C), followed by respiratory distress and severe dyspnoea. Terminally the horses are often recumbent with large quantities of frothy fluid discharged from the nostrils. In these cases, the prognosis is extremely poor, with mortalities commonly as high as 95%. The cardiac form, colloquially known as ‘dikkop’, generally presents with a fever (39 – 40 degrees Celsius) lasting for several weeks, subcutaneous oedema of the head (including the supraorbital fossae), neck and chest mainly, conjunctival congestion with possible petechiae and ecchymotic haemorrhages on the ventral surface of the tongue. Colic can be a complication in these cases, with mortality

rates approaching 50%. The mixed form is the most common form of AHS, being a combination of the pulmonary and cardiac forms. Death usually occurs within three to six days after the onset of fever, with mortality rates close to 70%. Horse sickness fever is a mild, non-lethal form of the disease; causing a moderate fever and oedema of the supraorbital fossae. This form is frequently encountered in African donkeys, zebras, horses with higher susceptibility or who are infected by a less virulent strain. This is also the most common form seen in partially immune (vaccinated) horses. In recent years it has been observed that most cases are a combination of the four forms and that there is no real distinction. This is most likely because they all relate to cardiac failure, which it has recently been theorized is the main pathophysiological lesion caused by AHSV (direct communication with Dr. M. Hewetson).

Subclinical infection with AHSV has been described in both immunised horses and outbreak situations. These cases tend to show no clinical or haematological abnormalities(Grewar *et al.*, 2013; Weyer *et al.*, 2013).

The differential diagnoses for AHSV include equine infectious anaemia (EIA), equine viral arteritis (EVA), equine encephalosis virus (EEV), West Nile virus (WNV), equine pneumonia due to Hendra virus infection, anaplasmosis, babesiosis or theileriosis, anthrax (*Bacillus anthraxis* infection) and purpura haemorrhagica. These possibilities are excluded on a basis of clinical signs and development of the disease, post-mortem examination, epidemiological factors (vector presence) and laboratory diagnosis (Mellor, 1994; Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004; Guthrie and Quan, 2009).

2.6 Macroscopic pathology

Post-mortem findings can often be pathognomonic; such as supra-orbital swelling, pulmonary oedema with fluid and foam accumulation in the trachea and bronchi (Laegreid, 1997). Pulmonary oedema, severe hydrothorax and mucosal petechiae predominate in the peracute (“dunkop”) form. Whereas severe hydropericardium with slightly yellowish, gelatinous oedema of the intermuscular connective tissues and subcutaneous tissues of the head and neck (severest around the nuchal ligament) are typical of the acute (“dikkop”) form (Kazeem *et al.*, 2008; Guthrie and Quan, 2009).

2.7 Clinical pathology

The clinicopathological changes induced by AHS is one of the areas least documented. Experimentally the following haematological changes have been noticed: leukopenia (most noted being a lymphopenia and neutropenia), thrombocytopenia with an increased haematocrit (Ht), red cell count (RCC) and haemoglobin (Hgb) concentration (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995). Some of the postulated and described mechanisms of leukopenia include margination of leukocytes within pulmonary vessels and increased leukocyte numbers within capillaries (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995), stress-induced corticosteroid release with subsequent lymphocyte sequestration and viral interference with leukocyte production (Latimer, Mahaffey and Prasse, 2003). The increased Ht, RCC and Hgb presents a polycythaemia, which is most likely due to compromised endothelial barrier function allowing a fluid shift from the intravascular to the extravascular compartments. Another reason for the polycythaemia may be febrile dehydration. Prolongation of coagulation times, noted by prolonged prothrombin, activated partial thromboplastin and thrombin clotting times, and elevated fibrin degradation products (FDPs) make up the haemostatic changes (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995; Sailleau *et al.*, 1997).

Thrombocytopenia is a conserved finding between all four forms of AHS (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995; Weyer *et al.*, 2013). There are four main mechanisms of thrombocytopenia: an increased consumption, sequestration, excessive loss or impaired production of thrombocytes (Latimer, Mahaffey and Prasse, 2003). Increased consumption is the most likely cause of thrombocytopenia in AHS, due to thrombocytes adhering to the damaged endothelium (Laegreid *et al.*, 1992; Roth, 1992). Thrombocytopenia is not a change specific to AHS, as horses with other viral infections also show a reduced thrombocyte count due to adherence of thrombocytes to damaged endothelium. Another postulated mechanism, shown in experimental cases of AHS, is vascular stasis as a result of haemoconcentration, which increased thrombocyte binding. Some authorities think that the disease outcome of AHS is related to the onset and

duration of thrombocytopenia, however, this requires further investigation (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar and Laegreid, 1995).

2.8 Diagnosis

A presumptive diagnosis of AHS is often made based on clinical signs, history and macroscopic lesions. It should, however, be noted that clinical signs are not necessarily specific to AHS and that some horses may be sub-clinically infected(Grewar *et al.*, 2013; Weyer *et al.*, 2013). Symptoms that can have multiple aetiologies include the pyrexia and subcutaneous oedema of the “horse sickness fever” form which can be confused with EEV; recumbency, per-acute death and fever associated with the “dunkop” form may be confused with WNV. WNV can be distinguished by the terminal neurological signs, which are not present in AHS. This emphasises the importance of confirmatory laboratory diagnosis. Multiple tests have been developed for identification of the infectious virus, viral antigens and specific antibodies to aid in AHS diagnosis.

2.8.1 Viral isolation and identification

Traditionally virus detection and identification were performed by virus isolation techniques. Ante-mortal diagnosis is made on blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes. Post-mortem samples include lung, spleen and lymph node specimens (Coetzer and Guthrie, 2004). These samples should be transported at 4 degrees Celsius. The use of these methods has decreased as faster and more efficient methods have been developed.

Two methods described for virus isolation are inoculation of cell cultures (e.g. Vero or BHK-21 cell line), which takes at least 7 – 14 days, and intracerebral inoculation of newborn suckling mice (Howell, 1962). When using the cell culture method, the cytopathic effect (CPE) of the virus is measured by refractivity and detachment of cells. This can appear two to eight days post-inoculation. When a virus is isolated it has to be confirmed as AHS, as the morphology and cytopathic effects of EEV are similar to AHSV (Crafford, 2001).

Enzyme-linked immunosorbent assay (ELISA) tests make use of enzymes that have been coupled to an antibody to mark immune complexes. The enzyme action on the substrate leads to a colour formation, which is quantifiable. Multiple

ELISA methods have been developed for AHS diagnosis and previously described, including the indirect sandwich ELISA used at the Equine Virology Laboratory, Faculty of Veterinary Science, University of Pretoria (Hamblin *et al.*, 1991; Crafford, 2001).

2.8.2 Virus serotyping

AHSV serotyping is mainly done using a plaque inhibition neutralization test (NT) using type specific antisera (Porterfield, 1960; Quan *et al.*, 2008).

2.8.3 Group-specific serology

Methods that can detect group-specific antibodies include complement fixation tests (CFT), agar gel immunodiffusion (AGID) tests, indirect immunofluorescent antibody (IFA) tests and ELISA tests.

The CFT uses a haemolytic system as an immunological indicator to detect immune complex formation. This allows for the detection of group specific antibodies. This system utilizes the idea that RBC's in the presence of hyperimmune serum will form immune complexes. The complement cascade will be triggered by these immune complexes, leading to lysis of the RBC. This is a visible reaction (Haig *et al.*, 1956).

Precipitation reactions in a semisolid medium are used by the AGID tests (Hamblin *et al.*, 1990). A crude antigen is used and often result in non-specific lines. These may interfere with accurate diagnostics. (House, Mikiciuk and Beminger, 1990).

A standard antigen and fluorescent antiglobulin are used in IFA tests to determine the presence of a specific antibody. Samples from vaccinated horses often have non-specific fluorescence. This complicates the interpretation of results and can lead to false positive (House, Mikiciuk and Beminger, 1990). Therefore, this test is best suited as a screening test.

Another good screening test is the indirect ELISA using recombinant VP7 protein, which detects IgG antibodies. Soluble AHSV antigen can also be used in this test, but the VP7 is advantageous in most areas. These include being more sensitive in detecting early immunological responses, due to its conservation among serotypes (Mellor and Hamblin, 2004; Guthrie *et al.*, 2009), making it ideal for

detecting declining maternal antibody levels. It also has good specificity (Maree and Paweska, 2005).

There is also a competitive ELISA available for group-specific antibody detection (Hamblin *et al.*, 1990).

2.8.4 Serotype-specific serology

The serum neutralisation test (SNT) is used to detect the presence of serotype specific antibodies and to determine antibody titres in sera as described by Guthrie et al. (2009). Notably, a four-fold increase in paired sample titres indicates seroconversion (Guthrie *et al.*, 2009). Because this test is serotype-specific it cannot identify new serotypes, limiting its use in outbreak situations (Crafford, 2001).

AHSV is however best confirmed by virus detection techniques instead of serology; as antibodies are only detectable 10-14 days post-infection and many horses will be dead before these antibodies are induced (Rodriguez, Hooghuis and Castano, 1992; Sailleau *et al.*, 1997). Another factor playing in this is that in areas with routine vaccination serological results can be difficult to interpret. However, these serological tests are not obsolete, and several are prescribed by the World Organization for Animal Health (OIE) for the purposes of animal trade (World Organization for Animal Health (OIE), 2012). These include complement fixation test (which are group-specific but lack sensitivity) (Tessler, 1972), serum neutralization test (where some cross-neutralization between AHSV serotypes has been observed, notably between serotypes 9 and 6, and to a lesser extent, between serotypes 1 and 2, 3 and 7, and 5 and 8 respectively) (Kanai *et al.*, 2014) and enzyme-linked immunosorbent assay (indirect and competitive ELISAs for AHSV detection have been developed; these show adequate sensitivity) (Hamblin *et al.*, 1991; Laviada *et al.*, 1992; Rubio *et al.*, 1998).

2.8.5 PCR

Virus identification was traditionally performed with serological techniques using isolated virus antigen (virus neutralization test). The major disadvantage of these VNT and VI is the timeframe, as these often take a minimum of two weeks to obtain a result (Hamblin *et al.*, 1991; Sailleau *et al.*, 2000), which is costly in an outbreak situation. The development of highly sensitive and specific real-time RT-

PCR assays has however replaced the serological tests in most laboratories. This is due to the high throughput and quick turnaround time of these tests. RT-PCR assays amplify conserved genomic sequences (1,3,5,7 or 8 dsRNA segments) for group diagnosis, or highly variable segments (segment 2 encoding VP2 in particular) for typing (Sailleau *et al.*, 2000; Agüero *et al.*, 2008; Quan *et al.*, 2008; Rodriguez-Sanchez *et al.*, 2008; Fernández-Pinero *et al.*, 2009; Maan *et al.*, 2011; Monaco *et al.*, 2011; Guthrie *et al.*, 2013; Bachanek-Bankowska *et al.*, 2014; Weyer *et al.*, 2015). Some PCR tests have been developed for the detection of AHSV targeting the VP3 (Aradaib *et al.*, 2006), VP7 (Zientara *et al.*, 1993; Zientara, Sailleau, Moulay, Wade-Evans, *et al.*, 1995; Guthrie *et al.*, 2013), NS1 (Mizukoshi *et al.*, 1994), NS2 (Stone-Marschat *et al.*, 1994) and NS3 (Zientara, Sailleau, Moulay and Cruciere, 1995) genes. PCR is also superior in that these test can be used on specimens that do not contain live virus (Coetzer and Guthrie, 2004; Agüero *et al.*, 2008), on specimens that contain attenuated strains or strains of low virulence (Sailleau *et al.*, 1997). Another advantage is that PCR can be used for earlier viral detection of viraemia than viral isolation (Stone-Marschat *et al.*, 1994).

The advantage of a real-time PCR is that it has greater analytical sensitivity and specificity, is quick to perform, as well as having a small potential for contamination (Quan *et al.*, 2010). RT-PCR techniques were also described by Bachanek-Bankowska *et al.* (2014), Fernández-Pinero *et al.* (2009), Bremer and Viljoen (1998), Bremer (2012), van Rijn *et al.* (2018) and Potgieter, Wright and Erasmus (2015).

In this study the group-specific (GS) real-time RT-qPCR method developed by Guthrie et al. (2013) at the VGL (UP) was used for GS-AHS diagnosis. The Guthrie et al. (2013) method was adapted from the Quan et al. (2010) method developed and optimised in the VGL laboratory; which used VP7-specific primers and probes and field and laboratory strains of all 9 AHSV types (Quan et al., 2010). The assay also includes proprietary Xeno4 primers and probes to target XenoRNA acting as a synthetic external control (EC) for all samples (Fang et al., 2007). The level of normalised fluorescence reached during a specific number of PCR cycles was used to classify a sample as positive or negative. With a positive sample having exceeded the 0.1 threshold in 40 PCR cycles in both samples replicates and a negative sample exceeding the 0.1 threshold in 33 PCR cycles for the Xeno4 assay

and not exceeding the 0.1 threshold in 40 PCR cycles for both replicates of the AHS assay. The quantification cycle (Cq) was defined as the cycle number in which the fluorescence threshold was reached. The median diagnostic specificity and sensitivity for this test is >99.3% and >97.8% respectively (Guthrie et al., 2013).

Methods described for AHSV typing are mainly focused on segment 2 of the viral genome encoding for the VP2-gene, with the majority using hybridized probes (Koekemoer et al., 2000; Sailleau et al., 2000; Koekemoer and Van Dijk, 2004; Koekemoer, 2008). AHSV type determination for this study was done with the AHSV type-specific (TS) RT-qPCR test developed by Weyer et al. (2015). Unique regions of the sequences encoding VP2 of each AHSV type are used as targets for primers and probes, designed by Primer Express v3 (Lifetech). Lifetech also synthesizes the oligonucleotides. There are 3 triplex reactions, each containing primer probe combinations for three AHSV types. Triplex one includes oligonucleotides for types 1,3 and 4 (the serotypes included in AHS1 of the OBP AHSV-LAV). Triplex two contains oligonucleotides for types 2,5 and 9 and triplex three included oligonucleotides for types 6,7, and 8. The triplexes were developed to ensure that AHS types which show cross-reaction on neutralization tests are separated (serotypes 1 and 2; 3 and 7; 5 and 9; 6 and 8). Group-specific RT-qPCR positive cases were tested with the TS Rt-qPCR described by Weyer et al. (2015) to determine the specific serotype present. The timeframe for this process is approximately two hours (Weyer et al., 2015).

2.9 Disease control

AHS control consists of a multimodal approach. Firstly, by protecting AHSV-free countries from the import of infected equids from an AHSV at-risk area.

The *Terrestrial Animal Health Code* (2014) of the OIE makes the following recommendations for the importation of equids from AHS infected countries or zones:

“Veterinary Authorities should require the presentation of an international veterinary certificate attesting that the animals:

1) showed no clinical sign of AHS on the day of shipment;

2) have not been vaccinated against AHS within the last 40 days;

3) were held in isolation in a vector-protected establishment:

a) for a period of at least 28 days and a serological test to detect antibodies against the AHSV group, was carried out with a negative result on a blood sample collected at least 28 days after introduction into the vector-protected establishment; or

b) for a period of at least 40 days and serological tests to detect antibodies against AHSV were carried out with no significant increase in antibody titre on blood samples collected on two occasions, with an interval of not less than 21 days, the first sample being collected at least 7 days after introduction into the vector-protected establishment; or

c) for a period of at least 14 days and an agent identification test was carried out with a negative result on a blood sample collected not less than 14 days after introduction into the vector-protected establishment; or

d) for a period of at least 40 days and were vaccinated, at least 40 days before shipment, against all serotypes whose presence in the source population has been demonstrated through a surveillance programme in accordance with Articles 12.1.12. and 12.1.13., and were identified in the accompanying certification as having been vaccinated;

4) were protected from Culicoides attacks at all times during transportation (including transportation to and at the place of shipment)". (OIE, 2014, p. 3)

If AHSV occurs in an area outside the AHS endemic areas, the control program implemented should include three components: quarantine, vector control and vaccination. Suspected AHS cases should be quarantined in an insect-proof enclosure.

As South Africa is an endemic country, control zones are in place to facilitate the export of horses to AHSV free countries. The area around metropolitan Cape Town has been free from AHS in the past. There have been isolated outbreak events

due to the introduction of AHSV positive horses from outside the free zone (Coetzer and Guthrie, 2004; Grewar *et al.*, 2013). To maintain this as an AHS free zone movement control of equids (horses, donkeys and zebra) into the area have been implemented by the South African State Veterinary Authorities since 1997 (Gazette and Notice, 1984; Guthrie, 1997; Grewar *et al.*, 2013). The Western Cape is currently divided into three zones: The Metropolitan Cape Town AHS free zone, the AHS surveillance zone and the AHS protection zone as illustrated in Figure 2-3. The rest of the country is considered endemic and is named the AHS infected zone. Strict movement control of equids from areas of lower control to areas of higher control aids in ensuring the limitation of disease spread.

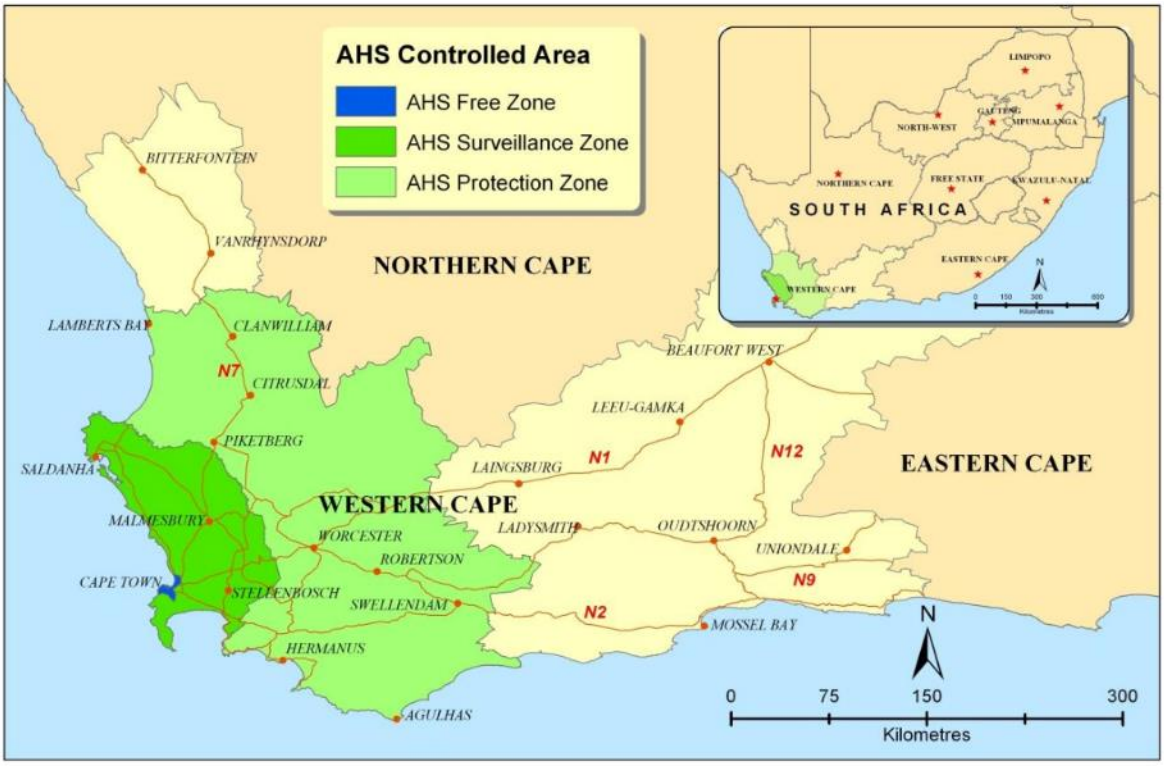

Figure 2-3: A map indicating the AHS zones of the Western Cape Province with the main towns in each zone and the main routes connecting these (Weyer, 2016).

Secondly, management, consisting mainly of vector control, is of major importance in at-risk areas. Management practices include shielding infected stables and treating them with authorized pesticides, such as alphacypermethrin, to reduce adult populations (Page *et al.*, 2014). Equids can further be protected from vectors by applying a pyrethroid or DEET (N, N-Diethyl-meta-toluamide) based spray at least twice a day or soaking fly sheets in these, stabling equines from two

hours before dusk to two hours after dawn (Barnard, 1997), putting fans in the stables (the midges are not strong flyers and a steady breeze will deter them) and reducing midge breeding areas (standing water or wet areas) by spraying these with an authorized insecticide or draining them.

Vaccination is a cornerstone of AHSV control. The first live attenuated AHS vaccine was developed in the 1930s and was based on virus strains attenuated by multiple passages in suckling mouse brain (Alexander, Neitz and Du Toit, 1936). The vaccine-induced solid immunity, but occasionally caused severe side effects, including fatal cases of encephalitis in horses and donkeys. To minimize the side effects, the vaccine strains were further attenuated by passage through cell culture(House, House and Mebus, 1992; von Teichman and Smit, 2008). These cell-culture-adapted viruses still form the basis of the currently available OBP-LAV (Lubroth, 1988). AHS types included in the vaccine are attenuated through cell culture passages followed by plaque purification to select mutants that are non-pathogenic but immunogenic (Erasmus, 1966; von Teichman and Smit, 2008). Even though the vaccine is economically priced and provides adequate immunity in general, it is still lacking in some areas. The potential for variable attenuation and a possible lack of immunogenicity exists; with variable immune responses in individual animals to each AHS type. This may be due to over attenuation of certain AHS types, or interference between types in the vaccine (Erasmus, 1978; Coetzer and Guthrie, 2004). It has been shown that the vaccine virus can revert to virulence and/ or reassort (Weyer *et al.*, 2016). It can also interfere with laboratory diagnostic tests, as none of the routinely available diagnostic tests can distinguish between the vaccine virus and natural infection in a horse (House, House and Mebus, 1992; House, 1993; MacLachlan *et al.*, 2007; Guthrie *et al.*, 2009).

Annual vaccination with the OBP polyvalent cell-culture-adapted LAV that was developed in the 1960s is currently the main means of controlling the disease in endemic areas (Erasmus, 1978). According to the Animal Diseases Act 34 of 1984, all equines, except horses that reside in the AHS free and surveillance zones, require compulsory yearly vaccination (South African Government, 1984). The current DAFF vaccination recommendations are indicated in Table 2-1. The vaccine is made up of two components: combination 1 (AHS1) and combination 2 (AHS2) which are administered at least 3 weeks apart (von Teichman and Smit, 2008).

AHS1 is trivalent and consists of serotypes 1, 3 and 4; AHS2 is tetravalent and consists of serotypes 2, 6, 7 and 8. AHS serotype 9 is not included in the LAV as it has, until recently, caused minimal disease in Southern Africa, and cross-protection is obtained from vaccination against AHS serotype 6 included in AHS2 (von Teichman and Smit, 2008). AHS serotype 5 was included in AHS1 in the past but was removed in October 1993 following suspected reassortment leading to a virulent reassortant strain causing infection and consequent disease of vaccinated horses (von Teichman and Smit, 2008). Von Teichman, Dungu and Smit (2010) reports that AHS serotype 8 gives cross-protection against serotype 5. As immunity to AHSV is serotype-specific, horses need to have immunity to all nine serotypes to be completely protected against the disease (Koekemoer, 2008). The use of polyvalent vaccines has not been a problem as most horses develop protective antibodies against all included serotypes. However, due to the difference in individual equine immune response to the vaccine or viral serotype interference some horses have not developed all-encompassing immunity after their first vaccination course. (Laegreid, 1997). This highlights the necessity for several vaccination courses to achieve full immunity. Erasmus (1978), Von Teichman and Smit (2008) and Guthrie and Quan (2009) all described that horses that had received three or more annual vaccination courses have sufficient immunity to be protected (Erasmus, 1978; von Teichman and Smit, 2008; Guthrie and Quan, 2009).

Table 2-1: The current DAFF vaccination schedule regulations and recommendations for use with the OBP AHS-LAV as per Table 2 of the Regulations of the Animal Diseases Act 1984 (Gazette and Notice, 1984; 'Notice of restriction of AHS vaccination period', 2015).

Vaccine	AHS Zone	Regulations and Recommendations
Initial vaccination	As per zone regulations	Weanlings between 6 and 12 months old.
Secondary vaccination	As per zone regulations	Yearlings between 12 and 18 months of age.
Annual vaccination	As per zone regulations	Every equine should be vaccinated annually.
Zone regulations	AHS Infected Zone	Recommended that horses should be vaccinated between 1 June and 31 October.
	AHS Protection Zone	All equines in this area must be vaccinated within the period of 1 June to 31 October yearly.
	AHS Surveillance Zone	Permission for vaccination will only be granted from 1 June to 31 October each year.
	AHS Free Zone	Permission for vaccination will only be granted from 1 June to 31 October each year.

A monovalent, attenuated AHSV-9 vaccine (National Laboratory, Spain) was used in West Africa, as this was the only known circulating serotype before the 2007 isolation of AHSV-2 and AHSV-7. During the1959 - 1961 outbreaks in the Middle East, the 1955 – 1956 outbreaks in North Africa and Spain, and the 1987 – 1991 outbreaks in Spain, Portugal and Morocco monovalent vaccination (AHSV-9 and AHSV-4) where used as part of a multifactorial approach to eradicate the virus. (Lubroth, 1988).

Research and development have also been put into other vaccine options; including inactivated vaccines used in AHS outbreaks in non-enzootic regions. These were safe vaccines but required repeated immunizations due to horses losing immunogenicity (Dubourget *et al.*, 1992; House, 1993). Virus like particles (VLPs) produced from recombinant baculoviruses, a recombinant vaccinia vectored vaccine were explored (Stone-Marschat *et al.*, 1996) and a DNA vaccine (Guthrie *et al.*, 2009). In 2009 Guthrie et al. developed a recombinant canarypox-vectored vaccine against AHS-4 (Guthrie *et al.*, 2009). This vaccine was safe and produced solid protective immunity against the specific AHS type. Synthetic genes encoding the VP2 and VP5 proteins of AHSV-4 inserted into a recombinant canarypox virus vector was used for the development of this vaccine.

Weyer et al. (2016) showed that virulent virus strains can originate from horses vaccinated with the polyvalent AHS-LAV through strain re-assortment and/or

reversion to virulence; so transmitting the virus to susceptible horses (Weyer *et al.*, 2016). Thus, DAFF restricted the period (season) in which AHS vaccination is allowed within the AHS controlled area which is explained in Table 2-1 ('Notice of restriction of AHS vaccination period', 2015). The vaccination period in the AHSV free, surveillance and protected zones is from 1 June until 30 October. In the rest of South Africa, it is recommended to vaccinate during this period to reduce the risk of potential vaccine virus transmission. Molini *et al.* (2015) showed in Namibia that the majority of horses develop neutralizing antibodies against multiple AHS serotypes only after approximately eight vaccination courses at six years old and onwards. In the same study, the authors noted that these neutralizing antibodies only seem to reduce the severity of the clinical disease; as vaccinated animals can still contract field infections, and the immune response differs greatly between different horses and to the different AHS serotypes (Molini *et al.*, 2015). A previous study in Zimbabwe has indicated that repeated immunization will result in more encompassing antibody response to the various AHS serotypes and higher individual titres (N.K. Blackburn and Swanepoel, 1988b). In this study, the old mouse-brain attenuated vaccine containing only 7 serotypes which were all included in a single bottle was used. Therefore, the results are not comparable to the current study. It should be noted that this has been the only previous study comparing vaccination status with disease outcome for AHSV. In their study Blackburn and Swanepoel (1988b) noted that some horses did not produce satisfactory response to one or more of the AHS serotypes, they did however not indicate the number of immunizations required to produce an adequate immune response that will be protective against a virulent field challenge (N.K. Blackburn and Swanepoel, 1988 b).

Early ingestion of colostrum after birth provides foals born to immune mares with acquired passive immunity. This immunity progressively declines to undetectable levels at four to six months of age (Alexander and Mason, 1941; Piercy, 1951; Coetzer and Guthrie, 2004; Crafford *et al.*, 2013). If foals are vaccinated before immunity declines the maternal antibodies will interfere with the production of antibodies against the vaccine. Therefore, vaccination is recommended from six months old and older.

In 2011 a study was conducted in Ethiopia investigated the seasonality, geographic distribution and prevalent AHS types of retrospective and outbreak cases (Ayelet *et al.*, 2013). Ayelet et al. (2013) found that the highest frequency of cases was from areas with the highest relative equine density, a geographical location conducive to the breeding requirements of the vector and in the months following the rainy season.

2.10 Gaps in the literature on African Horse sickness

Although quite a few studies have theorized about the number of vaccinations required to provide protective immunity to a field challenge, there are no studies that specifically evaluate this and therefore no reliable data (Blackburn and Swanepoel, 1988b; Molini *et al.*, 2015). This gap in the literature also includes antibody response post-vaccination. Other areas lacking in data on AHS include survival and case fatality, the prevalence of subclinical cases (Grewar *et al.*, 2013; Weyer *et al.*, 2013), clinicopathological changes and how these correlate to prognosis and the overwintering mechanism of the virus.

Chapter 3 Materials and methods

3.1 Study design and sample frame

A retrospective descriptive study design was used to describe the outcome of cases with a clinical suspicion of AHS that tested positive on real-time RT-qPCR from all cases submitted to the Veterinary Genetics Laboratory at the University of Pretoria.

Recruitment criteria were as follows: a) only horses with samples submitted from South Africa b) with a clinical suspicion of AHS c) submitted to VGL for diagnostic purposes d) that tested positive with real-time RT-qPCR and e) had a definitive disease outcome were included in this study.

All cases included in the data set were considered a positive AHSV infection per the OIE definition (OIE, 2014). Cases with a group cycle threshold (Ct) value of less than 33 formed part of the type specific (TS) real-time RT-qPCR population. This is due to the TS test being less sensitive than the GS test (Guthrie, personal communication).

Details obtained from submission forms and sample reports submitted to the VGL, Faculty of Veterinary Science, UP, Onderstepoort, for diagnostic AHSV testing that tested positive on real-time RT-qPCR (Guthrie *et al.*, 2013), were used to design a questionnaire in Google forms to further investigate submitted AHSV disease positive cases. The questionnaire was sent to every veterinarian or owner of an AHS disease positive case submitted to the VGL between 1 September 2017 and 30 June 2019.

This study was designed to evaluate the case fatality rate and survival rate correlated to the AHSV vaccination status of AHS cases. These were then be used to evaluate whether horses that were vaccinated were more or less likely to die of AHSV. Thus, the main question in the questionnaire was whether a horse was vaccinated against AHSV and whether it survived the AHSV episode or not.

Furthermore, the study design provided the opportunity to evaluate if other variables (e.g. Ct value, AHSV type, the order in which AHSV-LAV was administered, clinical symptoms observed, age, sex and time since last AHS

vaccination) affected disease outcome significantly, as well as addressing the issue of possible vaccine failure.

3.1.1 Study population and sampling

The study population included all horses from South Africa with clinical signs suspicious of AHSV whose samples were submitted to the VGL for diagnostic purposes, from 1 September 2017 until 30 June 2019, and tested positive for AHSV with real-time RT-qPCR. This effectively encompassed two AHS seasons.

3.2 Sample collection and laboratory testing

3.2.1 Sample collection

Sample collection and transport were done according to the standard procedure by private or state veterinarians that submitted the samples for diagnostic purposes. Thus, there was no direct control over sample collection and transport. Sample types included whole blood in EDTA, organs, body fluids and serum.

3.2.2 Sample processing and laboratory testing methods

Sample processing was performed by the VGL using the real-time RT-qPCR (Guthrie et al., 2013) accredited by the OIE for AHSV diagnostics (World Organization for Animal Health (OIE), 2012). The VGL is accredited by the South African National Accreditation Society (SANAS) to use this method for AHS identification for diagnostic and export purposes.

3.2.2.1 AHSV Group-specific and Type-specific real-time RT-qPCR

The GS real-time RT-qPCR method developed by Guthrie et al. (2013) at the VGL was used for GS-AHSV diagnosis.

AHSV type determination was done with the AHSV TS real-time RT-qPCR test developed by Weyer et al. (2015). Unique regions of the sequences encoding VP2 of each AHSV type where used as targets for primers and probes, designed by Primer Express v3 (Lifetech). After performing the GS real-time RT-qPCR on samples as described by Guthrie et al. (2013) plates containing the remaining eluates were stored according to standard operating procedures until the GS real-time RT-qPCR assays where completed. For the GS-AHS positive samples stored

nucleic acid extracts were then submitted to TS real-time RT-qPCR analysis. A sample was classified as positive if the normalized fluorescence for the AHSV TS real-time RT-qPCR assay exceeded a 0.1 threshold within 40 PCR cycles. The cycle number during which the fluorescence threshold was reached is defined as the quantification cycle (Cq). (Weyer et al., 2015).

3.3 Questionnaire development

Data obtained from the sample submission forms submitted to the VGL and the results reports were evaluated for completeness and relevance to the study question. A questionnaire was thereafter developed by the researcher and supervisors, based on information that was incomplete or lacking from the above-mentioned documents to allow the researcher to obtain a comprehensive history on each positive case. It was important to make this easily distributable, quick to answer yet comprehensive and understandable to non-veterinary people. Therefore, the questions consisted of multiple choice and multiple answer questions, with minimal fill in questions. Colloquial terms for the clinical signs and case-related information were used. The questionnaire was developed using Google forms and stored in a shared Google Drive folder. Test questionnaires were sent out to select private veterinarians for comments on completeness, relevance and user-friendliness.

3.4 Data collection

Data were obtained from the VGL sample submission forms and result reports, as well as the questionnaire sent to owners and veterinarians. In cases where the owner or veterinarian did not respond to the online questionnaire data were collected telephonically and the researcher completed the questionnaire for these cases.

3.5 Data recording and analytical programs used

Data were recorded in Microsoft Excel (Microsoft Office 2016). Basic analysis, including counts and proportions, was done using the built-in formulas in

Excel. Further data analysis was done using R (3.4.1 (2017-06-30 -- "Single Candle") and R-Studio (Version 1.0.153). CFR within the calendar year was evaluated with a z-test. The case fatality within the season as well as the correlation between case outcome and clinical signs, days since last vaccination, PCR Ct value and age were evaluated using logistic regression. The CFR per AHS type was evaluated using a chi-squared test. A chi-square test was used to evaluate the correlation between case outcome and vaccination order, PCR TS outcome, sex and vaccination status. The ANOVA test was used to determine the correlation between the case outcome and Ct value as well as horse age. The odds ratio was used for multiple analysis. Google Maps was used to link cases to the closest town based on GPS coordinates provided by the submitting veterinarian. Geographic presentation of the data was done using QGIS (3.6.2-Noosa).

3.6 Data and statistical analyses

Data gathered on cases included outcome of AHS episode, clinical signs observed, GPS coordinates of the location a case was reported at, date of last AHS vaccination before the AHS episode, order in which AHS vaccine components where administered at this date, AHS vaccination frequency throughout lifetime, whether cases were TS real-time RT-qPCR positive, how many different AHSV types one sample tested positive for, Ct value of diagnostic samples, age and gender.

An exploratory analysis was performed to explore the associations within the data for the cases that died versus those that did not. The case fatality rate was used to determine the proportion of cases that died. CFR was determined by dividing the number of study negatives (i.e. deaths) by all recruited disease positive AHS cases within each categorization.

3.6.1 CFR within each calendar year

A two proportions z-test with a two-tailed design and a significance level (alpha) of 0.05 was employed, to specifically evaluate whether the CFR in 2019 was greater than that in 2018. Only 5 of the 233 study cases evaluated where 2017 cases and was not considered in this analysis.

3.6.2 Fatality associated with season

Case outcome for each month of the year was determined to evaluate the seasonality of AHS. This was done by establishing the case outcomes for each month of the year (i.e. January through December), irrespective of the actual year in which the case occurred. Logistic regression was then used to evaluate this data establishing the log odds of horses dying in a specific month. The initial analysis did not show any indication of differences between months and further analysis of temporal fatality was not considered.

3.6.3 Geographical distribution and CFR for different areas

For geographical representation: data were aggregated to a 50 km hexagon grid to account for the resolution of the underlying data and to avoid human and/or geographic bias. The geographical location of cases was based on the closest town to the case location. CFR and case densities for the different geographical areas were determined and visually analysed.

3.6.4 Case outcome compared to PCR Ct value

A one-way ANOVA test and logistics regression test were used to evaluate the correlation between the Ct value and disease outcome. The ANOVA test evaluated the difference in means between the two categories (died or recovered) while the logistic regression evaluated the odds of death with the outcome variable (death or recovered) dependant on the Ct-value. It should be noted that this is an exploratory analysis; other factors such as sample type, sample quality and time since infection are likely to influence the Ct-value for each case.

3.6.5 AHSV type CFR

Case outcomes were compared for each AHSV type where the type was established for the dataset.

Study cases were classified as being positive for one or multiple AHSV types and the effect this had on disease outcome was evaluated. Instances, where one case tested positive for two or three AHSV types, were classified as multiple AHS types. A chi-square test was used to establish associations between differences in case outcome for these two categories.

3.6.6 Case outcome compared to TS PCR outcome

Cases were classified as AHS TS (i.e. whether a study case was TS RT-qPCR positive or not) positive or negative and evaluated whether this had any influence on the outcome. To evaluate the difference in case outcome for cases that tested positive with TS RT-qPCR versus those that did not test TS positive a 2x2 table was created and a chi-squared test was used to establish any associations. When evaluating the number of cases that were positive on TS RT-qPCR it should be kept in mind that the TS RT-qPCR sensitivity has not been validated as the GS RT-qPCR has, anecdotally it would also appear that certain of the TS assays likely have lower sensitivity than the GS assay (Guthrie personal communication). Therefore, samples with a GS Ct value of less than 33 were not submitted for the TS test.

3.6.7 Case outcome compared to age

The ANOVA test and logistics regression test were used to evaluate whether age influenced disease outcome. The difference in mean age between the two groups compared (dead and recovered) were evaluated with the ANOVA test while a logistic regression evaluated the odds of death with individual age as an independent variable.

3.6.8 Case outcome compared to sex

Only cases with known sex were included in this evaluation. Male subcategories (gelding, stallion and colt) where merged and cases were categorised by outcome and sex. A Pearson's Chi-squared test with Yates' continuity correction was used to evaluate the significance of the results.

3.6.9 Case outcome compared to observed clinical symptoms

Observed clinical symptoms associated with AHS were classified into five categories based on the type of clinical signs observed. These included pulmonary, cardiac, mixed, fever and unknown. Classification of clinical signs into these categories was based on descriptions from previous studies (Theiler, 1921; Coetzer and Guthrie, 2004). The following was included as possible clinical signs in the questionnaire, with the option for the veterinarian or owner to add other signs: swelling of the supraorbital fossae, swelling of the eyelids, swelling of the facial tissues, swelling of the neck, swelling of the thorax, swelling of the brisket and

shoulders, dyspnoea or difficulty breathing, spasmodic coughing, dilated nostrils with frothy fluid oozing out, pyrexia and redness of the conjunctiva. Depending on the clinical signs noted as present these were categorized as following into the different AHS forms: pulmonary form (dyspnoea, spasmodic coughing, dilated nostrils with frothy fluid oozing out, pyrexia and conjunctivitis), cardiac form (swelling of the supraorbital fossae, oedema of the eyelids, facial tissues, neck, thorax, brisket and shoulders, pyrexia and conjunctivitis), mixed form (all signs) and fever form (pyrexia and conjunctivitis). These data were analysed using logistical regression.

3.6.10 Case outcome compared to vaccination status

The main variable to be evaluated in this study was the vaccination status. This was evaluated by determining the outcome counts for each category of AHS vaccination status (unvaccinated, unknown, vaccinated, vaccinated by a layperson). Associations were tested using Pearson's Chi-squared test with Yates' continuity correction.

3.6.11 Case outcome compared to vaccination frequency

The relationship between the frequency of vaccination and disease outcome was evaluated. This was done by determining the case outcome for each category of vaccination frequency data (once-off, yearly, two-yearly and greater than two-yearly).

3.6.12 Case outcome comparted to number of days since the last AHS vaccination

The number of days between the AHS episode and the last AHS vaccination received was also evaluated by determining the case outcome for grouped periods between the case and last vaccination date. Periods were grouped as 0 – 100, 100 – 200, 200 – 300 and >300 days post-vaccination. Significance of these results was analysed using logistic regression.

3.6.13 Case outcome compared to vaccination order

To evaluate whether the order in which vaccination components where administered had significance the case outcome for the two categories (AHS1 then AHS2 and AHS2 then AHS1) was determined. Statistical significance was evaluated using Pearson's Chi-squared test with Yates' continuity correction.

Chapter 4 Results

All data gathered are represented in Appendix 2. In summary, of all the suspected AHS cases submitted to the VGL between 1 September 2017 and 30 June 2019 for diagnostic purposes, 688 tested positive with real -time RT-qPCR. After exclusion of cases that did not comply with the recruitment criteria, the data set consisted of 233 cases. The major cause of the significant reduction in the size of the final dataset was due to not obtaining the final disease outcome for positive PCR's. Depending on the factor evaluated some cases were included or excluded, based on whether they had a specific outcome for that factor (e.g. AHS vaccination status, AHS vaccination frequency, clinical signs, age, sex and AHS type). A total of 204 cases had a specific AHSV type diagnosed with TS RT-qPCR. Definitive vaccination status was obtained for 127 cases, with 79 cases having a definitive vaccination frequency. Sixty cases had a known vaccination component administration order. The sex was noted in 221 cases and 227 cases had known clinical symptoms.

4.1 Temporal distribution of cases

4.1.1 Outcome correlated to AHS date

Figure 4.1 illustrates the temporal occurrence of data set cases for the period 1 September 2017 - 30 June 2019. The seasonal increase in cases over summer months was well illustrated, with the maximum number of cases occurring in March each year (34 in 2018 and 45 in 2019), followed by April, May and February (in 2019 it was April, Feb and then May respectively). The tail of the 2017 – 2018 season ended in July, with no cases reported in August, September and October. Due to time constraints, this tail was not illustrated for the 2018 – 2019 season.

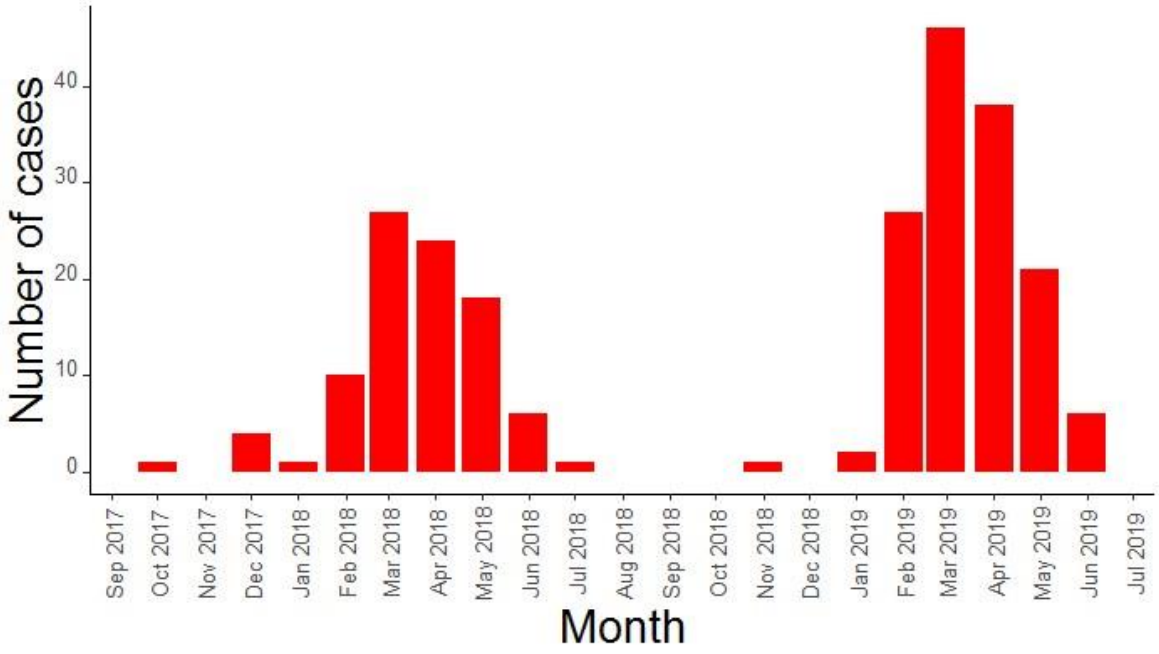

Figure 4-1: Epidemic curve showing the temporal occurrence of study cases between 1 September 2017 and 30 June 2019.

4.1.1.1 Evaluate whether there was a difference between case fatality rates between the years: 2017, 2018 and 2019

Case fatality rates for study cases was calculated for each year of the study period as illustrated in Table 4-1. The number of deaths increased substantially between 2018 and 2019. The CFR increased significantly between 2018 and 2019 ($p < 0.001$), with the lowest CFR of 0.523 in 2018 and the highest CFR of 0.771 in 2019.

Table 4-1: Case fatality rates (CFR) for study cases; categorized according to the year in which they occurred (2018 and 2019) with lower and upper confidence intervals (CI) indicated.

Year	Dead	Recovered	CFR	Low CI	Upper CI
2017	4	1	0.800	0.449	1.000
2018	46	42	0.523	0.418	0.627
2019	108	32	0.771	0.702	0.841

4.1.2 Outcome correlated to seasonality

Table 4.2 and Figure 4-2 illustrates the spread of the case outcomes and CFR over a year. The CFR from the three years observed (2017, 2018 and 2019) were combined to evaluate the CFR for each month of the year. The average

monthly CFR derived from this data was 0.465, with the highest CFR being November and December at 100% and the lowest CFR in July and October at 0%. In all these months, however, the number of cases was below 5. November and December were followed by March and April, with CFR of 0.74 and 0.74 respectively. Thereafter the CFR declined from February to June to May and finally to January with a CFR of 0.33. The CFR varied markedly from month to month, with only some similarity shown between consecutive months (March and April; May and June; November and December). The logistic regression test on this data did not show any month/s having a higher likelihood of having deaths compared to others. (p-value > 0.05 for all months).

Table 4-2: The case outcome and case fatality rate for each month of the year (January – December respectively) illustrating how outcomes and CFR differed through the year. Study cases from 2017, 2018 and 2019 were combined per month to give the average CFR per month. August and September had no definite disease outcomes.

Month	Dead	Recovered	Total	CFR
January	1	2	3	0.33
February	23	14	37	0.62
March	54	19	73	0.74
April	46	16	62	0.74
May	22	17	39	0.56
June	7	5	12	0.58
July	0	1	1	0.00
August	0	0	0	0.00
September	0	0	0	0.00
October	0	1	1	0.00
November	1	0	1	1.00
December	4	0	4	1.00

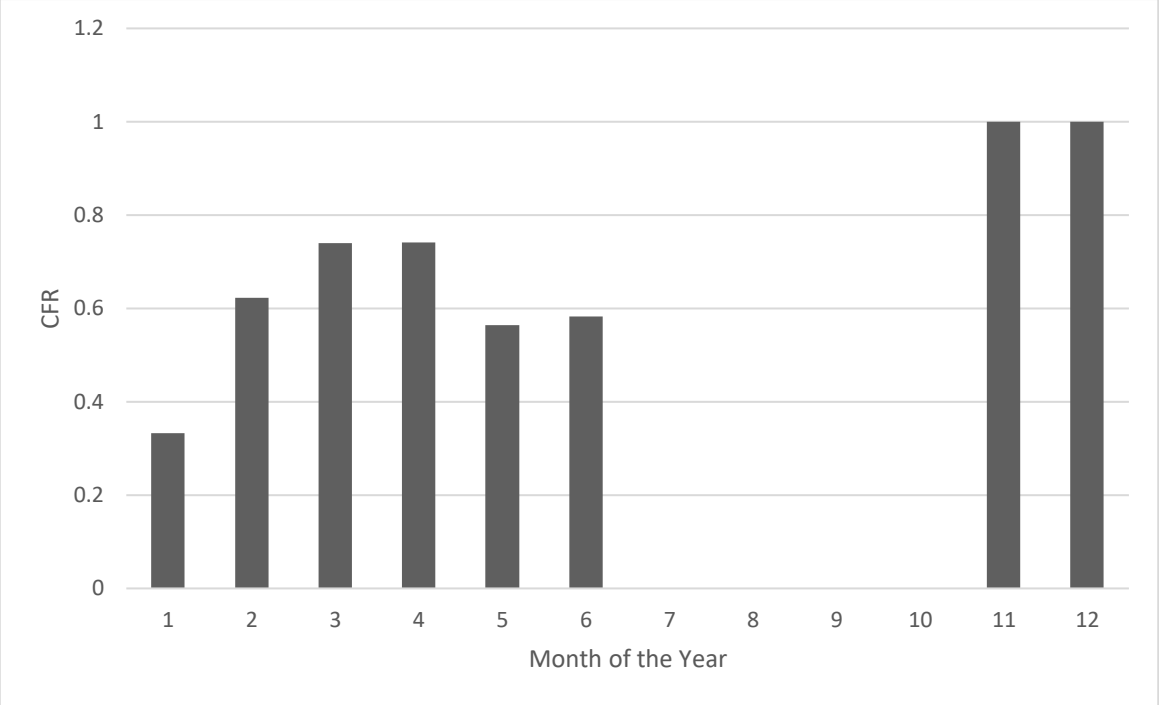

Figure 4-2: Case fatality rate (CFR) for each month of the year (January – December) illustrating CFR spread over a year. Study cases from 2017, 2018 and 2019 were combined per month to give the average CFR per month. August and September had no definite disease outcomes.

4.2 Geographical presentation of data

4.2.1 Geographical presentation of all cases observed during the study period

Study cases where represented geographically in Figures 4-3 to 4-6. As shown in Figure 4-3 the highest number of cases observed in one location for the entire period observed were 40 – 43 in the Eastern Cape. Thereafter 20 – 30 and 30 – 40 cases per area were observed at two locations in Gauteng. All the other locations that had AHS outbreaks reported fewer than 20 cases with most areas registering less than 10 cases.

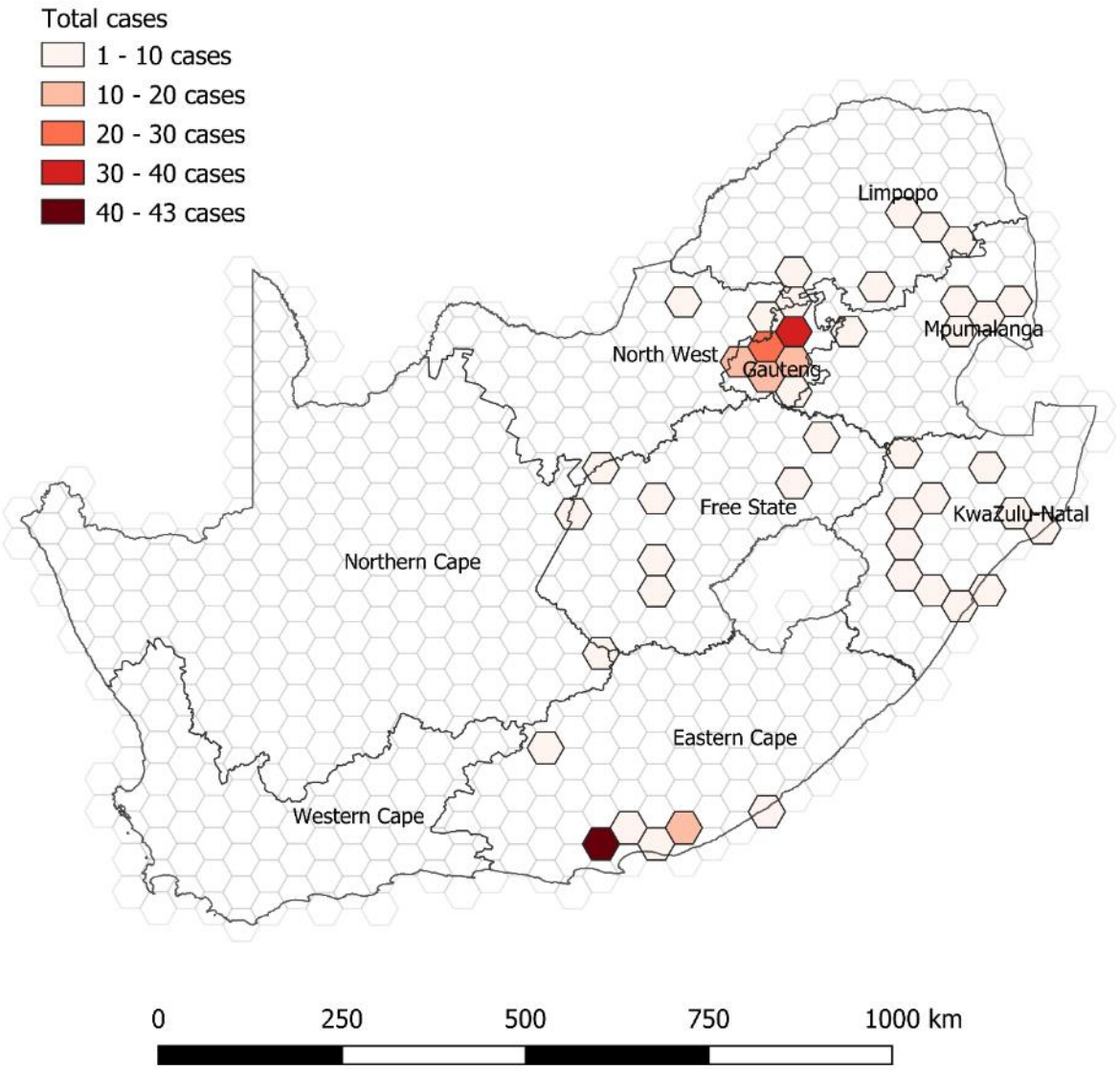

Figure 4-3: Map of the nine South African provinces illustrating the study cases per location for the period observed (1 September 2017 – 30 June 2019).

In 2017 the highest number of cases observed per location was in the 1 – 10 range. These outbreaks were limited to Gauteng, the Free State and Eastern Cape as illustrated in Figure 4-4.

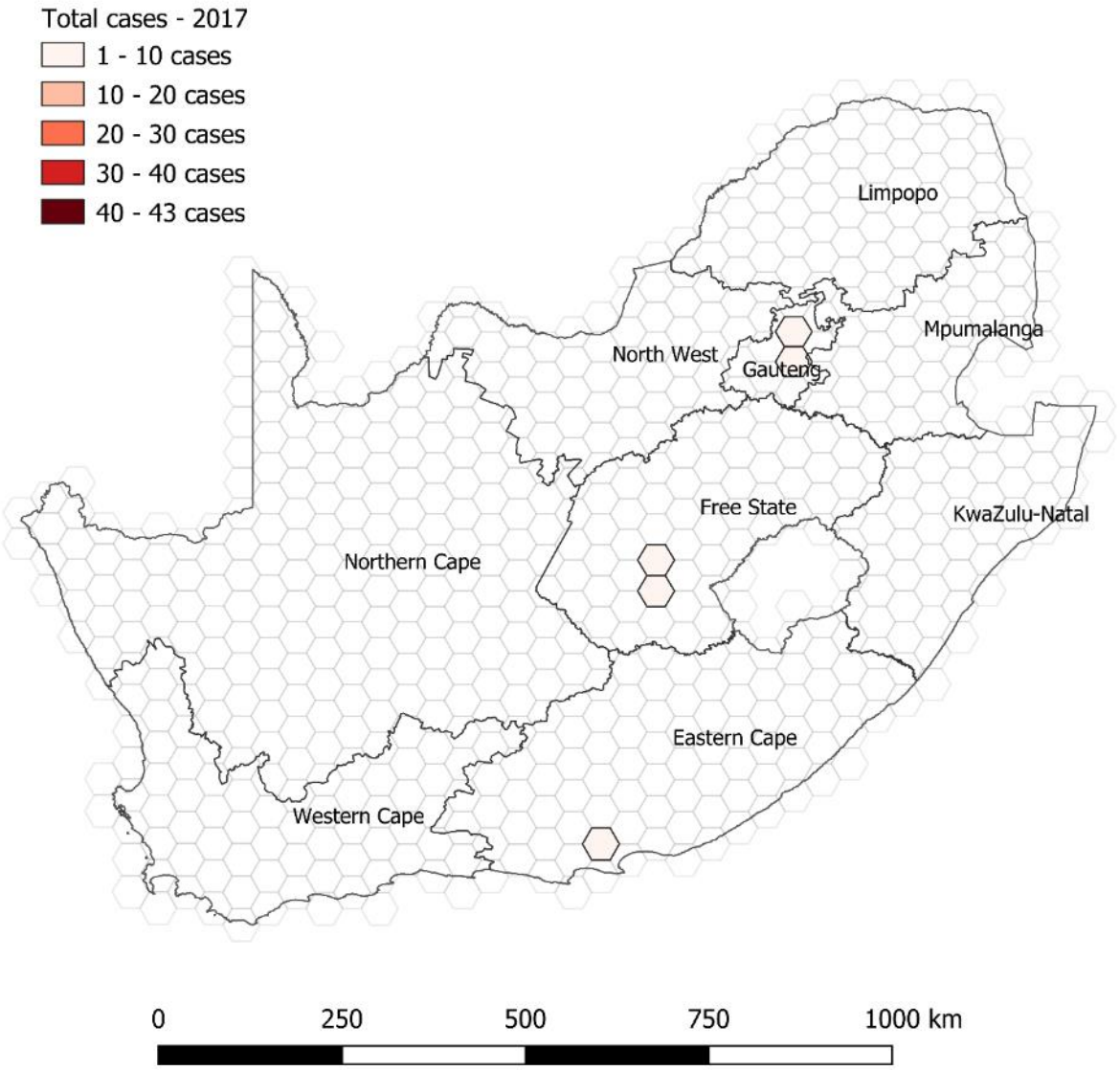

Figure 4-4: Map of the nine South African provinces illustrating the data set per location for the period observed in 2017 (1 September 2017 – 31 December 2017).

Figure 4-5 illustrates that during 2018 the highest case density was 30 – 40 cases per area, observed in the Eastern Cape. Even though there were multiple outbreaks in the rest of the country (Limpopo, Mpumalanga, North West, Gauteng, Free State, KwaZulu-Natal, Northern Cape borders), the case density for all of these was low (1 – 10 cases per area).

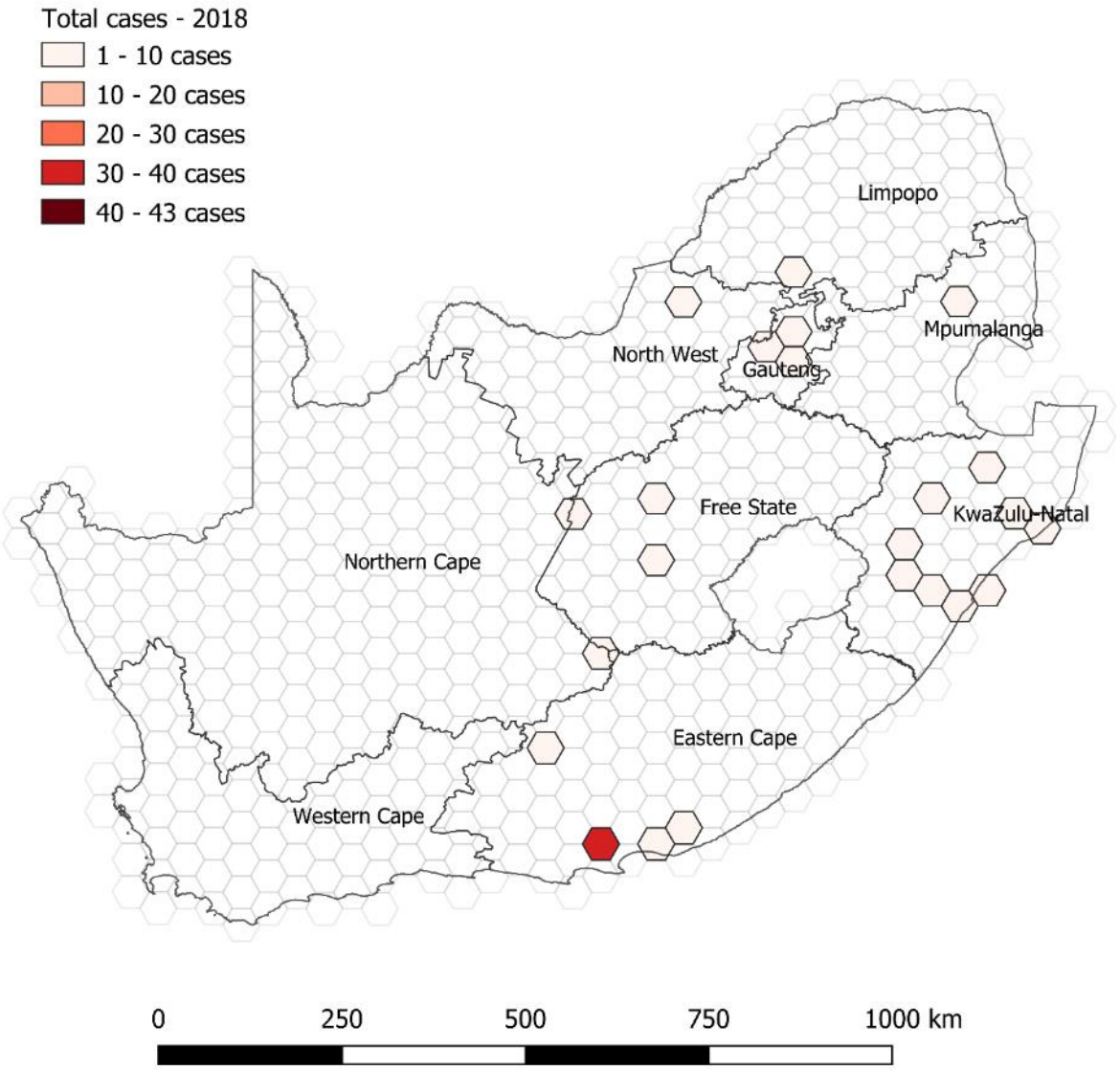

Figure 4-5: Map of the nine South African provinces illustrating the data set per location for 2018 (1 January 2018 – 31 December 2018).

In 2019 there was clustering of cases in the Gauteng area as is seen in Figure 4-6. The highest case density was also in this province, with 30 – 40 cases in one location. The rest of the provinces affected (Limpopo, Mpumalanga, KwaZulu- Natal, Eastern Cape, Free State, Northern Cape border and North-West border) had low case densities (1 – 10) throughout. In Limpopo, Mpumalanga and Eastern Cape there were some clustering of cases.

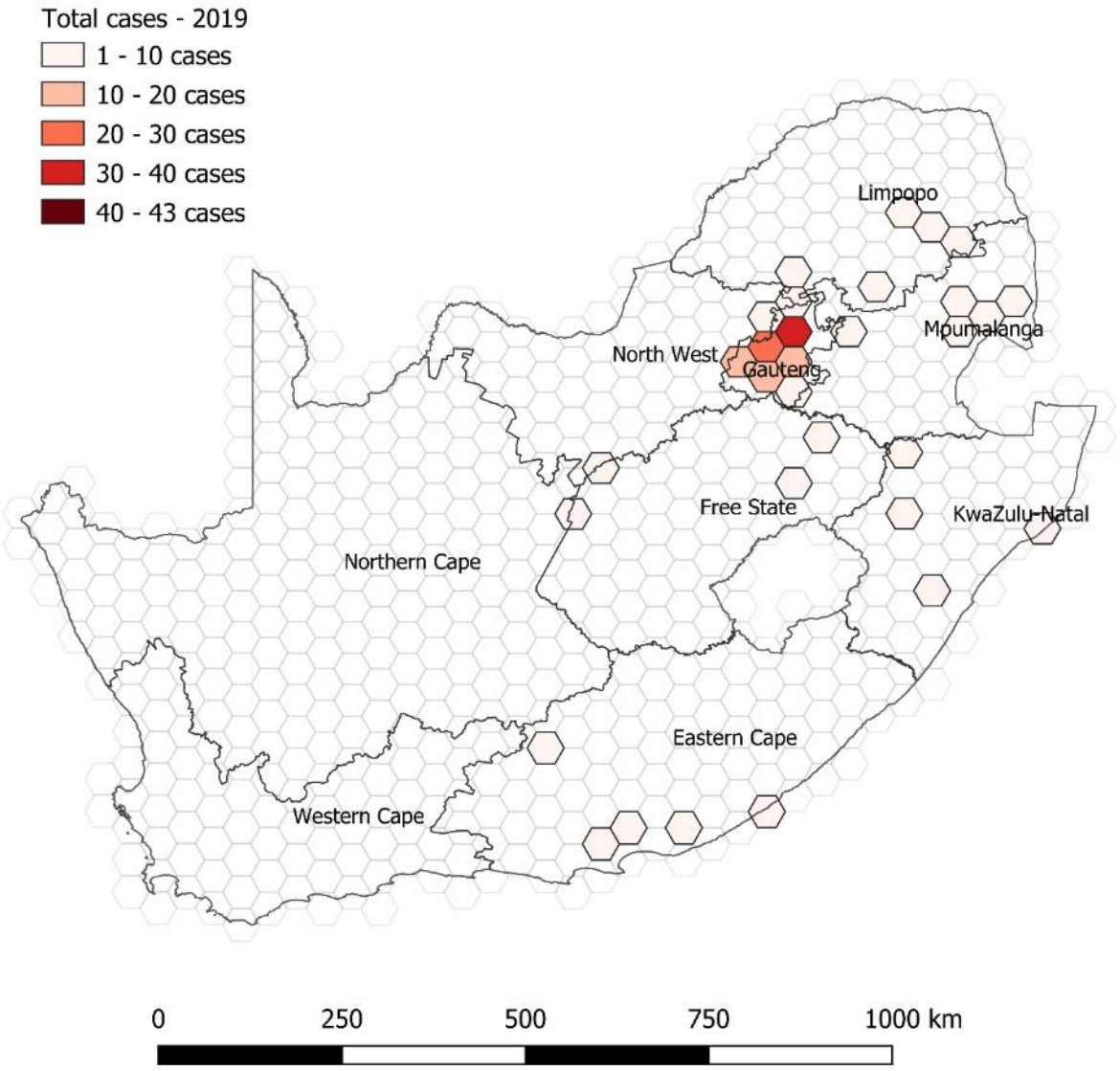

Figure 4-6: Map of the nine South African provinces illustrating the study cases per location for the period observed in 2019 (1 January 2019 – 30 June 2019).

4.2.2 Geographical presentation of CFR

The case fatality rate for the data set was represented geographically for the nine provinces of the Republic of South Africa in Figures 4-7 to 4-9. Firstly, the overall CFR for the period observed was noted. Thereafter, a separate map was constructed for each year observed (2017, 2018 and 2019).

Figure 4-7 illustrates that the lowest CFR per location was 0.0 – 0.2 in some coastal areas of the Eastern Cape, on the Eastern Cape - Northern Cape – Free State border, in Mpumalanga and northern KwaZulu-Natal. The highest CFR was

0.8 – 1.0 and occurred in Gauteng, Limpopo, KwaZulu-Natal, Mpumalanga, Free State, North West and Eastern Cape, with a decreasing number of areas with high CFR per province respectively. Most affected areas had a CFR of 0.8 or higher.

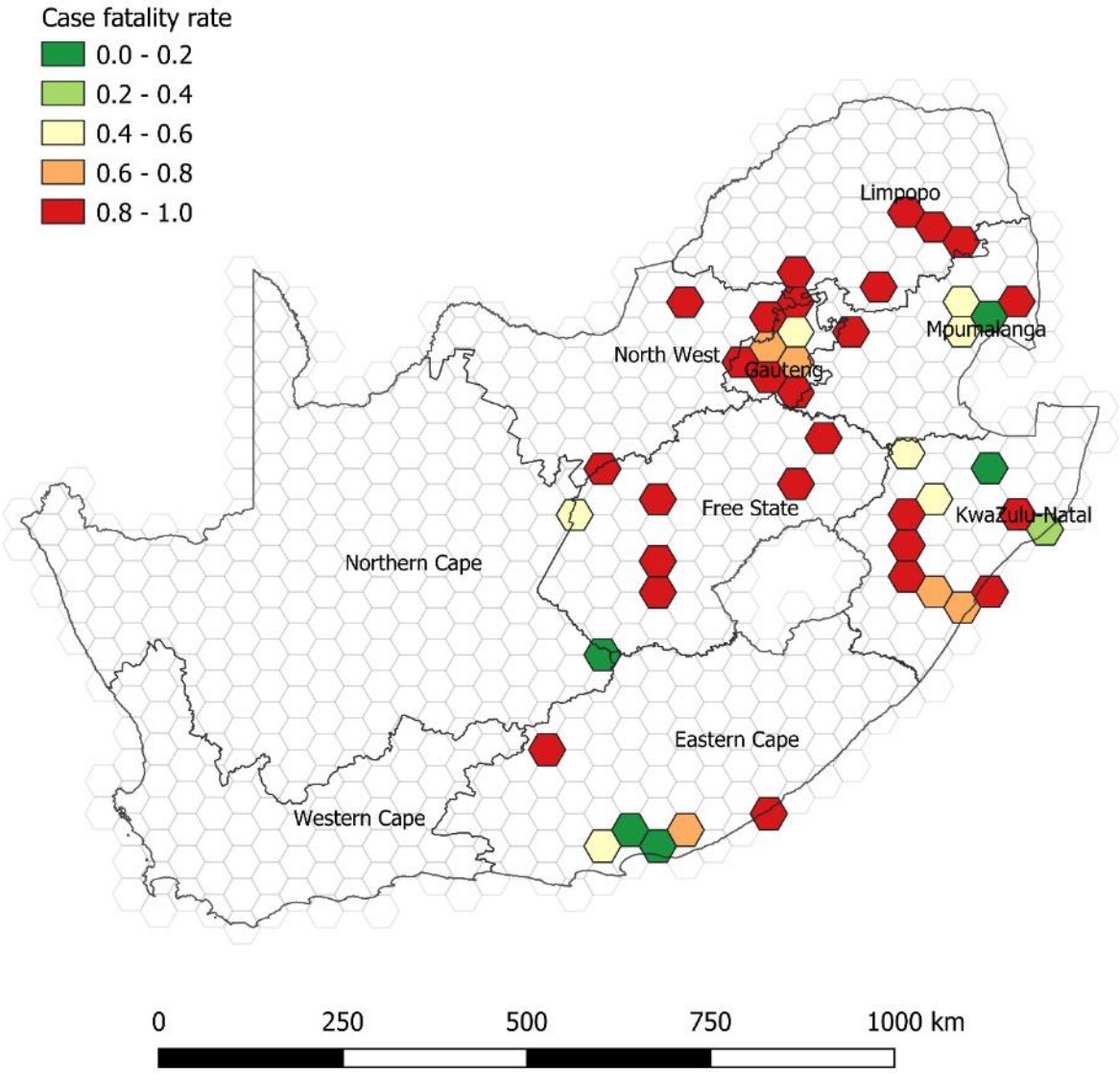

Figure 4-7: A map of the nine South African provinces illustrating the data set case fatality rate for the period observed (1 September 2017 to 30 June 2019).

As previously stated, due to data limitations for 2017 the individual geographical presentation is not truly representative

The 2018 data are illustrated in Figure 4-8. The highest CFR observed was 0.8 – 1.0, and occurred in KwaZulu-Natal, the Free State, Limpopo, North West and the Eastern Cape with a decreasing number of disease cases respectively. The

lowest CFR was 0.0 – 0.2, with multiple occurrences with CFR in between (0.2 – 0.8) occurring in all the provinces except the Northern Cape and Western Cape.

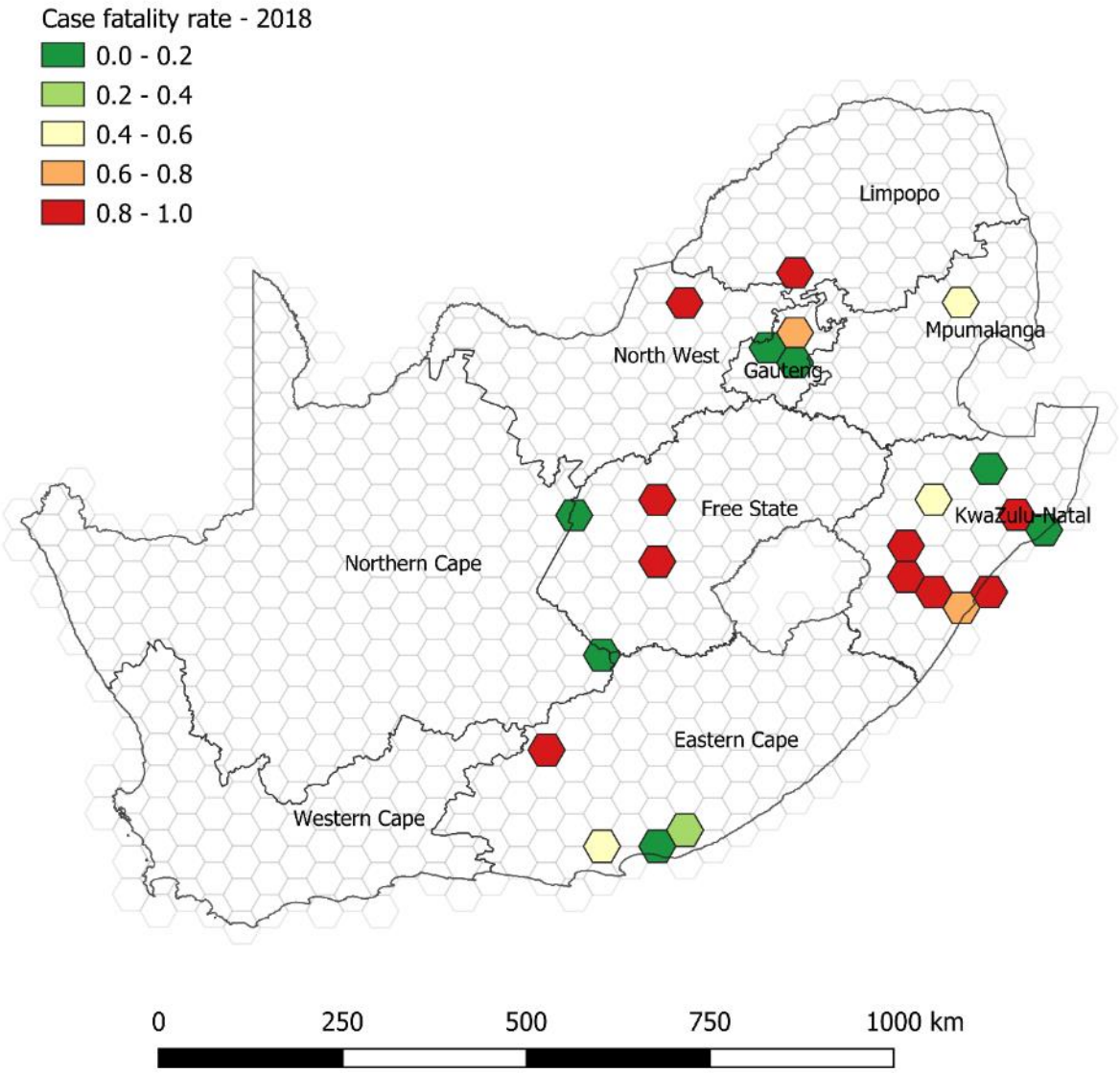

Figure 4-8: A map of the nine South African provinces illustrating the case fatality rate for study cases for 2018 (1 January 2018 – 31 December 2018).

The 2019 data are illustrated in Figure 4-9. During this time the highest CFR was 0.8 – 1.0 and occurred with high density in Gauteng and Limpopo. There were also some high CFR areas in the Free State, Free State – North West border, Eastern Cape, Mpumalanga and KwaZulu-Natal. Most of the other AHS occurrences

consisted of low CFR cases in the Eastern Cape and Mpumalanga.

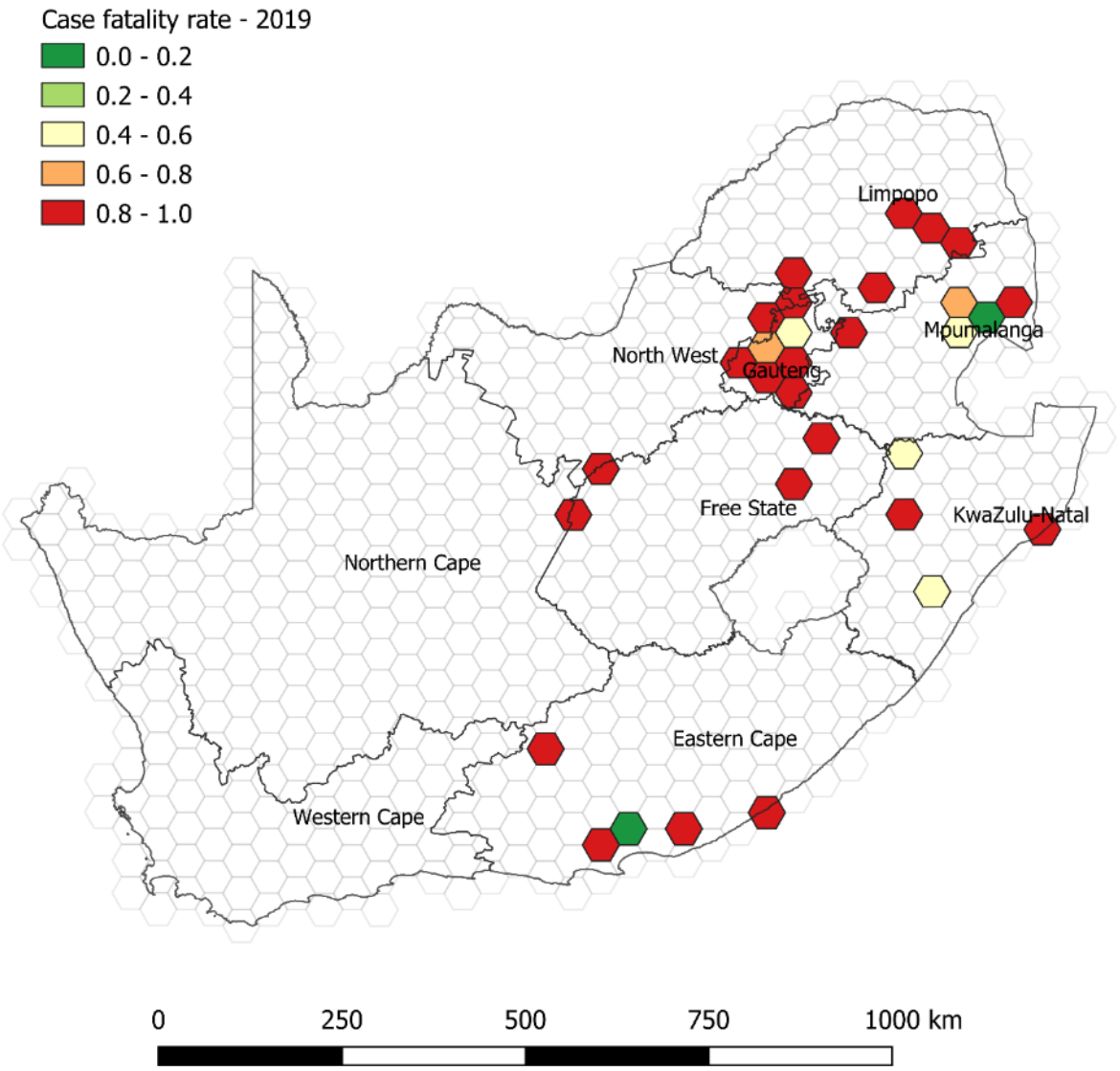

Figure 4-9: A map of the nine South African provinces illustrating the case fatality rate for study cases for the period 1 January 2019 until 30 June 2019.

4.2.3 Type specific case density distribution

Data on the specific AHSV types occurring in the nine different provinces of South Africa are presented in Table 4-3. Types 2 and 8 represented most of the TS cases observed during the study period, with 66 and 64 cases respectively. There were 25 cases of type 7, 19 cases of type 9, 15 cases of type 4 and 10 cases of type 1, with less than 10 cases for each of the remaining types (3,5 and 6). In the Eastern Cape type, 2 was the main AHSV type (51 cases) with four cases of type 7

and one case of type 5. Type 2, 6 and 9 occurred in the Free State (four, three and one cases respectively). Gauteng was the province with the most diverse AHSV types identified; with type 8 making up most of the cases (50), followed by type 9, 7, 4, 1, 2,3 and 5 (14, 9, 5, 2 and 1 cases respectively). In KwaZulu-Natal, there was relatively even spread between the number of cases for each AHSV type that occurred in the province, with types 1,2,4,5,7 and 8 occurring here at frequencies between three and seven. Limpopo had the fewest TS AHS positive cases (six) with types 4, 8 and 9 occurring here. In Mpumalanga type 7 had the most cases (seven) followed by type 1, 4 and 8 (three, two and one respectively). AHS types 8, 9 and 2 were the only ones occurring in the North West and Northern Cape, with frequencies of five, one and one respectively for the North West and one, two and six respectively for the Northern Cape. No samples were submitted to the VGL for diagnostic purposes of suspected cases of AHSV from the Western Cape during the period observed.

Table 4-3: A summary of the study cases indicating the TS RT-qPCR positive cases for each type of AHSV in each province of South Africa during the period observed (1 September 2017 – 30 June 2019).

	AHSV1	**AHSV2**	**AHSV3**	**AHSV4**	**AHSV5**	**AHSV6**	**AHSV7**	**AHSV8**	**AHSV9**
Eastern Cape		51			1		4		
Free State		4				3			1
Gauteng	2	1	1	5	1		9	50	14
Kwa-Zulu Natal	5	3		7	3		5	3	
Limpopo				1				4	1
Mpumalanga	3			2			7	1	
North West		1						5	1
Northern Cape		6						1	2
Western Cape									
Total	10	66	1	15	5	3	25	64	19

4.3 Outcome related to RT-qPCR test factors

4.3.1 Ct value correlated to disease outcome

Figure 4-10 is an illustration of the ANOVA test done to evaluate the correlation between Ct value and disease outcome. The mean Ct value for the 158 equines with a definitive outcome of death due to AHSV was 23.98, with a median

of 25.50. For the 75 cases with a definitive outcome of recovery from confirmed AHSV the mean Ct was 28.58 and the median 26.50 There was a larger spread for the Ct values of cases that died than those that recovered. Analysis indicated a difference in the mean Ct value between cases that died and those that recovered ($p < 0.001$). Logistic regression suggested a strong association between the Ct value and outcome, with the log odds of death decreasing by 0.35 for every unit increase in Ct.

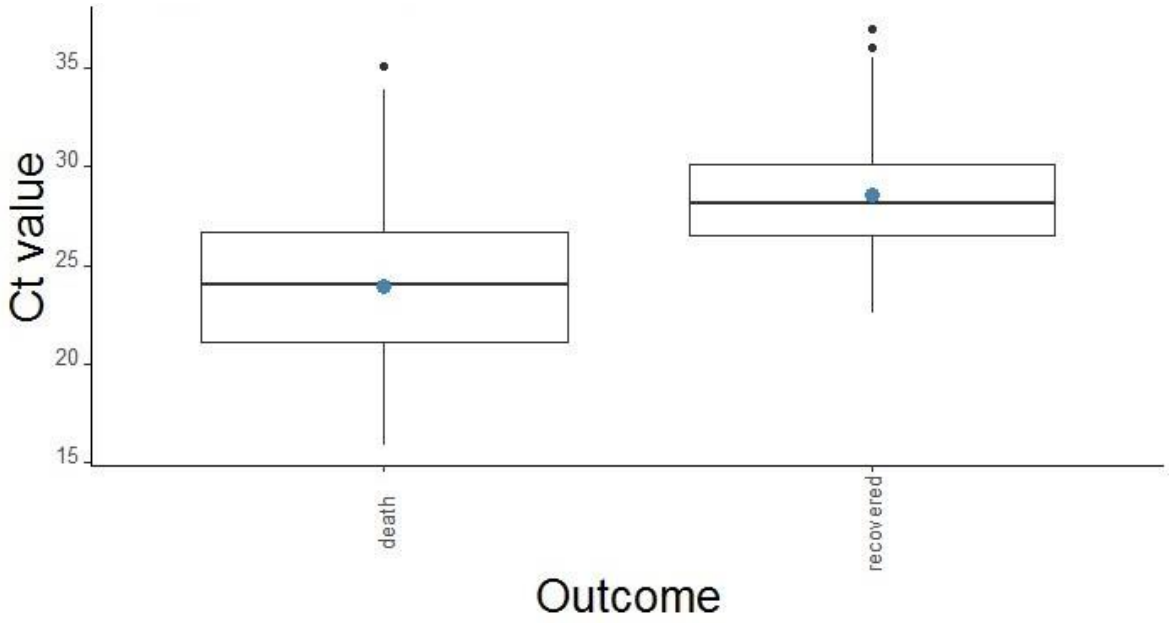

Figure 4-10: A box and whisker plot illustrating the mean and median Ct values for study cases. The results are categorized according to the definitive disease outcome: death or recovery. The blue dots indicate the mean values, the horizontal black line the median values and the black dots the outlier values.

4.4 Outcome related to AHSV variables

4.4.1 Outcome correlated to AHSV type

Table 4-4 illustrates the number of cases in total, disease outcome of cases and CFR for each category of the number of AHS types positive for with PCR. The largest proportion of cases was TS positive for one AHSV type (200), of which 141 died and 59 recovered. Of the four cases that typed positive for two AHSV types only one case survived. There were 29 TS negative cases, out of which 15 recovered. This group had the lowest CFR of 0.48.

Table 4-4: The CFR for study cases categorized according to the number of AHSV types each sample tested positive for and disease outcome.

AHS type class	Dead	Recovered	Total	CFR
Double	3	1	4	0.75
Single	141	59	200	0.71
Negative	14	15	29	0.48

The data obtained when grouping all the cases that were TS positive together (single, double or triple) compared to the cases that were not TS showed that out of the 204 cases that were TS positive for AHSV, 144 died and 60 recovered. For the cases that were TS negative (i.e. AHSV GS PCR positive, but not typed) the number that died (14) is almost equal to the number that recovered (15). The CFR for cases that were TS positive (0.71) was 1.5 times higher than that for cases that were TS negative (0.48). There was a significant association between the lab outcome of TS real-time RT-qPCR and case outcome, such that TS positive study case samples were more likely to die ($p = 0.028$).

4.5 Outcome related to individual equine variables

4.5.1 Horse age correlated to disease outcome

A definitive age was obtained for 168 of the 233 study cases. The mean age was 7.7 years old and the median 4 years old. The age distribution for study cases categorized according to disease outcome is shown in Figure 4-11 years old The ANOVA test on this data showed a mean difference in the age between horses that died and those that recovered with a p-value of <0.001. This indicates that there was a significant association between the age of an individual and the disease outcome. This should be interpreted with caution as vaccination status and previous disease exposure are possible confounders with age.

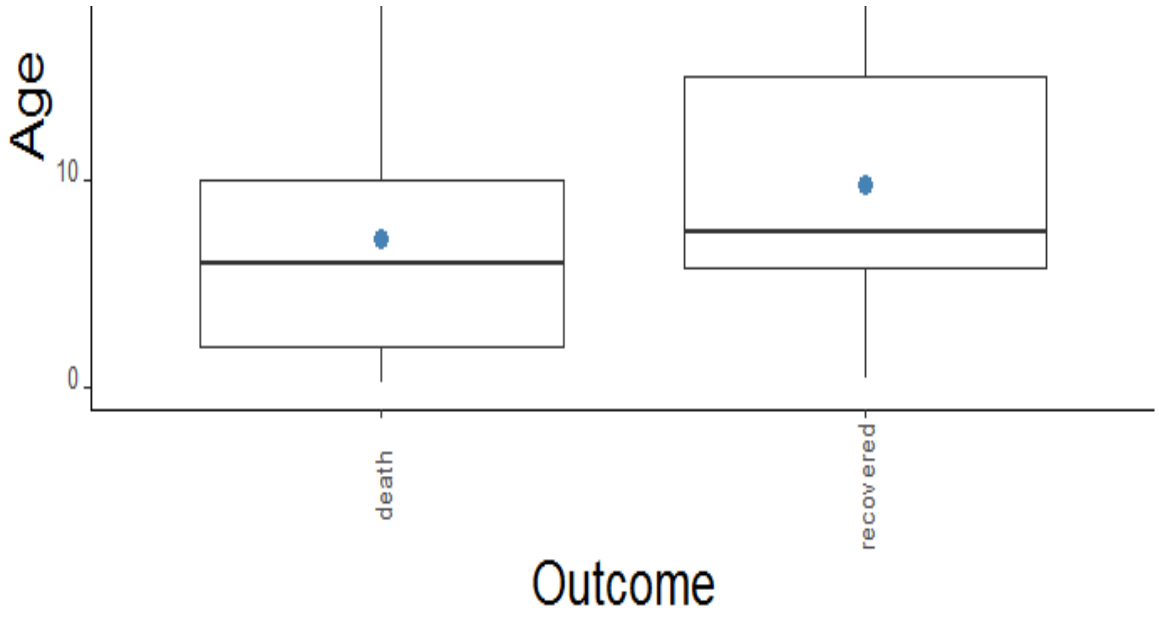

Figure 4-11: A box and whisker plot illustrating the distribution of age for study cases with a mean (blue dot) and median (horizontal black line) highlighted.

4.5.2 Horse sex correlated to disease outcome

There were 194 cases with known sex; of these 109 were females and 85 males. As shown in Table 4-5, 78 of the females died, with a CFR of 0.71. Of the 85 males, 56 died, with a CFR of 0.60. There does not seem to be a significant association between sex and case outcome ($p = 0.14$).

Table 4-5: Case outcome and CFR for study cases categorized according to sex (male – colts, stallions and geldings; female – fillies and mares).

Gender	Dead	Recovered	Total	CFR
Female	78	31	109	0.72
Male	56	29	85	0.66

4.5.3 Clinical symptoms correlated to disease outcome

Clinical symptoms associated with AHS were divided into categories according to the symptoms shown and the acuteness of onset. Out of the 233 study cases, 227 cases had described clinical signs. Many of these cases were of the pulmonary (peracute) form, with 88 out of 89 dead. The cardiac (acute) form was the second most common, with 28 of 65 cases dying. The AHS fever form appeared the least, with only 25 cases. The pulmonary form had a CFR of 0.99, the mixed

form one of 0.67, the cardiac form one of 0.43 and the AHS fever form a CFR of 0.16 as illustrated in Table 4-6. Analysis indicated that there was a high likelihood of the type of clinical presentation influencing the disease outcome. AHS fever appeared comparatively protective ($p = 0.02$), while there is an increase in odds of dying for pulmonary cases ($p < 0.001$) compared with the base clinical signs in this case, which was cardiac ($p = 0.008$). Overall being mixed ($p < 0.001$) or pulmonary imply an increased odds in death compared to cardiac cases.

Table 4-6: Disease outcome correlated to the type of clinical symptoms shown (as classified in rules and definitions) with the CFR for each class of symptoms.

Clinical signs	Dead	Recovered	Total	CFR
Cardiac	28.00	37.00	65.00	0.43
Fever	4.00	21.00	25.00	0.16
Mixed	32.00	16.00	48.00	0.67
Pulmonary	88.00	1.00	89.00	0.99
Unknown	6.00	0.00	6.00	1.00

4.6 Vaccination

Data on AHS vaccination status, frequency of AHS vaccinations, time from the last AHS vaccination and order in which vaccine components where administered was compiled for all study cases. There was a total of 78 unvaccinated cases, 118 vaccinated cases, 28 cases with unknown vaccination history and 9 cases vaccinated by laypeople.

4.6.1 Vaccination correlated to disease outcome

4.6.1.1 Vaccination status correlated to disease outcome

Cases were evaluated according to vaccination status and disease outcome. With the outcome being categorized as death or recovery and vaccination status as unknown, unvaccinated, vaccinated or vaccinated by a layperson (vaccinated_lay). The proportion of cases that died or recovered in each category was evaluated and is shown in Figure 4-12. With 0.78 out of 27 cases with unknown vaccination history dying and 0.22 recovering. Of the 81 unvaccinated cases, 0.8 died and 0.2 recovered. Out of 166 vaccinated cases, 0.40 died and 0.30 recovered. There where 9 cases vaccinated by lay people (vaccinated_lay) of which 0.67 died and 0.33 recovered.

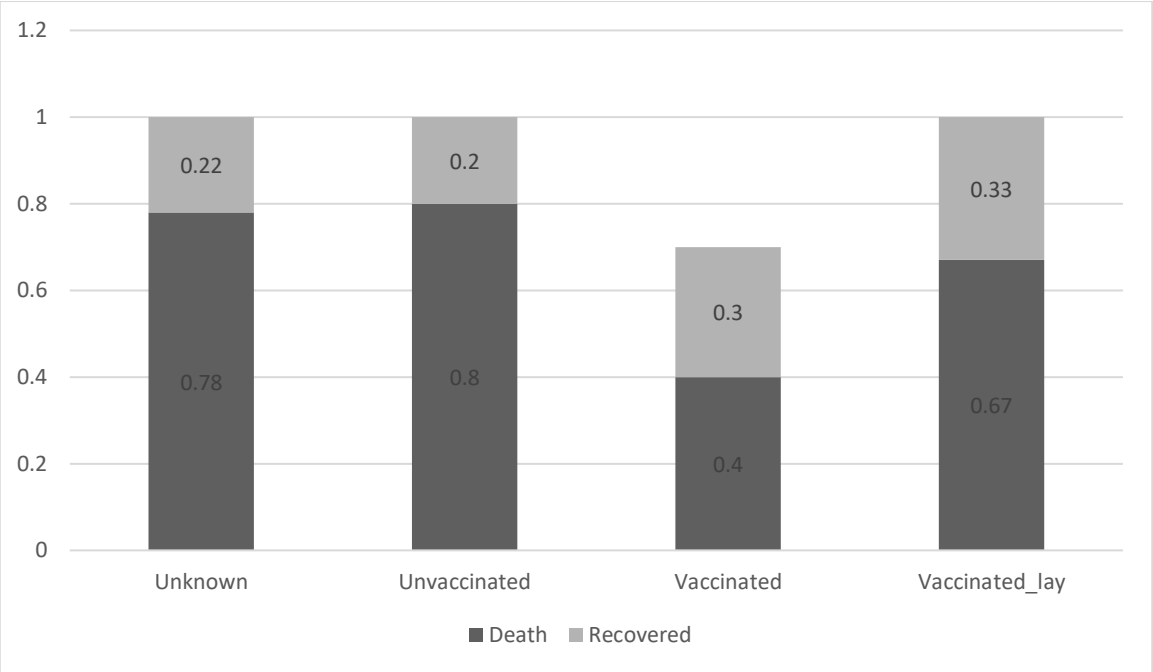

Figure 4-12: Bar chart of the vaccination categories (unknown, unvaccinated, vaccinated, vaccinated_lay) on the x-axis, with the proportion of dead and recovered for each category on the y-axis.

From here we evaluated whether vaccination plays a role in death and only the cases with a known status (vaccinated or unvaccinated) were used (total used: 197). Table 4-8 illustrates this data; with unvaccinated cases having a CFR of 0.80 and vaccinated cases a CFR of 0.57. Data analysis indicated a significant association between the vaccination status and disease outcome, with unvaccinated cases being more likely to die (p = 0.001).

Table 4-7: CFR for study cases categorized according to vaccination status (unknown, unvaccinated, vaccinated by a layperson (vaccinated_lay) and vaccinated) and a known disease outcome indicated as a proportion.

Vaccination Status	**Proportion Dead**	**Proportion Recovered**	**CFR**
Unknown	0.78	0.22	0.78
Unvaccinated	0.80	0.20	0.80
Vaccinated_lay	0.67	0.33	0.67
Vaccinated	0.40	0.30	0.57

4.6.1.3 Outcome correlated with the frequency of vaccination

To evaluate the relationship between AHS vaccination frequency and disease outcome of these cases vaccination frequency was categorized as unknown, once-off, annual, two-yearly and greater than two-yearly and illustrated in Figure 4-13. Due to the high number of unknown vaccination frequency study cases, only the proportions for each category was evaluated. Of the 76 cases with an unknown vaccination frequency, 0.82 died and 0.18 recovered. The proportions for the 11 cases with once-off vaccination was similar (dead = 0.82, recovered = 0.18). Annual vaccination (61 cases) had a death proportion of 0.34 and a recovery proportion of 0.66. There were only two cases with more than two years between AHS vaccinations and they both died.

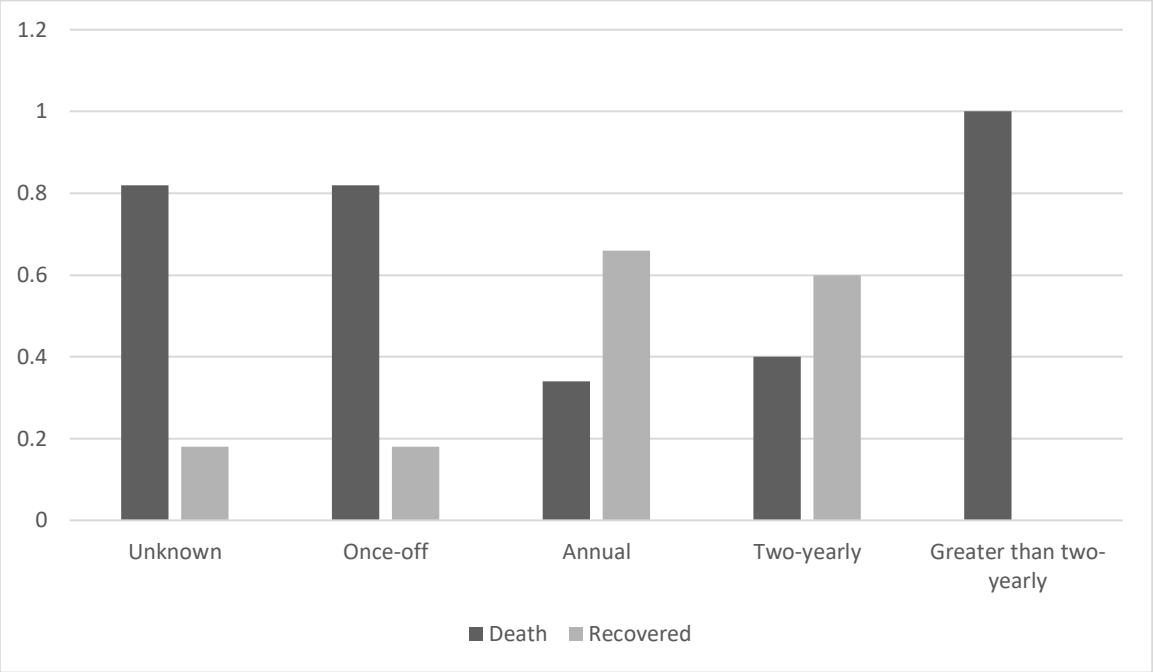

Figure 4-13: Bar chart illustrating the proportion of study cases that died and recovered for each category of vaccination status (unknown, once-off, annual, two-yearly and greater than two-yearly).

4.6.1.4 Outcome correlated to date of last AHS vaccination

As illustrated in Table 4-8 the group 0 – 100 days post-vaccination 8 out of 13 died. In group 100 – 200 days post-vaccination 13 out of 25 recovered. Out of 27 cases in the 200 – 300 days post-vaccination group 18 recovered. In the >300 days group 11 out of 19 cases recovered.

The CFR for cases in the 0 – 100 days since last vaccination bracket was the highest (0.62). Followed by cases in the 100 – 200 days group (0.48), then the 300 days to infinity group at 0.421 and lastly the 200 – 300 days since last vaccination group with a CFR of 0.33. There did not appear to be any significant association between the number of days since last AHSV vaccination and disease outcome, with p values for all categories being greater than 0.05.

Table 4-8: CFR when comparing the last AHS vaccination date categorized according to the number of days before case date vaccination was given and disease outcome.

Last vaccination group	Dead	Recovered	Total	CFR
0 - 100	8	5	13	0.62
100 - 200	12	13	25	0.48
200 - 300	9	18	27	0.33
300 - Inf	8	11	19	0.42

4.6.1.5 Vaccination order correlated to disease outcome

To evaluate whether the order in which the two components of the AHSV-LAV where administered affected disease outcome, the CFR for each category (death and recovered vs AHS1 then AHS2, AHS2 then AHS1) were determined and statistically analysed.

Table 4-9 illustrated this data, with 39 cases receiving component 1 first, out of these 17 died and 22 recovered. Twenty cases received component 2 first, of these four died and 16 recovered. There were 174 cases in which the order the vaccine components were administered in was unknown. Of these 137 died and only 37 recovered.

In cases that received the AHS1 first, the CFR was 0.436, with 17 out of a total of 39 dead. For cases that received AHS2 first, the CFR was 0.200, with only four of the 20 cases dying. Data analysis should be interpreted with caution due to the limited study cases that had a known outcome for this variable. The analysis did not indicate any significant association between the vaccination order and case outcome ($p = 0.13$).

Table 4-9: Disease outcome (dead or recovered) of study cases categorized according to the order in which vaccine components where administered (AHS1 then AHS2 or AHS2 then AHS1) or unknown vaccination status, with CFR for each category.

Vaccination order	Dead	Recovered	Total	CFR
AHS1 – AHS2	17.00	22.00	39.00	0.44
AHS2 – AHS1	4.00	16.00	20.00	0.20
Unknown	137.00	37.00	233.00	0.59

Chapter 5 Discussion

The purpose of this study was primarily to evaluate the case fatality of clinically suspected AHS cases that tested RT-qPCR positive when correlated to vaccination status.

This study is a first of its kind in South Africa. The only other study evaluating the relationship between vaccination status and death was a retrospective study done in Ethiopia and did not perform a statistical analysis of the data obtained (Aklilu *et al.*, 2014). It has been shown that vaccination protects against disease and most likely reduces the likelihood of death due to AHSV (Molini et al., 2015). However, this has not been quantified in previous studies. The samples included AHSV suspected cases for which samples were submitted to the VGL, that tested positive for AHSV with real-time RT-qPCR and had a definitive disease outcome.

It is important to note that this is not the only laboratory to which samples are submitted for AHSV diagnosis and that many cases are clinically diagnosed in the field, therefore this data set does not represent all the suspected AHS cases in South Africa.

During the outbreak in Gauteng, it became evident that there were multiple cases of sub-clinically infected horses in the same geographical area as clinically infected horses, confirming the findings by Weyer et al. (2013) (Weyer et al., 2013). There were 48 cases described as subclinical cases in this study. This represented 7.0% of the total AHS RT-qPCR positive cases observed during the study period. These subclinical cases showed no symptoms of AHSV yet tested positive with the RT-qPCR. In reflection, this would indicate that the positive case definition for this study should have included “samples from an equid epidemiologically related to a positive case” as defined by the OIE (OIE, 2014). Other samples that were excluded during the study include cases that tested positive and died, but not of clinical symptoms representing AHS. These cases were excluded on the basis that they did not comply with the recruitment criteria. It is worth noting that in some cases AHSV is an incidental finding in an animal that dies of another disease. However, this cannot always be determined clinically. Conditions that are known to occur concurrently with AHS more often include equine piroplasmosis (Theileria equi and Babesia caballi) and colic. Conditions that can be confused with AHSV include

congestive heart failure, EEV, EIA, EVA, and WNV (Mellor, 1994; Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004; Guthrie et al., 2009).

The epidemic curve of the data demonstrating the temporal distribution of study cases over the period observed showed that the tail of the 2018 season extended into July. Historic and current data would suggest this would be the same in 2019. Further study of an extended period would be beneficial as this may influence the way vaccination is approached. Notable in both the 2018 and 2019 seasons was the peak of occurrence in March. This would correspond with the South African weather patterns. Comparing weather patterns (rainfall, temperature and humidity) with AHSV outbreak dynamics would be of interest.

Seasonality and the concurrent CFR per season were evaluated. The study showed that there were increased cases between March and May as observed in previous studies (Theiler, 1921; Coetzer and Guthrie, 2004). However, the statistical analysis showed that even though the number of cases increased during that period case fatalities remained the same from month to month (p-value > 0.05 for all months of the year). The reason for the increased cases during March to May was that the weather was more favourable for increased speed in virus replication and vector replication, resulting in higher rates of infection during these warmer months (Paton, 1863; Wellby et al., 1996; Baylis et al., 1999). The constant CFR is indicative that mortality is not affected by the season. There was a significant difference between the 2018 and 2019 CFR, with 2019 having a significantly higher CFR ($p = 0.001$). The variable associated with this will warrant further investigation. It is notable that even though DAFF recommended vaccination between June and October (DAFF, 2015) based on historical disease occurrence it was noted in both 2018 and 2019 that cases already occurred in October and the tail of the outbreak extended into July. These vaccination recommendations were based on the weather patterns in the controlled area, which is not necessarily the same as other areas in the country. Further study of multiple seasons would be necessary to confirm a trend.

When interpreting the geographical distribution of cases (density) and CFR it should be noted that this most likely reflects the distribution of the equine population at risk. Similar to 2017 there were few areas with more than 20 cases observed per area during 2018. The only exception being in the Eastern Cape close to Port

Elizabeth, where a total of between 30 and 40 cases were observed for the year. This corresponds with the high CFR observed there during 2017 and 2019, illustrated in Figures 4-8 and 4-10. It raised the question of whether this was due to increased vector density due to favourable weather patterns, an increased population of susceptible equines or increased replication of the AHSV serotype due to a combination of factors. These variables would have to be further evaluated to determine the cause of the outbreak. During 2018 there were multiple areas of disease occurrence throughout the country, with notably more dispersal than in 2017. It is important to note that the whole of 2018 was observed, whereas only four months of 2017 were observed. In 2019 there was a shift of high-density cases from the Eastern Cape to Gauteng. With multiple areas having more than 10 cases in Gauteng. The 2018 outbreaks, however, had multiple locations with a low CFR (0.0 – 0.4) and most of the high CFR outbreaks in 2018 were at different locations than in 2017. It would be worth investigating whether this trend was related to change in weather patterns, the introduction of an infected case from one of the 2017 outbreak locations or other factors influencing viral vector spread. From Figure 4-10 it is evident that the clustering of high CFR observed in the map of the whole period was most likely due to the 2019 study cases. It was interesting to note that there were high CFR in the Eastern Cape again (similar and worse than 2017). With the major outbreak being in Gauteng and moving north-west towards Limpopo and Mpumalanga (which had very few cases during 2017 and 2018). A high CFR did not always correspond to a high case density though. Especially in the Eastern Cape, even though there was a very high case density, the CFR was lower when compared to other areas of the country. The outbreak in Gauteng combined a high CFR with a high case density.

AHS type 2 and AHS type 8 were the predominant outbreak types during the period evaluated. Positive cases due to AHSV type 8 were almost twice as many as those due to AHSV type 2. It would be worth studying more seasons to evaluate whether this was an area outbreak-related AHSV type prevalence or whether it was a seasonal AHSV type prevalence. Even though AHSV types 2 and 8 were predominant in the number of cases, they were not the types with the highest likelihood of death due to infection. This should be interpreted with caution due to the high number of unknown disease outcomes. A question that arose from this data

was whether protective immunity provided by AHSV-LAV to type 2 and 8 was less. From the results, it did appear that cases that were TS real-time RT-qPCR positive had a higher likelihood of death ($p = 0.028$). Due to the limited data, it was not possible to analyse whether being positive for more than one AHSV type had an increased risk of death.

The logistic regression indicated a strong association between the Ct value and outcome, with the log odds of death decreasing by 0.35 for every unit increase in Ct value. These results tie in with the observation that cases that were TS positive where more likely to die, as the cut-off Ct value for TS RT-qPCR were 33. Thus, a case that was TS positive was more likely to have a Ct value below 33, which makes it more likely to die, showing a significant correlation between laboratory outcome and case outcome. The conclusion that horses with a Ct value below 25 were more likely to die was made. The mean Ct value of recovered cases where close to 30, whereas that for cases that died were below 25. It should be kept in mind that the Ct value was affected by sample quality (e.g. autolysed samples were likely to have a higher Ct value than a fresh sample from the same horse). Blood, in general, gives a higher Ct value than an organ sample taken from the same horse at the same time. Furthermore, the sensitivity and specificity study of Guthrie et al (2013) was only done with blood so the values apply to blood samples specifically, although likely similar to organs. The association observed in this study does indicate that further investigation is warranted.

When evaluating the relationship between sex and disease outcome there was a proportional difference between females (mares and fillies) and males (stallions, colts and geldings). However, due to the limited data, these results should be interpreted with caution. It would be worth noting if the higher number of female deaths were truly a significant finding or whether there was just a larger proportion of female samples submitted due to more of these cases being epidemiologically related and in a closer spatial area (e.g. mares in a herd on a breeding farm). On the statistical analysis of the limited data, there did not appear to be a significant association between sex and disease outcome ($p = 0.14$).

The mean age of horses that died from AHS was less than ten years old, whereas the majority of those that recovered where ten years or older. There were some isolated cases of older horses with multiple vaccinations that died of AHS.

The reason for this has been speculated to be overall reduced immunity and compromise, thus succumbing to an infection that may have been overcome by a younger horse, however, this was not confirmed in this study, and further serological investigations of these cases would be needed to answer this question. It is important to note that although there are associations between age and disease outcome there may also be confounding factors, such as the number of vaccinations, which was generally related to age. Therefore, no conclusions will be made based on our data and further study in this area is recommended.

The different clinical syndromes were associated with different fatality rates. Data from this study was not sufficient to make any conclusions with regards to this factor, however, it indicates that this is a variable worth exploring and maybe a clinically relevant outcome on prognosticating cases.

Data did indicate that the proportion of cases that died that were vaccinated close to the AHS season was higher, however, this did not appear to be statistically significant with the data obtained. This could be due to vaccine reactions or young horses being vaccinated out of the recommended period to achieve a booster vaccine by 12 months. It could also be attributed to lack of time for the horses to develop an adequate immune response after vaccination before a field challenge. This may have to be more closely studied in a larger population to obtain a population-representative conclusion. From the study analysis, there did not seem to be a significant association between vaccination order and case outcome ($p = 0.13$).

Lastly, and most importantly, the study confirmed that increased case fatality rates were not due to vaccine failure, but rather due to an increase in unvaccinated horses. This was evident as unvaccinated cases and cases with a questionable vaccination history represented most cases that died (90 cases out of 115). Statistical analysis indicated a significant association between the vaccination status and disease outcome ($p=0.001$), and that unvaccinated cases were more likely to die. The CFR for unvaccinated cases was 0.80, compared to 0.57 in vaccinated cases. It was also shown that cases not vaccinated by a veterinarian (unvaccinated, unknown and vaccinated by a layperson) had a higher likelihood of being infected with AHSV. When evaluating the proportion of cases that died compared to the frequency of vaccination, the proportion of cases that died were

similar for cases of unknown vaccination frequency and those that had received a once-off vaccination. Cases that were vaccinated annually or every two years had a lower proportion of deaths. Thus, it can be postulated that a once-off vaccination does not provide sufficient immunity to protect against a field challenge. However, due to a large amount of unknown vaccination frequency data, this cannot be interpreted as statistically significant results and further study into this would be warranted.

Chapter 6 Conclusion

This study has shown a definite relationship between vaccination status and case outcome, with vaccinated horses being less likely to die. This suggests that the increased fatality was likely due to multiple variables, of which an unvaccinated status is one, and not due to vaccine failure per se. This study further showed that factors such as the number of days since the last AHS vaccination, age and sex can all influence case outcome. However, the limited data obtained did not allow for extensive analysis.

Questions still to be answered and areas that warrant further investigation include the role subclinical cases play in disease dynamics and the percentage of subclinical cases present in areas where the disease is active, changes in AHS seasonality related to weather patterns over the different areas of South Africa and prevalent AHSV types in the different South African provinces. A more controlled evaluation of the relationship between Ct value, clinical signs and disease outcome will provide clinically important information.

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Appendix 1. AEC and REC Certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V043-18

27 July 2018

Dr J Crafford
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(jannie.crafford@up.ac.za)

Dear Dr Crafford

PROJECT V043-18
A case fatality study correlating vaccination status of African Horse Sickness cases, confirmed with A RT- QPCR, and disease outcome over one African Horse Sickness season (ML Genis)

The application was discussed and approved by the Animal Ethics Committee of the University of Pretoria at the June 2018 meeting. The following legal conditions need to be applied to this study

1. When a client is invited to participate, they must be given the option to decline
2. Further, once the owners in the database consent to participation, it must be indicted to them if their identities will be protected and under which circumstances

If you have any questions, please feel free to contact the committee.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee
Coy ML Genis

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**A case fatality study correlating vaccination status of African Horse Sickness cases, confirmed with a RT-QPCR, and disease outcome over one African Horse Sickness season**
PROJECT NUMBER	**REC052-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Margaret L Genis**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Jannie Crafford**

APPROVED	Date 25 **September 2018**
CHAIRMAN: UP Research Ethics Committee	Signature

Appendix 2. Google forms questionnaire

African Horse Sickness Case History

Dear Horse owner/ veterinarian as you are aware the AHS testing is done at the ERC for free on the understanding that the sample results are used for our research. For us to accurately use the samples for research purposes we need an accurate back ground for the positive cases. We would really appreciate the form below be completed and returned to us. If you are unable to complete the online version for any reason please contact Dr. Louie Genis at louie.genis@gmail.com to get a form for manual completion.

* Required

1. **Please state your horse's name, owner name and horse case number (E number)** *

_____

2. **Age of horse (specify if other)** *
Mark only one oval.

- ◯ 0 - 1 years old
- ◯ 1 - 2 years old
- ◯ 2 - 3 years old
- ◯ 3 - 4 years old
- ◯ 4 - 5 years old
- ◯ 5 - 6 years old
- ◯ 6 - 7 years old
- ◯ 7 - 8 years old
- ◯ Other: _____

3. **Horse gender** *
Mark only one oval.

- ◯ Mare
- ◯ Stallion

4. **Date of the AHS episode** *

_____
Example: December 15, 2012

5. **Outcome of the AHS episode** *
Mark only one oval.

- ◯ Dead
- ◯ Recovered

6. **Specific clinical signs during illness (specify if other)** *
Check all that apply.

- [ ] Swelling of the supraorbital fossa
- [ ] Swelling of the eyelids
- [ ] Swelling of the facial tissues
- [ ] Swelling of the neck
- [ ] Swelling of the thorax
- [ ] Swelling of the brisket and shoulders
- [ ] Dyspnoea, difficulty breathing
- [ ] Spasmodic coughing
- [ ] Dilated nostrils with frothy fluid oozing out
- [ ] Fever
- [ ] Redness of conjunctivae (inside eyelids)
- [ ] Other: _____

7. **Complications during AHS episode**

_____

8. **Geographical location of horse during AHS episode** *

_____

9. **Last AHS vaccination before AHS episode** *

_____
Example: December 15, 2012

10. **Batch number of the last AHS vaccination** *

_____

11. **Did your horse have a reaction to the last AHS vaccination**
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Other: _____

12. **Order in which last vaccination was given** *
Mark only one oval.

- ◯ AHS combination 1 first - AHS combination 2 second
- ◯ AHS combination 2 first - AHS combintaion 1 second
- ◯ Other: _____

13. **Frequency of AHS vaccinations throughout lifetime** *
Mark only one oval.

- ◯ Yearly
- ◯ Every two years
- ◯ Other: _____

14. **Type of vaccination used (specify if other)** *
Mark only one oval.

- ◯ OBP
- ◯ DCA
- ◯ Other: _____

15. **Last movement of the horse prior to the AHS episode (location from and location to)** *

_____

16. **Date of last movement before AHS episode** *

_____

17. **Additional comments**

_____

_____

_____

_____

_____

18. **Please confirm that you give consent for the information provided to be used in a research study, with the understanding that no personal information will be published**
Check all that apply.

- ☐ Confirm

Appendix 3. Case classification according to the rules as indicated in this table.

Rule	Definition
1	In the questionnaire, any cases which had received no vaccination was completed as [date: 1990-01-01] and [batch: 888].
3	Age was classified numerically in years. When unknown it was completed as (0).
4	Vaccination status was assessed as unvaccinated (0)/vaccinated (1)/unknown (2)/uncertain (3). Uncertain included all cases in which the horse was vaccinated by a layperson or vaccination status was confirmed by word of mouth to the private veterinarian. Cases in which no response to the questionnaire was received was classified as unknown. If vaccinated the frequency of vaccination was classified numerically (single dose (0)/yearly (1)/every two years (2)/ more than two years (3)/ unknown (4)).
5	In cases where multiple samples for one horse was submitted the sample with the lowest Ct value was used and the sample type (e.g. blood EDTA/ organ/ fluid/ serum) recorded for all samples.
6	Reference of samples was done using the report reference number (E) and the individual sample number (EP) as some reports contained results for multiple horses.
7	The outcome was classified as recovered (1), dead or euthanized due to disease (0) and unknown (2). Cases that died or were euthanized due to other causes or reasons during the study was considered as recovered from AHS.
8	Concurrent infection with Equine Encephalosis Virus (EEV) was noted as yes (1) or no (0).
9	Clinical signs were classified as pulmonary (0), cardiac (1), mixed (2), fever (3) and unknown (4).
10	Classification of clinical signs into AHS forms was based on previous studies (Theiler, 1921; Coetzer and Guthrie, 2004). The following was included as possible clinical signs in the questionnaire, with the option for the veterinarian or owner to add other signs: swelling of the supraorbital fossae (1), swelling of the eyelids (2), swelling of the facial tissues (3), swelling of the neck (4), swelling of the thorax (5), swelling of the brisket and shoulders (6), dyspnoea or difficulty breathing (7), spasmodic coughing (8), dilated nostrils with frothy fluid oozing out (9), pyrexia (10) and redness of the conjunctiva(11). Depending on the clinical signs noted as present these were categorized as following into the different AHS forms: cardiac form (1,2,3,4,5,6,10,11), pulmonary form (7,8,9,10,11), mixed form (1-11) and fever form (10,11).
11	The vaccine used was classified as unregistered vaccines (0), OBP (1) and unknown (3). Thus, disregarding all unregistered vaccines as unvaccinated.
12	Gender was classified as gelding (0), filly/mare (1), colt/stallion (2) and unknown (3).

13	Injection order (AHS1 first or second) was classified as AHS1 then AHS2 (1), AHS2 then AHS1 (2) or unknown (3).
14	In cases where a case date was unknown a date three days before the submission date was used as a proxy.
15	In cases where the date of death was unknown, but death confirmed, the sample date was used as a proxy for date of death.