ADDIS ABABA UNIVERSITY
SCHOOL OF VETERINARY MEDICINE

OUTBREAK INVESTIGATION AND MOLECULAR EPIDEMIOLOGY OF AFRICAN HORSE SICKNESS VIRUS CIRCULATING IN CENTRAL, EASTERN AND SOUTHERN PARTS OF ETHIOPIA

By

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ABBREVIATIONS

AGID	Agar Gel Immunodiffusion
AHS	African horse sickness
AHSV	African horse sickness virus
AHSV-1, 2, 3, …, 7	African horse sickness virus serotype 1, 2, 3, …, 7
ALV	Attenuated Live Virus
APHRD	Animal and Plant Health Regulatory Directorate
AU-IBAR	African Union – Interafrican Bureau for Animal Resources
BHK	Baby Hamster Kidney
BP	Blocking Percentage
cDNA	Complementary DNA
C-ELISA	Competitive enzyme linked immunosorbent assay
CF	Complement Fixation
CI	Confidence Interval
CPE	Cytopathic effect
CSA	Central Statistical Agency
ct	Cycle threshold
Defra	Development for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded Ribonucleic acid
EDTA	Ethylene Diamine Tetra-acetic Acid
EEV	Equine encephalosisVirus
ELISA	Enzyme linked immunosorbent assay
ENSO	El Niño/Southern Oscillation
ETH	Ethiopia
EVA	Equine viral arteritis
FLAC	Full-Length Amplification of cDNAs
GMEM	Glasgow Minimum Essential Medium
IAH	Institute of Animal Health
I-ELISA	Indirect Enzyme Linked Immunosorbent Assay
IFA	Indirect fluorescent antibody assay
KC	Culicoides cell line

Mab	Monoclonal Antibodies
MoA	Ministry of Agriculture
MS	Monkey Stable
MVA	Modified Vaccinia Ankara
NS	Non Structural
NS-1, 2, 3, 3a	Non structural protein- 1, 2, 3, 3a
Nt.	nucleotide
NVI	National Veterinary Institute
OD	Optical Density
OIE	World Organization for Animal Health
PA	Peasant Association
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Ref	reference
RNA	Ribonucleic acid
RSArrah	IAH dsRNA virus collection number
RT-PCR	Reverse transcriptase - Polymerase Chain Reaction
S10	Segment 10
Seg.	Segment
SNNPRS	Southern Nation Nationalities and People's Regional State
SPANA	Society for the Protection of Animals Abroad
TAE	Tris base, glacial acetic acid, EDTA
UK	United Kingdom
UV	Ultra Violet
VN	Virus Neutralization
VP	Viral Protein
VP-1, 2, 3, ...	Viral Protein 1, 2, 3, ...
WAHID	World Animal Health Information Database

ABSTRACT

This study was conducted from September 2009 to December 2010 in central, eastern and western parts of Ethiopia to investigate and identify circulating serotypes of African horse sickness virus (AHSV). A total of ten outbreaks were investigated in the study period. Samples collected for virus isolation and identification were whole blood in ethylene diamine tetra-acetic acid (EDTA) from diseased horses and mules, and tissues from freshly dead horses. AHSV isolation on Vero cell line and detection of AHSV genomes using conventional RT-PCR were archived in the National Veterinary Institute (NVI) molecular laboratory, Debre Zeit, Ethiopia. Samples were sent for further viral isolation, identification, sequencing and phylogenetic analyses in the Non-vesicular Laboratories of Institute of Animal Health (IAH), Pirbright, United Kingdom (UK). A total of 336 sera were collected from apparently healthy equids that were either unvaccinated or have vaccination history of before three years in Cheta and Tikur Inchini districts and subjected to a serological test using a commercialized blocking enzyme linked immunosorbent assay (ELISA) kit. A questionnaire survey was administered to 178 equine owners and retrospective data of outbreaks for three years obtained from Animal and Plant Health Regulatory Directorate (APHRD) in the Ministry of Agriculture (MoA) were analysed to see the national picture and seasonality of outbreaks. A total of 48 horses died of African horse sickness (AHS) during the outbreaks. Ada'a district was the major outbreak site where the outbreak involved a number of towns and villages. A total of 81 horses and 3 mules were affected and 46 (54.8%) of them demonstrated cardiac form of the disease while the rest 30 (35.7%), 6 (7.1%), 2 (2.4%) were affected by horse sickness, pulmonary and mixed forms of AHS, respectively. Multiple serotypes of AHSVs were identified from the submitted samples by quantitative real-time RT-PCR and sequencing. AHSV-4, AHSV-6, AHSV-8 and AHSV-9 serotypes were identified. Identification of AHSV-4, AHSV-6 and AHSV-8 are reported for the first time in Ethiopia. AHSV-9 was the dominant serotype circulating in the outbreak areas. Serotypes identified from the outbreaks were phylogenetically compared with the reference strains in IAH, Pirbright, UK. A total of 147 (43.8%) of the sampled equids were found seropositive for antibodies against AHSV. The respective seropositivity of equids in Cheta and Tikur Inchini districts were 49.4% and 37.8% (p= 0.032). Statistically significant difference was not observed between risk factors of species, age and sex ($p > 0.05$). From the total 178 respondents 71 (40%) of them indicated that their indigenous knowledge on the disease, its

transmission and control measures is unsatisfactory while the rest (60%) were aware of the disease. Local names used for AHS were 'Derbetu' or 'Dibe Ferda' in Tikur Inchini and 'Horyo' in Cheta. Locally known equine flies were 'Bombisa' in Tikur Inchini and 'Gemo' and 'Yubo' in Cheta. A total of 482 outbreaks were recorded retrospectively from Oromia (80.1%), Amhara (10.6%), Southern Nation Nationalities and People's Regional State (SNNPRS) (7.7%) and the rest from Tigray, Dire Dawa and Afar regional states. AHS outbreaks were more frequently observed from September to December and the highest number of outbreaks was reported in 2008. Generally, the identification of multiple serotypes in the current study indicated increased risk and devastating outbreaks of AHS is a possibility. The production of polyvalent vaccine which confers protection against the identified serotypes and prophylactic vaccination of horses and mules are recommended.

Key words: African horse sickness virus, equine, Ethiopia, outbreak, RT-PCR, serotype.

1 INTRODUCTION

Ethiopia is believed to have the largest livestock population in Africa (CSA, 2011). Livestock contribute about 30–35% of agricultural gross domestic product of the country and more than 85% of farm cash income (Degefe and Nega, 2000). Equines are of great significance in the agricultural development particularly in the farming and transport systems of the country and are unlikely to be replaced in the foreseeable future (Fielding and Pearson, 1991; Demelash and Moges, 2006). They provide both economical and social benefits to the livelihoods of equine owning communities of the country (Admasu and Shiferaw, 2011 - unpublished). As per results from the livestock survey in the rural sedentary areas of Ethiopia, there are about 2.03 million horses, 6.21 million donkeys and 0.38 million mules (CSA, 2011). Despite their importance, equids are the most neglected and affected animals by a series of health and welfare problems (Biffa and Woldemeskel, 2006; Shelima *et al.*, 2007). Among infectious diseases which cause severe socioeconomic losses to the equine owning population and the national economy in general are African horse sickness (AHS) and epizootic lymphangitis (Leforban *et al.*, 1983; Zeleke *et al.*, 2005; Aklilu and Abebaw, 2010; Admasu and Shiferaw, 2011).

AHS is an insect-borne, infectious viral disease with extremely high mortality in horses and mules. The disease is manifested by pyrexia, inappetence and edema of subcutaneous, intermuscular and lung tissues; transudation into body cavities and haemorrhages, particularly of the serosal surfaces (Kazeem *et al.*, 2008; OIE, 2008). The disease is caused by African horse virus (AHSV), an *Orbivirus* in the *Reoviridae* family. AHSV has nine serologically distinct serotypes (Radostitis *et al.*, 2007). AHSV affects all species of *Equidae* family (horses, mules, donkeys and zebras) and primarily transmitted by a biting midges belonging to the *Culicoides* genus (Mellor, 1994a). Horses are the most susceptible to AHS with a mortality rate of 50–95% followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections (Guthrie, 2008; OIE, 2008).

AHS is confined to sub-Saharan Africa, although periodic epizootics have caused severe outbreaks of the disease outside enzootic regions. Serum neutralizing antibodies to all nine strains of AHSV were found in Kenya (Binepal *et al.*, 1992; Davies *et al.*, 1993). The

circulation of these multiple serotypes in neighboring countries has a big risk in transmission of new serotypes into Ethiopia. Increase in the number of serotypes identified from endemic areas was reported (MacLachlan and Guthrie, 2010). AHSV serotype 2 (AHSV-2) was isolated for the first time in the northern hemisphere from a widespread epidemic of AHS in Nigeria (Fasina *et al.*, 2008).

Ethiopian equids have long been affected by AHS and many outbreaks of AHS reported previously (Leforban *et al.*, 1983; Zeleke *et al.*, 2005). An epidemiological survey conducted between 1977 and 1981 indicated that majority of the previous outbreaks were due to serotype 9. However, serotype 7 has also been reported from one site (Leforban *et al.*, 1983). National Veterinary Institute (NVI), Debre Ziet, Ethiopia has been producing attenuated monovalent freeze dried AHS vaccine against this dominant circulating serotype of the virus (AHSV-9). The vaccine has been successful and confers equids protection from outbreaks until recently where reports indicated that some vaccinated horses got infected and died of the disease. Zeleke and co-authors reported that they identified AHSV-9 and AHSV-6 from outbreak which occurred between 2002 and 2003 in southern, western and central Ethiopia. Samples were sent to and viruses identified at the OIE's reference laboratory, Onderstpoort Veterinary Institute, South Africa. They stated that identification of AHSV-6 was a first report to the country (Zeleke *et al.*, 2005). However, the report on AHSV-6 was found to be a mistake as the submitted sample was a known serotype of laboratory vaccine strain isolate of AHSV-6 which was submitted for further confirmation and it was not an outbreak collected sample (Personal communication with Dr. Esayas Gelaye).

An outbreak report to the OIE in 2008 indicated that a total of 15 outbreaks occurred in Keficho Shekicho and Bench Maji zones of SNNPRS and Illubabor zone of Oromia and killed 2185 equids (Sabirovic *et al.*, 2008; WAHID Interface, 2008). Samples from these outbreaks were submitted to the same laboratory, Onderstpoort Veterinary Institute, South Africa and AHSV serotype 2 was identified for the first time in the country. This report has also indicated that equids vaccinated against serotype 9 were affected by the outbreak. Another report to the AU-IBAR in 2008 indicated that Ethiopia had the highest number of cases (1636) in 319 outbreaks and lost 748 horses due to AHS. The country has vaccinated 306,454 horses to stop the progress of the disease outbreaks and as prophylactic vaccination. However, the majority of these (90.2%) were vaccinations following outbreaks of the disease (Pan African Animal Health Yearbook, 2008).

A sero-epidemiological survey conducted using competitive ELISA (C-ELISA) in different agro-ecological zones of central Ethiopia indicated a prevalence rate of 10.4%, 29.7% and 10.3% from horses, donkeys and mules, respectively (Kassa, 2006). Seropositivity of 34% has been reported previously from a country wide survey using compliment fixation test and sero-neutralization technique on Vero cell line (Leforban *et al.*, 1983). The same report revealed that antibodies were found against all immunological serotypes of AHSV in the country. A recent seroprevalence study in Jimma zone indicated 51.1% in donkeys, 30.2% in mules and 28.3% in horses and an overall reported seroprevalence of 32.5% (Bitew *et al.*, 2011).

The wide spread occurrence of outbreaks and identification of new serotypes necessitated further investigation of outbreaks for possible isolation and identification of other serotypes of AHSV. For the NVI to shift into bi/polyvalent vaccine production as it is practiced in most countries where the disease is enzootic and multiple serotypes circulate, isolation and identification of dominant serotypes is essential.

Therefore, this study was conducted with the following major objectives:

- To investigate occurrence and nature of active outbreaks of AHS in central, eastern and southern parts of Ethiopia,
- To isolate and identify circulating serotypes of AHSV from clinically sick equines.

2 LITERATURE REVIEW

2.1 The disease

AHS is an infectious but non-contagious arthopodborne viral disease of *Equidae*. The clinical signs and lesions result from selective increased vascular permeability and are characterized by an impairment of the respiratory and circulatory systems (Brown and Torres, 2008; OIE, 2008)

AHS is an OIE listed disease and has been classified as a notifiable disease worldwide due to its socio-economic consequence and ability to expand rapidly and without apparent warning out of its endemic areas (Mellor and Hamblin, 2004; OIE, 2008; von Teichman and Smit, 2008).

2.2 Etiology

2.2.1 The AHSV virus

AHS is caused by African horse sickness virus (AHSV) which is a member of the genus *Orbivirus* in the family *Reoviridae* (Radostitis *et al.*, 2007). The genus *Orbivirus* includes 19 species established principally on antigenic (serologic) grounds but many of these placements have been supported by molecular analysis. The genus possesses 149 serotypes and 9 tentative or unassigned isolates (Clashier and Mertens, 1998). AHSV is morphologically similar to other orbiviruses such as bluetongue virus, epizootic haemorrhagic disease virus and equine encephalosis virus (EEV) which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges (Roy and Sutton, 1998; OIE, 2008).

AHSV is an unenveloped particle of about 70 nm in diameter and is made up of a two layered icosahedral capsid, which is composed of 32 capsomeres (Coetzer and Erasmus, 1994; OIE, 2008).

2.2.2 AHSV structural proteins

AHSV has a genome consisting of 10 segments of double-stranded RNA, which encode seven structural proteins (VP1–VP7) and four nonstructural proteins (NS1, NS2, NS3 and NS3a) (Grubman and Lewis, 1992). Most of the structural proteins have been completely sequenced for AHSV serotypes 4, 6 and 9 (Williams *et al.*, 1998; OIE, 2008). Most of the genome segments coded for only one protein (Grubman and Lewis, 1992). The structural outer coat proteins VP2 and VP5 are encoded by genome segments 2 and 5, respectively. Proteins VP3 and VP7 are the major inner capsid proteins which are highly conserved among the nine AHSV serotypes (Martin *et al.*, 1998). VP7 (encoded by genome segment 7) particularly is the major serogroup specific antigen common to all nine AHSV serotypes (Zientara *et al.*, 1998b).

Complete set of full-length VP2 gene clones and sequence data for all the nine AHSV serotypes have been generated (Vreede and Huismans, 1998; Williams *et al.*, 1998; Potgieter *et al.*, 2003). Multiple alignments of the VP2 protein sequences showed homology between all nine AHSV serotypes varied between 47.6% and 71.4%, indicating that VP2 is the most variable AHSV protein and is the principal responsible for AHSV serotypes and together with VP5 for virus neutralization (VN) activity (Martínez-Torrecuadrada *et al.*, 1999; Potgieter, 2003). The S10 gene of AHSV is also a variable gene and well suited for use in characterizing AHSV strains (Quan *et al.*, 2010).

The remaining proteins (VP1, VP3, VP4 and VP6) are components of the virus subcore and appear to be largely concerned with the fundamental organization, transcription, capping replication and packaging of the viral RNAs (Wilson *et al.*, 2009). Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins (Mertens *et al.*, 2005) (Figure1).

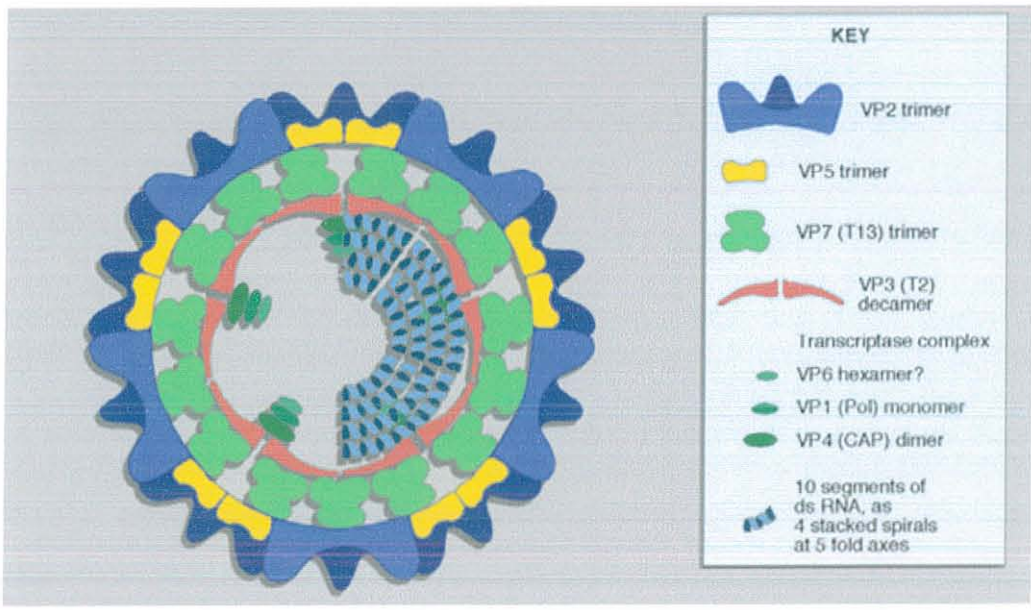


Figure 1. Diagram of an orbivirus structure

Source: Mertens *et al.*, (2005).

2.2.3 AHSV non structural proteins

The AHSV nonstructural proteins can be detected in the cytoplasm and membranes of infected cells (Stoltz *et al.*, 1996). NS1 forms tubular structures within the cell cytoplasm, while NS2 is a major component of the granular viral inclusion bodies that form within the cytoplasm of infected cells (Wilson *et al.*, 2009). AHSV genome segment 10 encodes the nonstructural proteins NS3 and NS3a. NS3 was found to vary as much as 36.3% across serotypes and 27.6% within serotypes indicating that it is the second most variable AHSV protein (van Niekerk *et al.*, 2001; Meiring *et al.*, 2009). It has been suggested that sequencing of the comparatively small NS3 gene in outbreaks of AHS may provide significant epidemiological information as to the origin of a virus involved in an outbreak. These NS proteins are believed to facilitate the processes of virus replication and cell exit through modifying membrane permeability. NS3 has many of the characteristics of lytic viral proteins that play a central role in viral pathogenesis (van Niekerk *et al.*, 2001).

2.2.4 AHSV life cycle

Variations in VP1, VP2, VP5, VP7 and nonstructural proteins NS1 and NS3 determine the range of host cell types which the virus is able to infect, thereby influencing the sites of virus replication, tissues in which the virus is concentrated, environmental conditions required for

replication and transmission, the degree of pathogenesis resulting from infection, and the identity of the virus from the perspective of the vertebrate immune system. Adaptation of the virus for vectorial transmission is likely to be largely restricted to the genome segments coding for these proteins (Wilson *et al.*, 2009). A recent finding indicated that AHSV VP5 is a membrane-destabilizing protein (Stassen *et al.*, 2011). NS3 acts in the AHSV life cycle as a viroporin by altering membrane permeability and facilitating virus release from infected cells. VP5 and NS3 are not the sole determinant of the cytopathic properties of the virus, it is likely that a number of viral and host factors play a role (Meiring *et al.*, 2009; Tracy *et al.*, 2009). VP2 plays a role in mediation of cell attachment and penetration during the early stages of infection (Martínez-Torrecuadrada *et al.*, 1999; Potgieter, 2003).

2.2.5 Serotypes of AHSV

Nine antigenically distinct serotypes of AHSV (AHSV-1, -2, -3, -4, -5, -6, -7, -8 and -9) with differing virulence have been identified the last being in 1960 (Coetzer and Erasmus, 1994). This suggests that, despite its segmented genome, the virus can be regarded as genetically stable and new serotypes do not readily develop. Some cross-reaction has been observed between AHSV serotypes 1 and 2; 3 and 7; 5 and 8; and 6 and 9, although there were no intratypic variations and cross-reactions with other known orbiviruses (OIE, 2008; von Teichman *et al.*, 2010). Phylogenetic analysis of the nine AHSV VP2s grouped together VP2s of serotypes that show serological cross reaction (Potgieter *et al.*, 2003) (Figure 2).

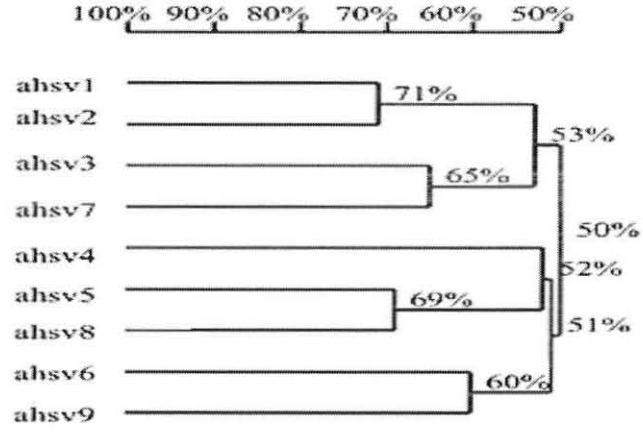


Figure 2. Homology trees compiled of the VP2 genes of all nine serotypes of AHSV Bootstrap values are indicated. Source: Potgieter *et al.* (2003).

2.2.6 Physio-chemical characteristics

The physio-chemical characteristics of AHSV are typical for the genus *Orbivirus*. The virus is acid sensitive, being readily inactivated at pH values below 6.0 but remains relatively stable at more alkaline pH values (7.0-8.5) (Mellor and Hamblin, 2004). Due to pH fluctuations, the virus is rapidly destroyed in carcasses that have undergone rigor mortis but putrid blood may remain infective for more than 2 years (Defra, 2008; OIE, 2009). It is resistant to lipid solvents and relatively heat resistant, particularly in the presence of protein. Infectivity is remarkably stable at 4^0C, particularly in the presence of stabilizers such as serum and OCG (5 g sodium oxalate, 5 g carbolic, 500 ml glycerine and 500 ml distilled water) in which it can remain infective for more than 20 years (Erasmus, 1998). The virus can be stored for more than 6 months at 4^0C in saline with 10% serum but it is labile between -20^0C and -30^0C (Coetzer and Erasmus, 1994; OIE, 2009). Vaccine strains survive well in lyophilised state at 4^0C. It is sensitive to different kinds of disinfectants at minimal concentrations (Shirai *et al.*, 2000; Brown and Torres, 2008; OIE, 2009).

2.3 Epidemiology

Epidemiology of AHS is highly dependent on the interaction between the infected host, a competent vector and susceptible non-infected equidae under favorable environmental conditions (Mellor, 1994a; Venter *et al.*, 2006; Defra, 2008). Epidemics of AHS tend to occur at cyclic intervals and are often seasonal because of the seasonal variations in the number of *Culicoides* spp. present. Epidemics are usually associated with drought followed by heavy rain. These weather patterns are more common during the warm phase of the *El Nino* Southern Oscillation (ENSO). Authors suggested that during such times, breeding sites of the insect vector may be altered or during drought, the virus reservoir (zebra) may congregate near the few remaining sources of water where they are in contact with and infect more vectors (Baylis *et al.*, 1999; OIE, 2009).

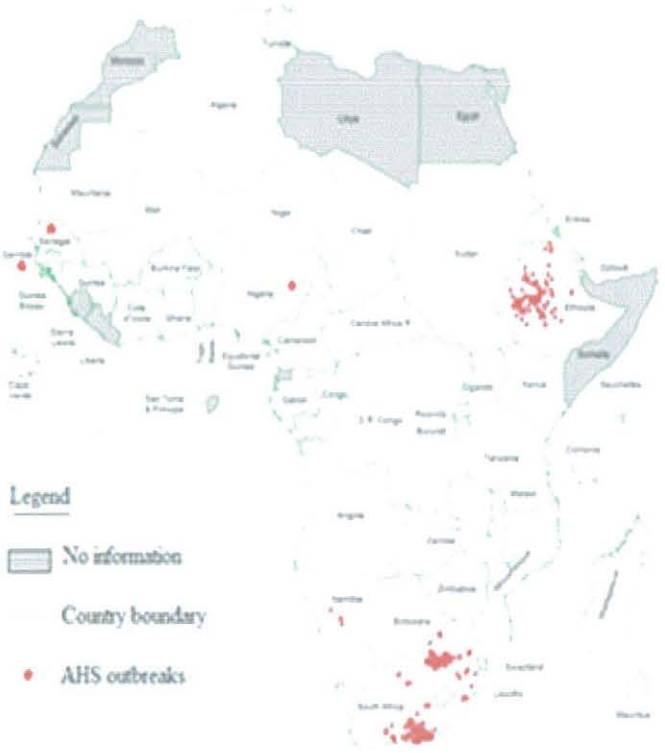
Midges are most active just after sunset and at about sunrise and their activity is favored by temperatures between 12.5^0C and 29^0C (Coetzer and Erasmus, 1994; Wittmann and Baylis, 2000; van der Rijt *et al.*, 2008). It was suggested that several cool and cold episodes are necessary to kill all or most vectors (Mellor, 1994a). Local factors including topography

influence the distribution of midges within their overall range and therefore the disease has a geographical distribution. Areas most severely affected are low lying and swampy (Baylis *et al.*, 1997; Venter *et al.*, 2006). Some authors speculate that global warming could increase the risk for spread of AHS as *Culicoides imicola* has made incursions into North Africa and southern Europe (Wittmann and Baylis, 2000; Zell *et al.*, 2008).

2.3.1 Geographic distribution

AHS is an ancient African disease. It is enzootic in tropical and sub-tropical areas of Africa south of the Sahara occupying between eastern and central Africa and spreads regularly to northern South Africa (Coetzer and Erasmus, 1994; Guthrie, 2008). The Sahara desert is supposed to provide a geographical barrier which has prevented the virus from establishing itself permanently in northern Africa or beyond (Mellor and Hamblin, 2004; Brown and Torres, 2008).

Occasional outbreaks of the disease were seen in northern African countries. In 1965, AHS appeared first in Morocco then spreading into Algeria and Tunisia. The disease has also occurred outside Africa on a few occasions, the most notable of which were outbreaks in Saudi Arabia, Syria, Lebanon, Jordan, Iraq, Turkey, Cyprus, Iran, Afghanistan, Pakistan, India and the Yemen (Coetzer and Erasmus, 1994; Mellor and Hamblin, 2004). The disease was reported to end in Asia after massive vaccination campaign and the death of over 300,000 equids. AHS also occurred in Spain in 1966 and recurred in 1987 and in Portugal in 1989. The disease had spread throughout Spain and Portugal in 1990 but was eliminated a year later (Mellor, 1994a; Mellor and Hamblin, 2004). Reports from member African countries to AU-IBAR indicated the main foci of AHS are western, eastern and southern parts of Africa (Pan African Animal Health Yearbook, 2008) (Figure 3).



Country	Number of outbreaks in 2008
South Africa	401
Ethiopia	309
Senegal	3
Gambia	2
Namibia	3
Nigeria	1
Total	729

Figure 3. Map to show the spatial distribution of AHS outbreaks in Africa in 2008. Source: Pan African Animal Health Yearbook (2008)

2.3.2 Susceptible host range

AHS causes clinical disease in horses, donkeys, mules and dogs and only infection to zebras. From equidae family, the more severe disease occurs in horses, with mules and donkeys and zebras showing lesser degrees of susceptibility in that order. Other hosts species including elephants, camels, cattle, sheep, goats and predatory carnivores may demonstrate antibodies to infection (Binepal *et al.*, 1992; Clashier and Mertens, 1998).

Mortality rate in horses ranges from 50 to 95%, followed by mules with mortality around 50% (OIE, 2008). In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. African donkeys (*Equus asinus africanus*) are reported to be resistant to experimental infections and showed no signs of AHS even when immunosupppressed (Fassi-Fihri *et al.*, 1998). Zebras are markedly resistant with no clinical signs, except fever. Dogs may die from peracute infection after eating infected horse meat (Salama *et al.*, 1981; OIE, 2009).

Equids that are vaccinated or recovered from the disease develop a solid humoral immunity against the homologous serotype and a partial immunity against heterologous serotypes at least for a year (Burrage and Laegreid, 1994). However, previous exposure of one serotype may not prevent initiation of infection with a second but the presence of sufficiently high levels of antibodies to the first unrelated serotype may prevent or reduce the level of viremia with the second type thereby precluding the animal from playing any further part in the epidemiology of the disease (Hamblin *et al.*, 1998). Foals from immune dams derive passive immunity from the colostrum and are immune for 3 to 6 months of age (Radostits *et al.*, 2007; Brown and Torres, 2008).

2.3.3 The role of reservoirs in the epidemiology of AHS

AHSV is maintained in endemic areas as a result of a continuous cycle between insect vectors and wild or domestic equines or other wild reservoir hosts. Zebras, which are often asymptomatic, are thought to be the natural reservoir hosts in most regions of Africa (Defra, 2008). The first cases of AHS occurred in the horses of hunters who entered zebra territory in South Africa. Nowadays, most of the free-living zebras have been confined to the northeastern part of South Africa which are now the only areas in the country where AHS is endemic. The prevalence of AHSV antibodies in Zebra and of AHS in unprotected horses is similar. The minimum size of zebra population necessary to maintain a focus of AHSV is unknown (Bernard, 1998). It was suggested that dogs do not play a significant role in the maintenance or spread of AHSV as viraemia is thought to be low level and transient and vectors are rarely attracted to them (Braverman and Chizov-Ginzburg, 1996). Antibody to AHSV has been found in sera collected from camels, African elephants and black and white rhinoceros, but their role in epizootiology is unlikely to be significant (Mellor and Hamblin, 2004; Brown and Torres, 2008). Similarly, complement fixing antibodies were found in sheep, goats, dogs, camels, buffaloes and cattle in an Egyptian survey but they have no reported reservoir activity (Awad *et al.*, 1981).

Because of the high mortality rate frequently recorded in horses and occasionally in mules, they are regarded as accidental or indicator hosts (Erasmus, 1998; Mellor and Hamblin, 2004; Defra, 2008). In addition, failure of AHSV to become established outside tropical and sub-tropical regions of Africa, where zebra principally reside, indicated that horses, mules and donkeys are not long-term reservoirs of AHSV and they do not remain as carriers after

recovery (Hamblin *et al.*, 1998). Therefore, they are not involved in the permanent persistence of the virus. However, AHSV-9 survives perfectly well in West Africa where zebra no longer exist (Wilson *et al.*, 2009).

2.3.4 Mode of transmission

AHSV is transmitted by the bite of blood-sucking insects including midges (*Culicoides* spp.), ticks (*Hyalomma dromedarii and* the brown dog tick, *Rhipicephalus sanguineus*), mosquitoes and large biting flies in the genera *Stomoxys* and *Tabanus* (Mellor, 1994a; Radostits *et al.*, 2007; Wilson *et al.*, 2009). Midges are by far the most important vector in the spread of the spontaneous disease. *C. imicola* is the principal vector responsible for the transmission of AHSV within its enzootic area and possibly during epizootics (Cetre-Sossah *et al.*, 2004). Other species of *Culicoides* including *C. bolitinos, C. varipenis*, *C. pulicaris*, and *C. obsoletus* are probably competent vectors. The role of other vectors in the epizootiology of the disease is regarded as absolutely minimal compared with that played by the *Culicoides* species (Erasmus, 1998; Meiswinkel and Paweska, 2003).

In areas in which AHS is enzootic, the virus persists by cycling between mammalian host and vectors year round. Midges are infected with AHSV but transovarian transmission of infection between generations of midges does not occur (Radostits *et al.*, 2007). Mosquitoes have also been implicated as biological vectors. Transmission by other vectors is thought to be mechanical (Brown and Torres, 2008). Analysis of field observations on the progression of outbreaks indicates that midges disperse only a few kilometers from their breeding sites, but it has been postulated that they can be borne for longer distances up to 700 km on air currents over water and 150 km overland. Movement of infected equidae was reported to be a means for farther long-distance jumps of infection (Radostits *et al.*, 2007; OIE, 2009). The outbreak in Spain in 1987 was apparently caused by the introduction of a number of sub-clinically infected zebra from Namibia into a safari park (Sánchez-Vizcaíno, 2006).

Equidae in the febrile phase of the disease are most likely to serve as a source of the virus for transmission to other susceptible equidae. The virus is commonly found in viscera and blood of infected equids. It can also be found in semen, urine and nearly all secretions and body fluids during viraemia, but no studies have documented transmission. Viraemia usually lasts 4–8 days in horses but may extend up to 21 days and in zebras it may last up to 40 days (OIE,

2009). In an experimental inoculation of AHSV to donkeys and mules, viral RNA was detected consistently up to day 47 and intermittently thereafter (el Hasnaoui *et al.*, 1998b). However, recovered animals do not remain carriers of the virus (OIE, 2009). Mechanical transmission of the virus on contaminated surgical instruments and needles should be considered a possibility (Radostits *et al.*, 2007).

2.3.5 Molecular epidemiology

All 9 serotypes of AHSV occur in eastern and southern Africa; however there recently has been an increase in the number of serotypes present within the northern limits of the virus range in sub-Saharan Africa (MacLachlan and Guthrie, 2010). The distribution of AHSV serotypes varies with regions (OIE, 2009; Sánchez-Vizcaíno, 2006). Serotypes 1 to 8 are typically found only in restricted areas of sub-Saharan Africa except for occasional excursions into North Africa or the Arabian Peninsula. Only AHS serotype 9 and 4 have been found in West Africa from where they occasionally spread into countries surrounding the Mediterranean (Sánchez-Vizcaíno, 2006). However, type 9 is more widespread and has been responsible for virtually all epidemics outside Africa the only exception being outbreak of AHS in Spain, Portugal, and Morocco due to AHSV-4. Serotype 2 has recently been reported from Ethiopia, Nigeria and Senegal (Kazeem *et al.*, 2008). In outbreaks which occurred between 2006 and 2008 in Namibia, serotypes of AHSV-1, AHSV-2, AHSV-4 and AHSV-9 were identified (Scacchia *et al.*, 2009). All nine serotypes were identified from submitted field samples in South Africa with a range of 3–60 isolates per serotype which were detected using AHSV S10 (Quan *et al.*, 2010).

2.3.6 AHSV serotypes identified in Ethiopia

Extensive studies have not been conducted in Ethiopia and the so far isolated and identified serotypes may not represent the actual situation. Serotype 9 was found as the dominant serotype circulating in Tigray, Dire Dawa, Hawassa, Jimma, Addis Ababa and Debre Zeit. AHSV-7 has also been reported in Jimma (Leforban *et al.*, 1983). Recently AHSV-2 was identified from outbreaks in Oromia (Bench Maji and Illubabor) and SNNPRS (Keficho Shekicho) (WAHID Interface, 2008). In another investigation, AHSV-9 was identified from outbreaks in the southern, western and central Ethiopia (Zeleke *et al.*, 2005).

2.4 AHS pathogenesis

AHSV is the most pathogenic of all equine viruses (Venter *et al.*, 2006). AHSV types 1-8 are considered to be highly pathogenic for horses and infection results in high mortality while type 9 AHS virus appears to be less pathogenic and infection may result in lower mortality (Coetzer and Erasmus, 1994). Another report from recent outbreaks demonstrated that AHSV-9 is capable of causing severe signs and death to unvaccinated horses (Scacchia *et al.*, 2009).

The primary sites of replication following vector inoculation are not known but they are thought to be lymph nodes, lungs and spleen and other lymphoid tissues and certain endothelial cells of small blood vessels, particularly capillaries and in large mononuclear cells resembling macrophages or reticular cells of lymphatic vessels (Burrage and Laegreid, 1994; Wohlsein *et al.*, 1998). Following primary replication, viremia is responsible for dissemination of AHSV throughout the body of the animal. Viral infection was commonest in the endothelial cells of myocardial vessels, followed by those in the lung, whereas in the spleen and liver, endothelial cell infection was rare and, in the case of the liver, limited to the interstitial capillaries. Viral replication in endothelial cells results in vascular damage and an increase in vascular permeability leading to hemorrhage and edema (Gomez-Villamandos *et al.*, 1999). Activation of pulmonary intravascular macrophages was also found to contribute to vascular changes by releasing chemical inflammatory mediators (Carrasco *et al.*, 1999). Virus multiplication in other body tissues and organs gives rise to a secondary viraemia, which is of variable duration and titre dependent upon a number of factors including host species (Burrage and Laegreid, 1994).

In experimentally infected horses, highest AHSV titers rapidly accumulated in the spleen, lungs, caecum, pharynx, choroid plexus and most lymph nodes with positive titers preceding detectable viremia which suggests that lymphoid organs and lung represent possible sites of primary replication. In the blood, the virus is associated with the cellular fraction of both red blood cells and the buffy coat, while very little is present in the plasma (Mellor and Hamblin, 2004).

2.5 AHS clinical forms

AHS exist in four different forms: the peracute (pulmonary) form, the subacute edematous (cardiac) form, the acute (mixed) form and horsesickness fever (mild) form. Symptomatic infections occur most often in horses and mules. However, symptoms are less likely in mules than horses. The pulmonary and mixed forms usually predominate in susceptible populations of horses. The pulmonary form is also the most common form in dogs. The mildest form, horsesickness fever, tends to be seen in horses with partial immunity, mules and donkeys in areas outside endemic areas. African donkeys and zebras are unlikely to show clinical signs of the disease (Coetzer and Erasmus, 1994). The primary determinant of the form of disease expressed by naïve horse is the virulence and tissue tropism of the infecting AHSV serotype (Burrage and Laegreid, 1994). Recent studies indicated that subclinical cases of AHS occur in naturally infected horses in endemic areas (Weyer *et al.*, 2010). This was defined by detection of AHSV using real-time RT-PCR in the absence of clinical signs.

Duration of incubation period depends on virulence of the virus and dose of virus received (Guthrie, 2008) but infective dose in natural conditions is not known. Experimentally it has been shown to vary between 2 and 21 days (Mellor, 1994b). In horses viraemia usually lasts for only four to eight days and has not been detected beyond 21 days. In donkeys and zebra the levels of viraemia are lower but may extend for up to four weeks. The longest recorded viraemia is about 40 days in the case of zebras (OIE, 2008).

2.5.1 The pulmonary form or peracute form

The pulmonary form is peracute and may develop so rapidly (3–5 days) that an animal can die without previous indication of illness. Usually there will be marked depression and fever (39–41^{0}C) followed by onset of respiratory distress and severe dyspnoea. Respiratory rate may increase to 60 or even 75 breaths/minute (OIE, 2008). The animal may be observed to stand with its forelegs spread apart, its head extended and its nostrils fully dilated. Profuse sweating is common and spasmodic coughing may be observed terminally, with frothy serofibrinous fluid exuding from the nostrils. The prognosis for horses suffering from this form of AHS is extremely grave and mortality rates commonly exceed 95%. Death usually occurs within a few hours after the first clinical signs are observed. This form is usually observed in completely susceptible animals, animals infected with a highly virulent strain of virus, or

animals that are worked during the febrile stage of the disease (Mellor, 1994b; Radostits *et al.*, 2007).

2.5.2 The cardiac or subacute edematous form

The cardiac form of AHS usually begins with a fever that lasts for 3 to 6 days. It has an incubation period of 7 to 14 days (OIE, 2008). The main clinical finding is subcutaneous oedema, particularly of the head, neck and chest but also of the supraorbital fossae eyelids. These swellings later spread to involve the cheeks, lips, tongue, intermandibular space, laryngeal region, and sometimes the neck, shoulders and chest but not to the lower legs. Other clinical signs, usually seen in the terminal stages of the disease, can include severe depression, colic, ecchymoses on the ventral surface of the tongue and petechiae in the conjunctivae. Difficulty in swallowing due to paralysis of the oesophagus is also seen. Death often occurs from cardiac failure and mortality rate is 50 to 60 %. If the animal recovers, the swellings gradually subside over the next 3 to 8 days. This clinical form of AHS is usually associated with infection by virus strains of low virulence or is encountered in immune animals infected by heterologous virus strains or may be a function of biological variation in the infected animal (Mellor, 1994b; Radostits *et al.*, 2007; OIE, 2008).

2.5.3 The acute or mixed form

The mixed form is often the most common form of AHS and is a combination of the cardiac and pulmonary forms of disease. It has an incubation period of 5 to 7 days. The mortality rate is approximately 70% and death usually occurs within 3 to 6 days after onset of fever. In most cases, the cardiac form is subclinical and is followed by severe respiratory distress. Occasionally, mild respiratory signs may be followed by edema and death from cardiac failure. The mixed form of AHS is rarely diagnosed clinically, but is often seen at necropsy in horses and mules. The mortality rate is around 70% (Mellor, 1994b; Radostits *et al.*, 2007; OIE, 2008).

2.5.4 Horsesickness fever or mild form

Horse sickness fever is invariably mild usually involving only mild to moderate fever (39–40^{0}C) of the remittent type, with the lowest temperatures in the morning rising to a high peak

in the afternoon, lasting for 3–8 days. The incubation period varies from 5 to 14 days. Apart from this, other clinical signs are rare and are frequently overlooked in natural outbreaks. The conjunctivae may be slightly congested, the pulse rate may be increased, and a certain degree of anorexia, depression and oedema of supraorbital fossae may be present. There is no mortality. This form of the disease is usually observed in partially immune animals or in resistant species, such as the donkey and zebra and following infection with less virulent strains of virus (Mellor, 1994b; Radostits *et al.*, 2007; OIE, 2008).

2.6 Diagnosis

2.6.1 Clinical diagnosis

A wide variety of emerging techniques have been applied for the improved detection of transboundary animal diseases that cause huge economic losses worldwide and are constantly spreading and affecting new geographic areas including AHS (Rodriguez-Sanchez *et al.* 2008b). Clinical signs combined with an appropriate history and epidemiological information may be sufficient for a presumptive diagnosis (Kazeem *et al.*, 2008; OIE, 2008). Some clinical signs and lesions are characteristic but in the horsesickness form and during the early febrile phase of other forms of AHS, a field diagnosis may be virtually impossible and laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. This is of particular importance whenever outbreaks occur outside the enzootic regions (Laegreid, 1994; Brown and Torres, 2008).

2.6.2 Specimen for laboratory diagnosis

The most important aspect of the diagnosis of AHS is the selection of samples and their transportation to the laboratory. In live animals, AHS virus may be isolated from blood of affected equidae during the early febrile stage (OIE, 2008). Blood samples for virus isolation should be collected into tubes containing anticoagulant (heparin or EDTA). From animals that have died, necropsy samples for virus isolation should include small pieces (2–4 g) of the spleen, lung and lymph nodes. The samples for virus isolation should be kept at 4^0C during transportation and storage and not frozen (Ozawa and Bahrami, 1968; Mellor, 1994b; OIE, 2008).

Serum is required for serological examination of antibodies and serum neutralization test. Horses that survive infection develop specific antibodies within 10-14 days after infection that reach a peak about 10 days later. Paired serum (acute and convalescent phase) samples are recommended and are particularly important in areas where the disease is endemic (Mellor, 1994b). Serological tests can demonstrate AHS antibodies for 1 to 4 years after infection (Erasmus, 1998).

2.6.3 Agent detection tests

AHS can be diagnosed by isolating the virus or detecting its nucleic acids or antigens. Several techniques are already available for AHS viral identification such as cell culture, inoculation of newborn mice, sandwich ELISA and polymerase chain reaction (PCR) tests. It is recommended to use more than one test to diagnose an outbreak of AHS, especially the index case (OIE, 2008).

Successful direct isolation of AHSV has been performed on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) mammalian cell lines and on *Culicoides* and mosquito insect cell lines (*Aedes albopictus*) C6/36 cells and *Culicoides* (KC) cells (Hazrati and Ozawa, 1965; Mellor and Hamblin, 1994). The virus can also adapt and grow in embryonated hens eggs following intravenous inoculation (OIE, 2008). A cytopathic effect (CPE) may appear between 2 and 10 days post-infection with mammalian cells. Three blind passages should be performed before considering the samples to be negative. However, the insect cell lines do not show CPE and are usually used only as a sensitive intermediary to amplify virus as a prelude to virus isolation in mammalian cells (OIE, 2008).

Intracerebral inoculation of 2 to 4 day old suckling mice is another preferred method for primary isolation of AHSV. In positive cases, animals develop nervous signs between 3 and 15 days post-inoculation. The brains from sick animals may be collected, homogenized and re-inoculated intracerebrally into at least six 1–3-day-old mice. This second passage should present a shortened incubation period (2–5 days) and 100% infectivity. Virus may be typed directly from mouse brain by conventional VN test or by RNA extraction and sequencing (OIE, 2008).

The indirect sandwich ELISA is also useful for the rapid identification of AHSV antigen in solid tissues taken from animals that have died following an acute infection. Two serogroup-specific sandwich ELISAs have been developed. One technique uses polyclonal antibodies to the AHSV and the other uses monoclonal antibodies against VP7. Both methods have been demonstrated to be adequate for the diagnosis of AHS, due to their high sensitivity and specificity and the availability of results in only 2–4 hours (Laviada *et al.*, 1992). This method can also be used for detection of the virus in insect vectors (Hamblin *et al.*, 1991).

AHSV can be identified directly in whole blood and other tissues using molecular probes and RT-PCR using group specific primers for segment 3 and VP7. An RT-PCR assay for the specific detection of AHSV genome in blood, homogenized equid or mouse tissue or cell culture fluids has been developed. Primers correspond to the 5' end (nucleotides 1– 21) and 3' end (nucleotides 1160–1179) of RNA segment 7, amplifying the complete segment 7 of the genome (Aradaib *et al.*, 2006).

Western immunoblotting assay has been used to identify at least four distinct AHSV proteins, including VP5, NS1, NS2 and NS3/NS3a. By using AHSV non-structural proteins as 'markers', this test can be used to discriminate between animals vaccinated with a purified, inactivated AHSV and those either naturally infected or vaccinated with a live attenuated vaccine (Bougrine *et al.*, 1998).

Experiments on cases of AHS revealed that RT-PCR testes detected AHS in horses prior to the onset of clinical signs (Weyer *et al.*, 2010). The recent development of a type-specific RT-PCR for identification and differentiation of the nine AHSV serotypes provided a method of confirming the identity of AHSV in tissue samples within a few hours which provides a perfect agreement with the VN test. Nine pairs of primers were designed in the virus genome segment 2 for each specific serotype (Sailleau *et al.*, 2000). A new conventional technique and a real-time RT-PCR technique have been developed using primers from a highly conserved region of AHSV segment 7, to achieve better sensitivity and rapidity in the diagnosis of AHS (Monacco *et al.*, 2011).

Typing of the nine AHS serotypes has also been carried out with probes developed from a set of cloned full length VP2 genes and can be an alternative to amplification of genome segment 2. The recent development of a type-specific RT-PCR has now provided a method of

confirming the identity of AHSV in tissue samples within 24 hours. A significant advantage of this method is that it can be used to type very low levels of AHSV antigen in samples that do not contain live virus (Sailleau *et al.*, 2000).

There are many other recently developed effective molecular tools that can be used to detect of all the nine serotypes of AHSVs. These include conventional gel-based RT-PCR and real-time PCR (Rodriguez-Sanchez *et al.*, 2008a), TaqMan-MGB RT-PCR (Fernández-Pinero *et al.*, 2009), nested RT-PCR (Aradaib, 2009), duplex real-time RT-PCR (Quan and Guthrie, 2009) and fluorogenic real-time RT-PCR (Aguero *et al*, 2008). More recently a rapid, analytically sensitive and specific duplex real-time RT-PCR assay targeting the VP7 and NS2 genes of AHSV was developed and optimized. The assay is quantitative and suitable for mathematical modelling and pathogenesis studies which will allow for a greater understanding of AHS (Quan *et al.*, 2010).

2.6.4 Antibody detection tests

In non-enzootic regions, at the start of an outbreak and when the AHSV serotype is unknown, the group specific ELISA can provide a rapid and reliable confirmation of clinical diagnosis (Rubio *et al.*, 1998). Group specific antibody against AHSV can be detected using several diagnostic assays that are directed primarily towards the recombinant VP7, including complement fixation, agar gel immunodiffusion (AGID), immunofluorescence and ELISA. VP7 is the most stable protein and has been used as antigen for AHSV antibody determination with a high degree of sensitivity and specificity (Roy *et al.*, 1994). The indirect ELISA and complement fixation tests are the prescribed tests for international trade (OIE, 2008). The earliest serologic markers correspond mainly to VP5, VP6, and NS2 and to a lesser extent to VP3, NS1 and NS3. VP7 specific antibodies are not detectable for about two weeks and not appropriate for early detection of the virus (Martinez-Torrecuadrada *et al.*, 1997). A group-specific recombinant protein VP7 of AHSV-3 was obtained by using purified baculovirus-expressed VP7 crystals from infected insect cells and an I-ELISA for the detection of IgG-antibodies to VP7 in horse sera was validated (Mareea and Paweska, 2005).

Indirect and competitive ELISAs using either soluble AHSV antigen or a recombinant protein VP7 have proved to be good methods for large-scale investigations and can be used for detection of all the nine different AHSV serotypes. I-ELISA is reported as a more sensitive

test in detection of earliest immunological response in infected equids and declining level of maternal immunity in foals and recommended as a highly accurate diagnostic tool for disease surveillance and control programs (Maree and Paweska, 2005). I-ELISA that uses the AHSV serotype 4 nonstructural protein NS3 as antigen is available and can be used to distinguish between naturally infected animals or vaccinated with the live vaccine and those vaccinated with purified virions, inactivated vaccine (Lavida *et al.*, 1995; el Hasnaoui *et al.*, 1998b). This test is also a preferred test to distinguish between antibodies to other Orbiviruses affecting equids (Williams *et al.*, 1993). C-ELISA for detection of AHSV in tissues and cell culture supernatants by the use of 2 monoclonal antibodies (Mab) directed against VP7 is a sensitive and specific test and can also be used for testing wildlife as species specific anti-globulin is not required with this method (Hamblin *et al.*, 1990; OIE, 2008).

VN, CF, and AGID tests are not suitable for extensive epidemiological studies. The VN test is used to detect serotype-specific antibody using type specific antisera and has been the method of choice for serotyping AHSV isolated from the field mainly in endemic areas where multiple serotypes are likely to be present. This technique takes five or more days before results are obtained and it is extremely laborious and requires standardized stocks of reference viruses, serotype-specific antisera and tissue cultures (Hamblin *et al.*, 1991; Maree and Paweska, 2005). It is also sensitive to serum toxicity which inhibits cell growth and would require 9 separate tests to ascertain the negativity of one sample (House *et al.*, 1990).

The CF test has been used extensively and is an OIE designated test method. However, due to the anti-complementary effect of sera from donkeys and mules and shorter duration of complement fixing antibodies as well as the good results obtained with the ELISA, its use is decreasing (Williams *et al.*, 1993; el Hasnaoui *et al.*, 1998b). The CF test is frequently used for the demonstration of group-specific antibodies against AHSV.

Indirect fluorescent antibody assay (IFA) can be used and is rapid and sensitive. It detects antibodies as early as 7-10 days after primary exposure to live virus; however, all positive results should be confirmed by other techniques (House *et al.*, 1990; el Hasnaoui *et al.*, 1998a).

2.7 Differential diagnosis

AHS must be differentiated from other diseases causing sudden death, respiratory distress or oedema (Anonymous, 2006). The clinical signs of AHS, particularly when not fully developed, may be confused with those caused by a closely related *Orbivirus*, equine encephalosis virus (OIE, 2009). They have a similar geographical distribution and vertebrate host range and the same vector species of *Culicoides*. As a result both can occur simultaneously in the same locations and even in the same animal (Mellor and Hamblin, 2004).

Other differential diagnosis includes equine viral arteritis (EVA), equine infectious anemia, Hendra virus infection, purpura hemorrhagica, trypanosomosis (surra) and the early stages of equine piroplasmosis (*Theileria equi* and *Babesia caballi*) particularly when the parasites are difficult to demonstrate in blood smears. The haemorrhages and oedema reported in cases of purpura haemorrhagica and equine viral artritis may be similar to those seen in the pulmonary form of AHS, although with AHS the oedema tends to be less extensive and the haemorrhages are less numerous and widespread. Chemical and plant poisonings, toxins, anthrax and other causes of sudden death, as well as diseases that result in severe respiratory distress, should also be considered (OIE, 2009).

2.8 Socio-economic impact of AHS

Despite the huge direct losses due to mortality of equids which is common in several African countries, there could be losses from suspension on the export of equids to Europe and other non endemic countries of the world (Venter *et al.*, 2006). There is no evidence that humans can become infected with any field strain of AHSV but certain neurotropic vaccine strains may cause encephalitis and renititis in humans following transnasal infections (Erasmus, 1998; Radostits *et al.*, 2007).

2.9 Control of AHS

There is no specific treatment for AHS. Affected animals should be provided with supportive therapy, nursed and rested as the slightest exertion may result in death. Animals that survive should be rested for at least 4 weeks following recovery before being returned to light work (Guthrie, 2008).

In enzootic regions of Africa, vaccination has been used essentially as a method of preventing animal losses and infection of vector insects being a secondary consideration while in non-enzootic regions the official policy has been to eradicate the virus as quickly as possible (House, 1998). At present, there is a minimum 60-day quarantine period for horses. If AHS is detected in naïve country, a strict quarantine zone and movement controls should be established. Euthanasia of infected and exposed animals may be considered. Whenever possible, all *Equidae* should be stabled in insect-proof housing particularly from dusk to dawn, the period when *Culicoides* are most active. Vector control measures such as modifications of *Culicoides* breeding areas, insect repellents and targeted applications of insecticides or larvicides may also be useful. Some countries used combination of methods to eradicate AHS. Average annual vaccination coverage of 92% was carried out as one of the strategies in eradicating AHS in Morocco (Benazzou *et al.*, 2006).

In endemic areas, the use of prophylactic vaccination with polyvalent attenuated live virus (AHS-ALV) vaccine will remain the main preventative and control measure against AHS (von Teichman *et al.*, 2008; von Teichman *et al.*, 2010). In South Africa, it is compulsory to vaccinate all horses, except those in the currently declared free and surveillance zones in the Western Cape Province with the AHS-ALV vaccine (von Teichman and Smit, 2008). However, there are reports of some concerns associated with modified live virus AHS vaccines which includes occurrence of vaccine reactions, including death; variable response to vaccination; difficulty in immunizing young horses because of interference from maternal immunity and the potential for reversion to virulence (infectivity) of vaccine virus (Oke, 2010).

A formalin-inactivated vaccine was developed commercially during the outbreak in Spain, Portugal and Morocco. Such inactivated vaccines have the advantage that they do not contain a live and potentially dangerous agent which prevents infection of insects. However, they may

be expensive to produce and multiple inoculations may be required to elicit and maintain high levels of protective immunity. It may also be difficult to ensure complete vaccine inactivation. However, vaccination with AHSV-4 inactivated viral vaccine given as 2 separate doses provided full protection against subsequent homologous challenge (House, 1998). Although this vaccine proved to be reasonably efficacious, it was withdrawn shortly after the eradication of AHSV from Europe and is no longer available. A high level of protection was generated against a heterologous virus serotype using purified VP7 as a subunit vaccine (Wade-Evans *et al.*, 1998). Baculovirus expressed AHSV outer capsid proteins, VP2 and VP5 have been reported to protect against death caused by AHSV challenge (Roy and Sutton, 1998).

Extensive work is presently under way to develop potent recombinant vaccines. A recent report indicated that a new safe and effective recombinant canarypox vectored vaccine has been developed and preliminarily tested as effective vaccine against serotype 4 of AHSV (Guthrie *et al.*, 2009). In another study recombinant modified vaccinia Ankara (MVA) vaccines expressing VP2, VP7 or NS3 genes of AHSV were used to stimulate immune responses against AHSV antigens in the horse. Results of the study indicated that vaccination with MVAVP2 induced a strong AHSV neutralizing antibody response and MVAVP7 also induced AHSV antigen–specific responses, detected by western blotting. NS3 specific antibody responses were not detected (Chiam *et al.*, 2009).

3 MATERIALS AND METHODS

3.1 Study area

Outbreaks of AHS were investigated in central, eastern and southern parts of Ethiopia. In central Ethiopia, outbreaks in East Shewa (Ada'aa, Adama, Wonji, Awash Melkassa districts) and West Shewa (Tikur Inchini) zones and Addis Ababa (Akaki sub-city) were investigated. Outbreaks in eastern Ethiopia in zone 3 of Afar region (Awash Fentale district) and in Kaffa zone (Cheta district) of the Southern Nation Nationalities and People's Regional State (SNNPRS) were investigated (Figure4).

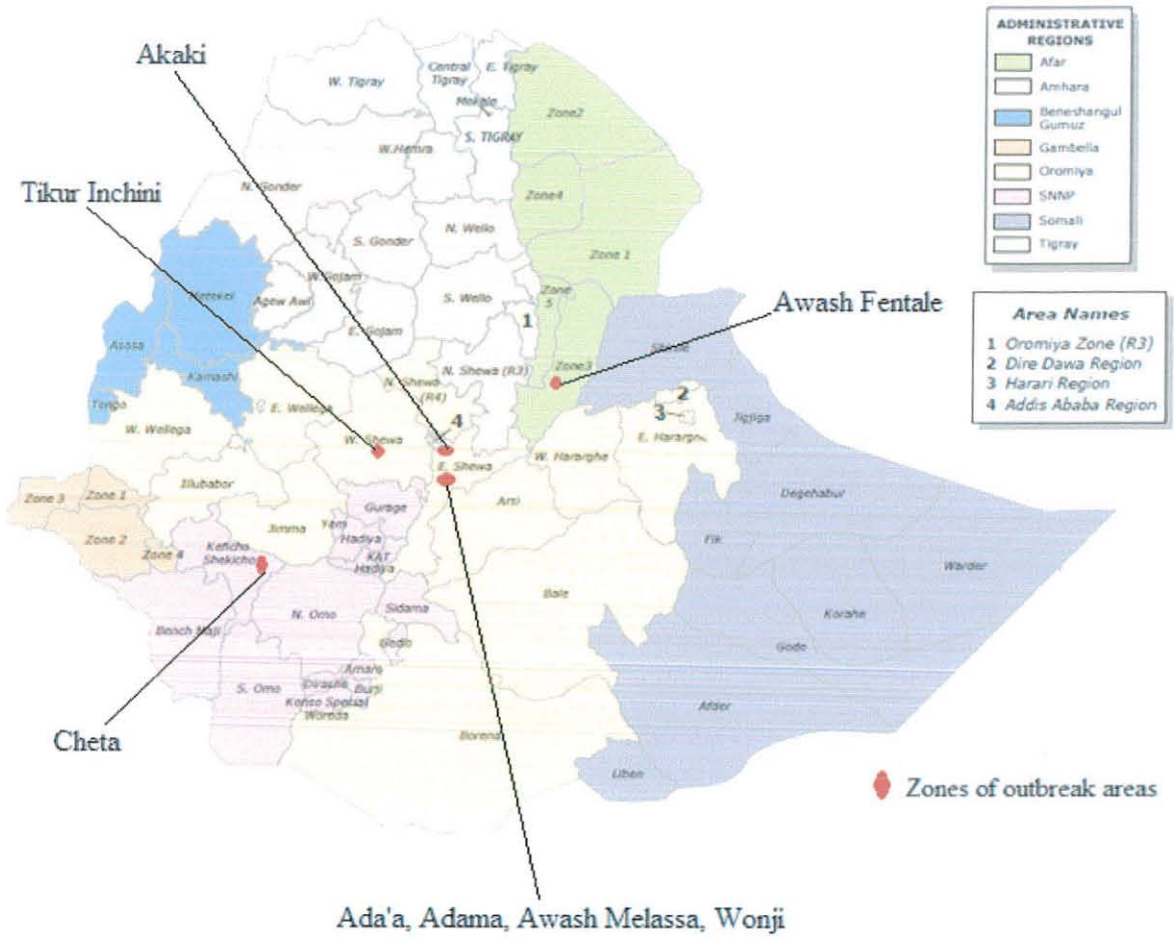


Figure 4. Map indicating outbreak sites in Ethiopia

In East Shewa zone, Ada'a was the main outbreak district. Among the peasant associations (PAs) and towns involved in Ada'a district, Debre Zeit is the biggest town and located 45km from Addis Ababa with an altitude of 1,850 meters above sea level. It has warm and humid weather. Several other towns and PAs were involved in the outbreaks of Ada'a district within a radius of about 20km from Debre Zeit. These were Dalota, Dembi, Erobe, Filtino, Gichi, Godino, Gote, Hidie, Koftu, Kurkura and Yerer. The other outbreak sites in East Shewa were Adama, Wonji and Awash Melkassa. Adama is located at 99 km southeast of the capital and has an altitude of 1,566 meters above sea level. Another outbreak site in Oromia was Tikur Inchini which is located in West Shewa and has an altitude of 2,480 meters above sea level. Akaki is a subcity of Addis Ababa located at 25km to the southeast direction. Its altitude is 2,120 meters above sea level. Awash Fentale is located in zone 3 of Afar region and has an altitude of 916 meters above sea level. Other study area was Cheta district located in Kaffa zone of SNNPRS. Cheta has an altitude of 1,650 meters above sea level.

3.2 Target population

The target population was equids in the outbreak areas. East Shewa possesses 14,861 horses, 255,548 donkeys and 5,338 mules. West Shewa possesses 163,650 horses, 175,126 donkeys and 9,454 mules. Kaffa zone of SNNPRS possesses 81,050 horses and 12,646 mules. Zone 3 of Afar region possesses 22,264 donkeys (CSA, 2011). Number of horses, mules and donkeys in study sites are indicated below (Personal communication with the respective MoA offices) (Table 1).

Table 1. Numbers of horses, mules and donkeys in outbreak areas

Region	Zone	District	No. of horses	No. of mules	No. of donkeys	Total no. of equids
Addis Ababa	Akaki sub city	Akaki area	6,414	2,061	23,712	32,187
Afar	Zone 3	Awash Fentale	150	0	500	650
Oromia	East Shewa	Ada'a	3,720	994	28,044	32,758
		Adama	2,890	112	32,517	35,519
		Wonji	1,200	80	6,000	7,280
		Awash Melkassa	300	108	3,000	3,408
	West Shewa	Tikur Inchini	18,801	1,313	11,978	32,092
SNNPRS	Kaffa	Cheta	1600	280	16	1,896
Total			35,075	4,948	105,767	145,790

3.3 Study methodology

3.3.1 Study design

When an active outbreak of AHS was encountered or reported, a field investigation was conducted at the specific site and epidemiological information was gathered by interviewing equine owners, district animal health workers and development agents. Clinical and epidemiological data were recorded and samples were collected for serological test, virus isolation and serotyping.

3.3.2 Retrospective data analysis

A three-year retrospective data of previous outbreaks of AHS reported to the Animal and Plant Health Regulatory Directorate (APHRD) office from all over the country were obtained from the office and analyzed to see the national picture of AHS outbreaks and months of the year when the disease more commonly occurred.

3.3.3 Questionnaire survey

A structured questionnaire was used to gather information from respondents in Cheta district in the SNNPRS region and Tikur Inchini district in Oromia (Annex 4). Information on indigenous knowledge of equine owners on the disease and its ways of transmission was gathered from 178 respondents.

3.3.4 Clinical examination

Equids were clinically categorized as AHS suspected when they manifested all or combinations of the following signs: pyrexia, oedema, hyperaemia of serosal surfaces and dyspnoea. The febrile state was determined whenever the rectal temperature exceeded 38.5^{0}C accompanied by in-appetence or anorexia. Epidemiological information was gathered from veterinarians, animal health assistants and equine owners from each outbreak sites including date of onset of AHS outbreaks, number of affected and dead animals and vaccination status of equids in the area while collecting samples. Information on history of vaccination, date of

commencement of the disease, clinical signs and whenever possible postmortem findings associated with AHS were recorded for each sampled equid.

3.3.5 Sample collection

For virus isolation, about 5 ml of whole blood in EDTA vaccutainer tube were collected from the jugular vein of equines suspected of AHS. Identification number was given to each sample and labeling included district, PA, species of animals, sex and age of the sampled animal. The blood was then gently mixed with the anticoagulant and kept in ice until delivery to the NVI laboratory. Once the samples arrived at NVI, they were stored at $+4^0$C until processed. Samples for virus isolation were collected from 81 horses and 3 mules (Table 2). About 10 grams of tissue samples (lung and spleen) were collected from three horses freshly died of AHS in the SPANA clinic in the compound of the School of Veterinary Medicine of Addis Ababa University. At postmortem examination in the SPANA clinic, pathological lesions were recorded and tissue samples were collected from the lung and spleen.

Table 2. Months of the index case and number of equids that demonstrated signs of AHS per outbreak sites

Month/year of index case	Districts	Horses	Mules	Total sample collected
Sept/2009	Cheta	12	2	14
Oct/2009	Adama	1	0	1
Oct/2009	Tikur Inchini	9	0	9
Nov/2009	Awash Fentale	6	0	6
Apr/2010	Cheta*	1	0	1
Jun/2010	Adama*	2	0	2
Jun/2010	Awash Melkassa	2	0	2
Jun/2010	Ada'a**	31	1	32
Jun/2010	Wonji	9	0	9
Jun/2010	Akaki sub-city	8	0	8
	Total	81	3	84

* Second outbreak ** Several villages and towns involved

A total of 336 sera samples were collected from apparently healthy horses, mules and donkeys in two localities (Cheta and Tikur Inchini) for determination of risk factors. Equids with history of vaccination within the last three years were not included. About 5ml of blood sample was collected from the jugular vein of each animal using plain vaccutainer tube and kept protected from direct sun light in slant position until the blood clots and then the serum was separated and transferred to a labeled sterile cryovials. The serum was then kept in ice and transported to NVI laboratory and stored at -20^{0}C until assayed.

3.3.6 Seropositivity determination

AHSV-specific antibodies were detected using a blocking ELISA according to manufacturer's instructions. The ELISA kit used was Ingezim Compac Plus 14.AHS.K3, Spain. The kit uses VP7 recombinant protein from the AHSV serotype 4 obtained using baculovirus expression system. Washing solution, conjugate and substrate were reconstituted in the laboratory following manufacturer's instruction.

Seropositivity of samples collected was determined following the manufacturer's instructions. The test procedures are indicated on Annex 1. Validation of the test result was done when the optical density (OD) value of the positive control was lower than 0.2 and that of the negative control was higher than 1.0. Determination of the Blocking Percentage (BP) of each sample was done by the following formula:

$$BP = \frac{\text{Abs (Control-) - Abs(sample)}}{\text{Abs (Control-) - Abs(control+)}} \times 100$$

Samples which gave BP value lower than 45% were considered negative for antibodies to AHSV and samples showing BP value higher than 50% were considered positive for antibodies to AHSV. Values between these two ranges were considered doubtful and needed to be retested.

3.3.7 Virus isolation on Vero cells

Virus isolation was conducted in virology diagnostic laboratory of the NVI, Debre Zeit, Ethiopia. The collected samples were kept at +4^{0}C until processed. The washed and lysed

blood cells and tissue lysates were cultured on confluent monolayers of Vero cells grown in 25cm^2 plastic tissue culture flasks (Annex 2). When CPE was not observed in three blind passages, then the sample was considered negative for virus isolation and results recorded.

3.3.8 Serotype identification by RT-PCR

About 2 ml from each of the samples which demonstrated CPE was taken from the cultures after three consecutive freezing and thawing. This was then transferred to the RNA extraction unit in the NVI molecular diagnostic laboratory (Annex 3). Viral double-stranded RNA extraction procedure was done using the commercial RNeasy Mini Kit (Qiagen) and one Step RT-PCR kit (Qiagen) was used for AHSV nucleic acid amplification using the extracted RNA sample as a starting template.

All samples were first tested with RT-PCR using universal primer obtained from Eurofins MWG Operon, University of Liverpool. Sequences of the primers (5'-3') were: forward primer (GGCTCCAACACTCACAAGATG-21) and the reverse primer (GGCGGATTA-ATAGGCTGCATA-21) designed to identify the serogroup of AHSV (OIE, 2008); positive isolates were serotyped by nine pairs of sequence specific primers designed for RT–PCR amplification of the serotype-specific genome segment 2, forward and reverse for each serotype of AHSV as described by Sailleau *et al.*, (2000).

However, optimized result couldn't be obtained and therefore the samples were sent to a Non-vesicular reference laboratory, Institute for Animal Health, Pirbright, UK for further virus isolation and identification, sequencing and phylogenetic analysis.

3.3.9 Sequencing and phylogenetic tree analysis

In the Institute for Animal Health, the serotype(s) present in each of the samples were initially investigated using recently developed real-time RT-PCR assays for serotype-specific detection of AHSV segment 2. The results generated were confirmed by FLAC amplification (Maan *et al.,* 2007) of Seg-2 and sequencing using conserved 'terminal primers'. If these methods failed (due to mixed serotypes or amplicons), Seg-2 was amplified by conventional RT-PCR, using AHSV type-specific primers, followed by sequencing of the resulting specific

amplicons. In each case the sequence data was compared to data for Seg-2 of AHSV reference strains. Phylogenetic tree analyses were made by comparing the isolated serotypes with respective reference strains and IAH AHSV-1 reference strain. The level of sequence relatedness is expressed as percentage identity. The sequence of AHSV-1 reference strain was used as an out-group in the analyses.

3.4 Data management and analysis

Records from seropositivity test, virus isolation and retrospective data were first coded into Microsoft Excel and analyzed using Statistical Package for Social Sciences (SPSS) software version 15. Chi-square test and descriptive statistics was used to analyze the data with determined confidence level of 95% and $p < 0.05$ for significance.

The molecular sequences generated in this study were done at IAH, Pirbright, UK. Phylogenic tree analysis was made to see the relationship among identified serotypes. The nucleotide data was analyzed by phylogeny reconstruction. For the tree inference Neighbor-Joining method was used. It was implemented with pairwise deletion for gaps and/or missing data. Substitution model used was nucleotide: p-distance and substitutions to include were done by d: transitions and transversions. Pattern among lineages were homogeneous and uniform rates were used among sites.

4 RESULTS

4.1 Retrospective data analysis

The retrospective data analysis for the past three years between January 2007 and December 2009 indicated that a total of 482 outbreaks were reported to APHRD in the MoA, Ethiopia. The results indicated that AHS was reported from six regions of the country, namely Afar, Amhara, Dire Dawa, Oromia, SNNPRS and Tigray. Higher frequency of outbreaks were recorded from Oromia (386) followed by Amhara (51) and SNNPRS (37) during the specified period (Table 3). The highest number of outbreaks were recorded in 2008 (178) followed by 2007 (166) and 2009 (138). The overall highest frequency of outbreaks was observed from August to December in all the three years (Figure 5).

Table 3. Occurrence of AHS outbreaks in the different regions from 2007-2009

	Number of outbreaks in the three years			
Regions	2007	2008	2009	Total
Afar	1	0	0	1
Amhara	15	26	10	51
Dire Dawa	0	2	0	2
Oromia	138	126	122	386
SNNPRS	11	22	4	37
Tigray	1	2	2	5
Total	166	178	138	482

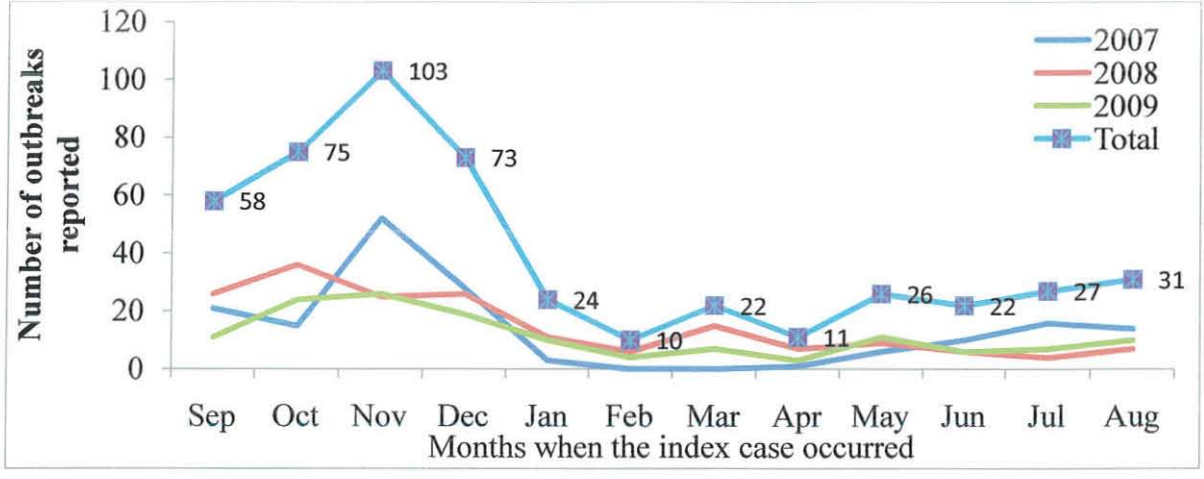


Figure 5. Occurrence and seasonality of AHS outbreaks from 2007-2009

4.2 Questionnaire survey

Results from interviewing 178 equine owners revealed that 59 (63.4%) of respondent owners in Tikur Inchini and 12(14.1%) in Cheta districts did not recognize AHS by its name as well as its clinical signs. Moreover, 76 (81.7%) and 15 (17.6%) were not aware of modes of transmission of AHS in Tikur Inchini and Cheta, respectively (Table 4). Zebras were not known to be present in both areas. Vaccination was practiced in both sites and 54.8% of the total respondents mentioned that their horses and mules were vaccinated at least once within the past two year's time and all vaccinations followed the occurrence of outbreaks but none of them vaccinated their donkeys.

Table 4. Summary of questionnaire survey administered in Cheta and Tikur Inchini districts

Gathered information		Districts				
		Cheta	%	Tikur Inchini	%	Total (%)
Number of respondents		85	47.8	93	52.2	178 (100)
AHS	• Do not know	12	14.1	59	63.4	71 (39.9)
	• Local name	'Horyo'		'Derbetu', Dibe Ferda'		
	• Months when AHS was more prevalent	May to September		September to November		
Transmission						
	• Do not know	15	17.6	76	81.7	91 (51.1)
	• Contact and smell	10	11.8	3	3.2	13 (7.3)
	• Biting insects	60	70.6	14	15.1	74 (41.6)
	• Local name of equine biting insects	'Gemo', Yubo'		'Bombisa'		

Local name of AHS was different in different areas. It is named as 'Derbetu' and 'Dibe Ferda' in Tikur Inchini (Oromia region) and 'Horyo' in Cheta (SNNPRS). Similarly, equine biting insects were known to be abundant in these areas and the suspected vectors according to the respondents are named 'Bombisa' in Tikur Inchini region and 'Gemo' and 'Yubo' in Cheta. 10 (11.8%) respondents in Cheta and 3 (3.2%) in Tikur Inchini mentioned contact and smell as means of transmission of AHS. None of the respondents used fly proof stables. The respondents also stated that AHS commonly occurred as an epidemic during the months of

September to November in Tikur Inchini and May to September in Cheta districts. Equids in both localities were used primarily for transportation of goods and people from place to place.

4.3 Data from the outbreak investigation

A total of ten outbreaks were investigated in two durations. Four of them occurred between September and November 2009 in Cheta, Adama, Tikur Inchini and Awash Fentale. The rest six outbreaks occurred between April and June 2010 in Cheta, Adama, Awash Melkassa, Ada'a, Wonji and Akaki. Cheta and Adama experienced two outbreaks. 7 out of the 10 outbreaks occurred in Oromia region while the rest outbreaks were occurred in Afar (1), (1) regions.

Based on the information gathered from equine owners, veterinarians and animal health assistants working in the outbreak sites, a total of 48 horses died of the disease. Contribution of each outbreak sites in the recorded death in decreasing order were 21 (43.8%) in Ada'a, 7 (14.6 %) in Awash Fentale, 6 (12.5%) in Awash Melkassa, 4 (8.3%) in each of Cheta and Wonji districts, 3 (6.3%) in Akaki, 2 (4.2%) in Tikur Inchini and 1 (2.1%) in Adama (Figure 6).

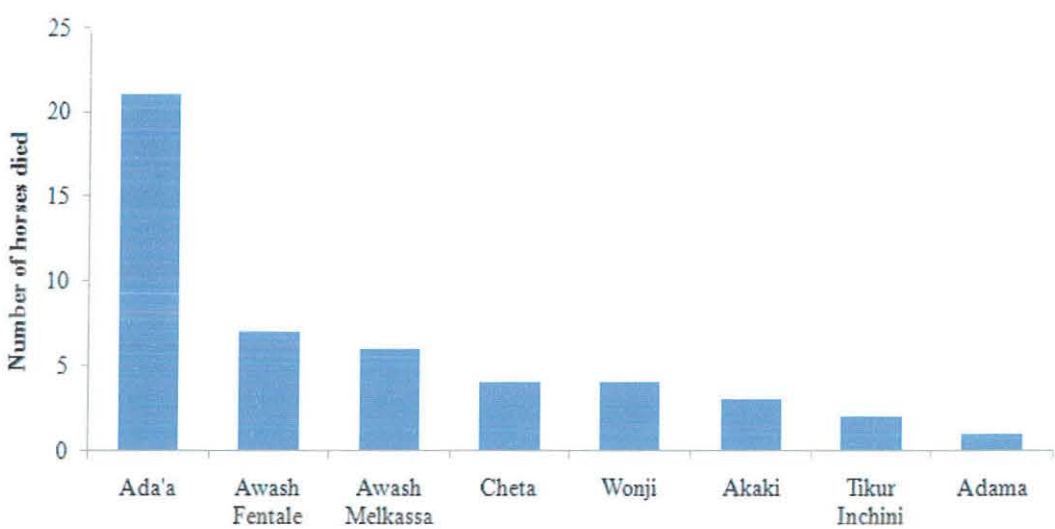


Figure 6. Number of horses died of AHS in the outbreak sites

A total of 84 equids have manifested clinical signs suggestive of AHS and 81 (96.4%) of them were horses while the remaining 3 (3.6%) were mules. All the four forms of AHS were observed in the outbreaks. 46 (54.8%) equids showed signs of cardiac form, 6 (7.1%) showed

signs of pulmonary form, 2 (2.4%) showed signs of mixed form and the rest 30 (35.7%) showed only pyrexia and mild edema suggesting horse sickness fever form of AHS (Table 5). Most commonly observed signs of AHS on clinically ill equids were oedema, ecchymoses of serosal surfaces, dyspnoea, pyrexia and inappetence. Rectal body temperatures were ranging from 38.5^{0}C to 41.5^{0}C. AHSV was not isolated from 49 (58.3%) equids showing clinical signs.

Cardiac form of the disease revealed a common finding of oedema of the head region including bilateral swelling of supraorbital fossae, ecchymoses of the ventral side of the tongue and depression. The pulmonary form was observed with evident respiratory distress, tachypnoea and exudation of slightly yellowish foamy discharge from both nostrils at close to the death of affected horses. All the three mules were affected by cardiac form of the disease. Three horses died (two with cardiac form and one with pulmonary form) were examined for postmortem findings in the SPANA clinic.

Table 5. Clinical form of AHS cases observed versus serotypes identified

	Identified AHSV serotypes						
Forms of AHS	2	4	6	8	9	-ves	Total
Cardiac							
Count	1	2	0	2	20	21	46
% of total	1.2%	2.4%	0	2.4%	23.8%	25.0%	54.8%
Pulmonary							
Count	0	0	1	0	3	2	6
% of total	0	0	1.2%	0	3.6%	2.4%	7.1%
Mixed							
Count	0	1	0	0	1	0	2
% of total	0	1.2%	0	0	1.2%	0	2.4%
Mild							
Count	0	2	0	0	2	26	30
% of total	0	2.4%	0	0	2.4%	31.0%	35.7%
Total							
Count	1	5	1	2	26	49	84
% of total	1.2%	6.0%	1.2%	2.4%	31.0%	58.3%	100.0%

Three horses died from full course of the disease were opened for postmortem examination. Postmortem findings from horses died of the cardiac form revealed hydropericardium and ecchymoses of serosal surface of the tongue and the heart. Observed postmortem findings from the pulmonary form were hydrothorax, edema of the lung, yellowish frothy discharges along the respiratory tract particularly in the trachea, bronchi and bronchioles (Annex 7). Other horses died of AHS were inaccessible and very much delayed to get the actual necropsy findings. Death of equids from horse sickness fever form was not reported or observed in the outbreaks.

4.4 Seropositivity and risk factors for AHS

Blocking ELISA test was used to determine seropositivity of equids in Cheta and Tikur Inchini. From a total of 336 sera samples collected from Cheta and Tikur Inchini districts, 147 (43.8%) equids were found seropositive for antibodies against AHSV. Seropositivity of equids in Cheta was 49.4% and that of Tikur Inchini was 37.8%. The seropositivity of equids in the two districts showed statistically significant difference (p=0.032). As depicted in Table 6, statistically significant difference was not observed for species, sex and age groups of equids in the two districts.

Table 6. Seropositivity of equids for and risk factors for AHSV infection

Risk factors	Number of samples	AHSV +ve	Seropositivty (%)	95% CI	χ^2	p-value
Site	336	147	43.8	0.38 - 0.49	4.60	0.032
Cheta	172	85	49.4	0.42 - 0.57		
Tikur Inchini	164	62	37.8	0.30 - 0.46		
Species	336	147	43.8	0.38 - 0.49	0.44	0.801
Horse	218	94	43.1	0.36 - 0.50		
Donkey	39	19	48.7	0.32 - 0.65		
Mule	79	34	43.0	0.32 - 0.55		
Sex	336	147	43.8	0.38 - 0.49	1.36	0.244
Male	250	114	45.6	0.39 - 0.52		
Female	86	33	38.4	0.28 - 0.50		
Age	336	147	43.8	0.38 - 0.49	0.61	0.436
Adult	209	88	42.1	0.35 - 0.49		
Young	127	59	46.5	0.37 - 0.56		

4.5 Virus isolation

Thirteen out of the total 84 samples were cultured on confluent monolayer Vero cells. In most of the cultures CPE was observed in 3 to 7 days post inoculation. Few cultures required two to three blind passages to develop obvious CPE (Figure 7B). CPE was also observed on cultures of lung and spleen tissue samples collected from dead horses. Both cultures with obvious CPE and whole blood samples were used for identification of virus serotypes using molecular techniques.

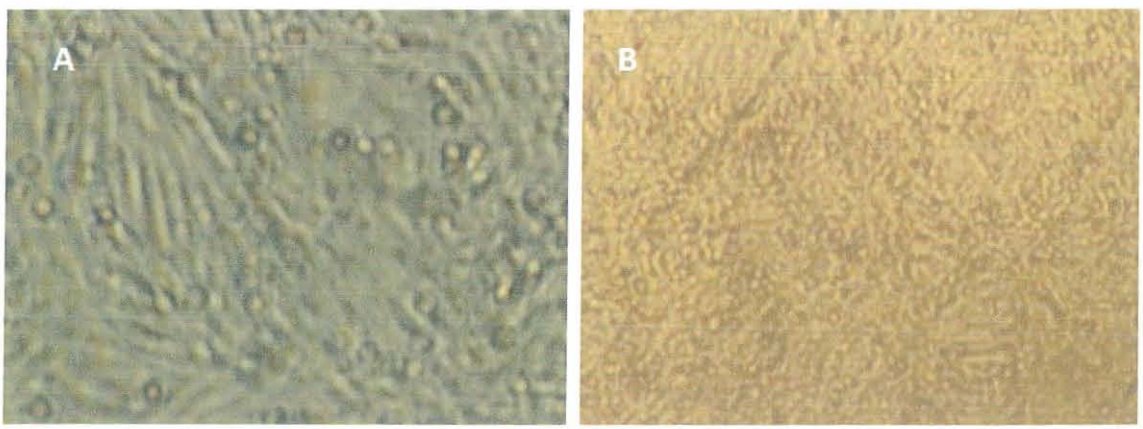


Figure 7. Vero cell line with and without CPE
(A) Confluent monolayer Vero cells (control) and (B) Vero cells with CPE from AHSV on day 3 of post-inoculation

4.6 Detection of AHSV genomic RNA by RT-PCR

Detection of AHSV genomic RNA was achieved using conventional RT-PCR (Figure 8) in the NVI virology and molecular laboratory. However, for various reasons optimization of the techniques for exact serotyping of AHSV could not be achieved. Therefore, the samples were submitted to IAH, Pirbright, UK for serotyping and further sequencing and phylogenetic tree analysis.

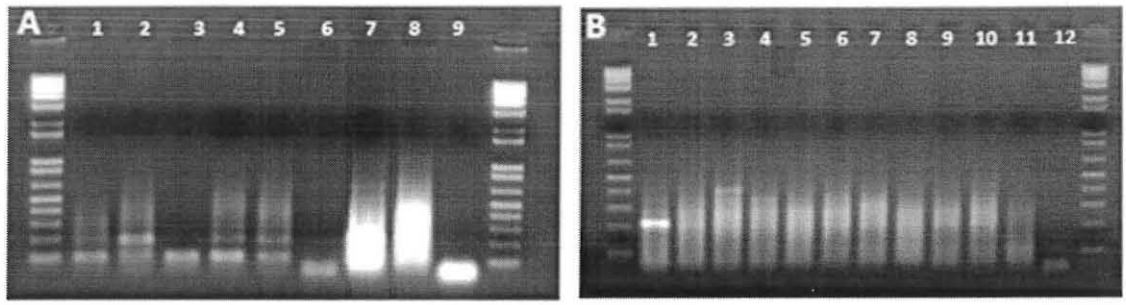


Figure 8. Electrophoresis of the PCR products from various samples obtained by conventional gel-based RT-PCR in a 1.5% agarose gel

(A) PCR amplified RNA using Universal primer for AHSV. Lanes 1–8 show the band belonging to the amplified 210 bp fragment of NS1 gene from AHSV serotypes. Lane 9 is control. (B) PCR amplified RNA using Primer for serotype 9. Lane 1 is NVI vaccine strain (AHSV-9) and Lane 12 is control.

Samples collected from the first outbreaks in Awash Fentale (6), Cheta (14), Adama (1) and Tikur Inchini (9) were not found to have live AHSV although we detected viral RNA with the conventional RT-PCR. Therefore, they were not included in the analyses and considered as AHSV negative. 54 whole blood samples, 6 tissue samples and 5 cultures on Vero cells which showed CPE were submitted after the occurrence of other outbreaks in different areas and in Cheta and Adama. Details of the samples and results are indicated on (Annex 5 and 6).

The Non-vesicular reference laboratories of IAH, Pirbright has identified the serotypes of viruses circulating in the outbreak areas (Table 7 and Figure 9). Serotype 9 was the dominant serotype identified from submitted samples of the outbreaks. It was identified from 26 (74.3%) of 35 positive samples (Figure 9). It was isolated from horses which were died from both cardiac and pulmonary forms. The other identified serotypes were serotype 2, 4, 6, and 8 and they were identified from 1 (2.9%), 5 (14.3%), 1 (2.9%) and 2 (5.7%) of the 35 positive samples, respectively. The AHSV positive samples had ct-value of between 19.71 and 37.46 (Annex 5 and 6).

Identification of serotypes AHSV-4, AHSV-6 and AHSV-8 is the first report for the country. Culture samples from Cheta district in SNNPRS region 'ETH2010/17 isolate' and Godino in Ada'a district of Oromia 'ETH2010/14' were identified as AHSV-9. Other culture samples

were found negative for AHSV isolation. Including the first submitted 30 samples, a total of 49 (58.33%) of the total submitted samples were detected negative.

Table 7. Summary of samples submitted to IAH, Pirbright, UK and obtained results

Sampling site	From First outbreaks	Result	From second outbreaks	No. of +ves	Results
Adama	1	-ve	2	0	All -ve
Tikur Inchini	9	All –ve	0	-	-
Cheta	14	All –ve	1	1	AHSV-9
Awash Fentale	6	All -ve	0	-	-
Ada'a	0	-	32	25	AHSV-2, 4, 6, 8, 9
Wonjii	0	-	9	3	AHSV-4, 9
Awash Melkassa	0	-	2	2	AHSV-4, 9
Akaki	0	-	8	4	AHSV-9
Total	30		54	35	AHSV-2, 4, 6, 8, 9

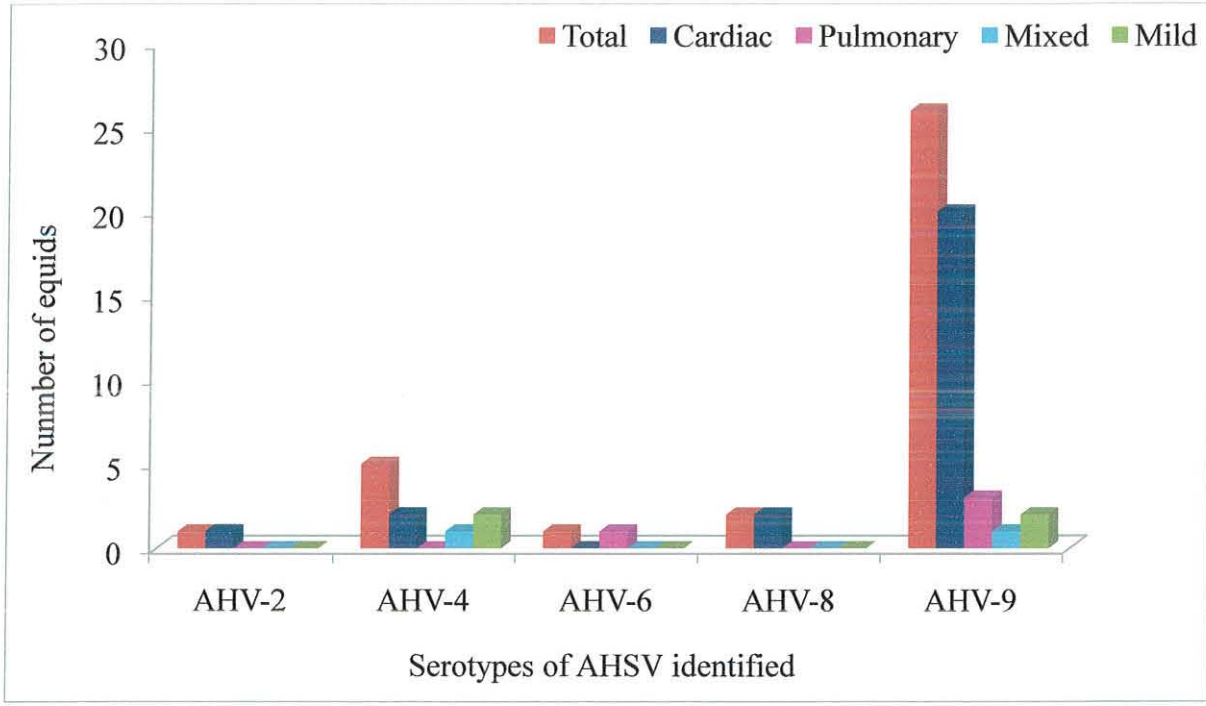


Figure 9. Number of equids which demonstrated the different forms of AHS versus serotypes identified

4.7 Phylogenetic analysis

Sequencing and phylogenetic analyses indicated multiple serotypes circulating in the outbreaks (Table 8). AHSV serotypes 2, 4, 6, 8 and 9 were detected in virus isolates from outbreak sites of Ethiopia. Most of the serotype identifications were done after isolation on KC cells at IAH. Serotypes were then identified by Seg-2 type specific real time RT-PCR. For AHSV-6, identification as AHSV was first made by group-specific real time RT-PCR and then identified as AHSV-6 by sequencing Seg-2.

The sequence data indicated that the isolate of AHSV-2, IAH ref collection number ETH2010/01, is directly derived from a vaccine strain (represented by ETH2010/02). Nt 1038/3218 of Seg-2 from this isolate was sequenced and compared, identifying it as AHSV-2. Nt 1038/3218 of Seg-2 from ETH2010/01 was sequenced and compared showing 96% identity to Seg-2 of the reference strain of AHSV-2, identifying it as this serotype. Seg-2 of ETH2010/01 also shows 100% identity to one of the two sequences that was derived from the Ethiopian vaccine strain (ETH2010/02), indicating a very recent 'common origin' to the AHSV-2 component of this vaccine (Table 8).

Nt 1967 onwards of Seg-2, were sequenced from the Ethiopian AHSV sample, IAH ref collection No. ETH2010/05, has revealed a close relationship to the AHSV-4 reference strain, identifying it as AHSV-4. It also has a very close relationship with field isolate ETH2010/07 consistent with being derived from the same lineage/outbreak. However, it shows a more distant relationship with the Ethiopian AHSV-4 vaccine (ETH2010/08), indicating that it was not derived from the vaccine strain (Table 8).

A sample from Yerer PA in Ada'a district, IAH ref collection number ETH2010/19, was identified as AHSV-6. Nt 3202/3202 of Seg-2, were sequenced from this ETH2010/19, showing 91.5% sequence identity with Seg-2 from the reference strain of AHSV-6, identifying it as this serotype (Table 8).

A sample from Filtino village in Ada'a region, IAH ref collection number ETH2010/10) was identified as AHSV-8. NT 3217/3217 of Seg-2 were sequenced from this isolate, showing very high levels of identity to Seg-2 of another field isolate ETH2010/11, consistent with being derived from the same lineage/outbreak. ETH2010/10 also shows high levels of

similarity in Seg-2 (97.5%) to the reference strains of AHSV-8, identifying it as this serotype (Table 8).

A culture of sample collected from Cheta district, IAH ref collection number ETH2010/17, was identified as AHSV-9. NT 1266/3202 of Seg-2, were sequenced from this isolate, showing high levels of similarity in Seg-2 to the reference strains of AHSV-9 (96.6%) identifying it as this serotype. The level of identity between the two field strains ETH2010/17 and ETH2010/16 (95.4%) indicates that the strains originated from different lineages and may represent different outbreaks (Table 8).

Table 8. Comparison of most related viruses for Seg-2 of AHSV reference strains

Virus type / name	Reference collection No.	No. nt compared/ segment length	% Identity
AHSV-2 Ethiopia 2010 field strain	ETH2010/01	1038/segment length not determined	100%
AHSV-2 Ethiopia 2010 vaccine strain	ETH2010/02	1038/segment length not determined	100%
AHSV-2 reference strain	RSArrah/02	1038/3221	96%
AHSV-4 Ethiopia 2010 field strain	ETH2010/07	1967 / Seg-2 length not determined	99.6%
AHSV-4 reference strain	RSArrah/04	1967 / 3229	97.9%
AHSV-4 Ethiopia 2010 vaccine strain	ETH2010/08	1967 / 3229	91.4%
AHSV-6 Ethiopia 2010 field strain	ETH2010/19		
AHSV-6 reference strain	RSArrah/06	3203/3203	91.5%
AHSV-8 Ethiopian 2010 field isolate	ETH2010/11	3217/3217	99.8%
AHSV-8 reference strain	RSArrah/08	3217/3217	97.5%
AHSV-9 Ethiopian 2010 field isolate	ETH2010/16	1266/3202	95.4%
AHSV-9 reference strain	PAKrrah/09	1266/3202	96.6%

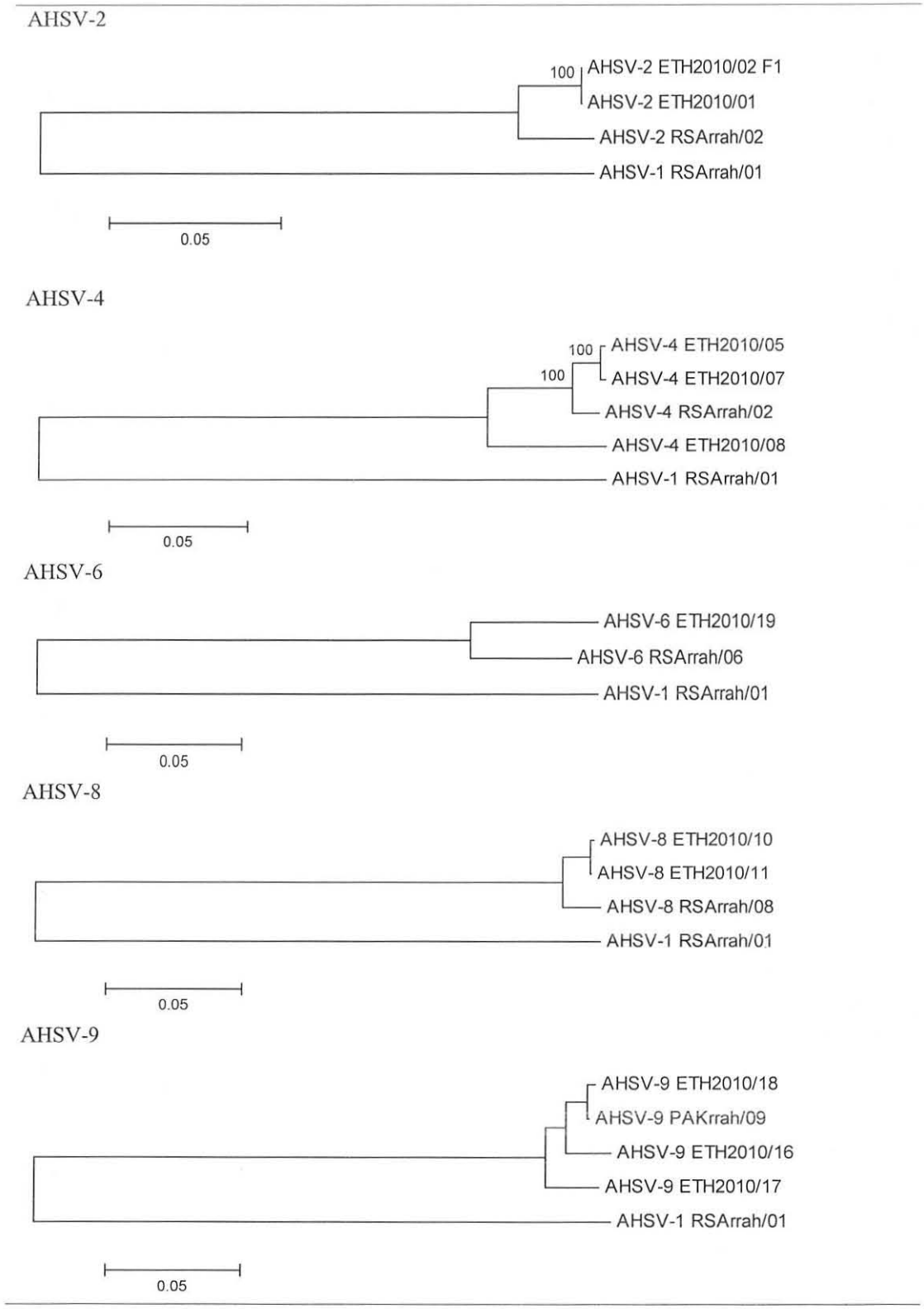


Figure 10. Comparison of AHSV-2, 4, 6, 8 and 9 identified from Ethiopia to respective IAH AHSV reference strains and IAH AHSV-1 reference strain

5 DISCUSSION

Outbreak investigation was carried out on 10 outbreaks which occurred in two periods. Four of them occurred between September and November and the rest six outbreaks occurred between April and June 2010. This is in agreement with the fact that outbreaks of AHS occurs after the rainy seasons (Nawathe *et al.*, 1981; Leforban *et al.*, 1983; Baylis *et al.*, 1999).

The study indicated the existence of all forms of AHS in the outbreaks which primarily affected horses. Majority (7/10) of the outbreaks investigated occurred in Oromia region. A previous report indicated that the disease exists in all regions of low and medium altitudes of the country (Leforban *et al.*, 1983). The cardiac form was the most common (54.8%) form followed by the horse sickness fever (35.7%), pulmonary (7.1%) and mixed forms (2.4%). This finding is in agreement with the work of Kazeem *et al.* (2008) in Nigeria. In all cases, typical clinical and postmortem findings were obtained as described elsewhere (OIE, 2008; Radostits *et al.*, 2007). The cardiac form demonstrated oedema of the head region and hyperaemia of serosal surfaces while the pulmonary form showed dyspnea, yellowish foamy nasal discharge and tachypnoea. Postmortem lesions from this form included hydropericardium and pettechial hemorrhages on serosal surfaces of the tongue and the heart. In the pulmonary form hydrothorax, edema of the lung, yellowish frothy discharges along the respiratory tract were observed. In the affected mules there was no death and only the cardiac and horse sickness forms were seen suggesting that they are more resistant species (Radostits *et al.*, 2007).

An attempt made to identify the AHSV serotype responsible for causing the outbreaks using RT-PCR method in the NVI was unsuccessful and only detected the presence of AHS viral genome in the samples. This could be due to failure in optimization of the test. Thus, the outbreak samples submitted to the IAH were examined for virus isolation, identification of serotypes, sequencing and phylogenetic analyses. A total of 49 (58.33%) of the total submitted samples were detected negative. Similar outcomes happened in an experimental infection stating that a viraemic horse after 6 to 14 days post infection remained consistently negative by RT-PCR and AHSV viral RNA was detected by RT-PCR in the blood of 4 donkeys and 4 mules up to 55 days after their infection despite the absence of any detectable

infectious virus (Zientara *et al.*, 1998a). The same scenario could have resulted in negative results of equids with suggestive clinical signs of AHS.

Five serotypes of AHSV have been identified. These are AHSV-2, 4, 6, 8 and 9. The identification of AHS-4, AHSV-6 and AHSV-8 are the first report to the country. Leforban *et al.* (1983) has reported the existence and abundance of serotype 9. Their work also included a report of AHSV-7 in Jimma district. AHSV-9 (74.3%) was the dominant serotype identified in the current study as well. However, AHSV-7 was not identified at the current investigation. AHSV-9 was isolated from horses which were died of both cardiac and pulmonary forms indicating its fatal consequences as a pathogenic organism. A study in Namibia also indicated that the virus has a capacity to kill unvaccinated horses (Scacchia *et al.*, 2009). A recent report to OIE has revealed the identification of AHSV-2 from SNNPRS and west Oromia in 2008 after killing 2,185 equidae (WAHID Interface, 2008).

In most outbreak areas multiple serotypes were isolated and identified. Ada'a district had all the isolated serotypes while equids in Wonji and Awash Melkassa both were affected by AHSV-4 and AHSV-9. In Cheta, the only involved site from SNNPRS, AHSV-9 was identified. This site was also involved in the outbreak of AHS by AHSV-2 in 2008 (WAHID Interface, 2008). In Akaki sub city AHSV-9 was identified. Previous serological studies demonstrated serogroup-antibodies against AHSV in this and other sites (Kassa, 2006).

Sequencing and phylogenetic analyses indicated multiple serotypes circulating in the outbreaks. The existence of 100% identity between the isolate of AHSV-2 from Ada'a (Hidie) of collection number ETH2010/01 and a submitted NVI vaccine strain (ETH2010/02) may be due to accidental laboratory contamination of the sample with the vaccinal strain. Since it was only identified from one sample, it was impossible to compare with other field isolates and hence, its existence in the outbreak sites may necessitate further investigation and it is excluded from the current report.

AHSV-4 was identified from five horses (ETH33/10, ETH34/10, ETH35/10). This is a first report of AHS-4 in Ethiopia. Three of the isolates were identified from neighboring towns in Ada'a district while the other two were also relatively close to each other. It was interesting to note the close identity between samples collected from Debre Zeit (ETH34/10) and Awash Melkassa (ETH50/10) with a distance of over 70 kilometers. However, it had been reported

that *Culicoides* spp. can travel a distance of 700 km at temperatures ranging from 12 to 35^{0}C (Mellor *et al.*, 2000). AHSV-6 was identified from a relatively further locality in the Ada'a district (Yerer). It showed 91.5% sequence identity with Seg-2 from the reference strain of AHSV-6 (ETH08/10). AHSV-6 is identified for the first time in Ethiopia from a field sample as the previous report was made mistakenly from a laboratory isolate sent from confirmation (Personal communication with Dr. Esayas). The identification of AHSV-8 (ETH37/10) from Debre Zeit and ETH31/10 from Filtino in Ethiopian horses is also a first report. Both isolates were identified from horses in Ada'a district. The two field isolates showed very high identity indicating that they originated from one outbreak.

The most abundant serotype occurring in all positive outbreaks was AHSV-9. The level of identity between the two field strains of ETH56/10 and ETH70/10 (95.4%) indicates that these two strains originated from different lineages and may represent different outbreaks. That could be due to geographical reasonable distance as Cheta is located in the SNNPRS and Dembie is located in Ada'a district of Oromia. Moreover, the occurrence of outbreaks had a minimum of two months difference.

Seropositivity of equids in Cheta and Tikur Inchini ditricts was determined using blocking ELISA. The test result indicated that there was statistically significant difference in seropositivity of the two districts (p=0.032). Equids in Cheta district (49.4%) had a higher seropositivity than Tikur Inchini (37.8%). This can be justified as Cheta had higher incidence rate of infection every year. Kassa (2006) has reported agro-ecological based variation of seropositivity with the highest seropevalence in lowlands followed by midland and highland areas. Bitew *et al.* (2011) recently reported a completely reversed rank of seropositivity. In the current study, Tikur Inchini was found to have a higher altitude than Cheta. However, statistically significant difference was not observed between seropositivity of equids and risk factors of species (p=0.801), sex (p=0.244) and age (p=0.436). The recorded seropositivity in horses, mules and donkeys were 43.1%, 48.7% and 43.0%, respectively. Kassa (2006) reported a statistically significant difference in seropositivity among the three species of equids with the highest seropositivity in donkeys (29.7%) followed by horses (10.4%) and mules (10.3%). Bitew *et al.* (2011) has reported the highest seropositivity in donkeys (51.1%) followed by mules (30.2%) and horses (28.3%). These inconsistent findings among studies were probably due to lack of reliable history on vaccination status of equids and/or differences in vector activities among the geographically distinct study sites. This may

question the use of the seropositivity tests as a means of detection of infection in such endemic areas as the tests does not discriminate between infection and vaccination with the available live attenuated vaccine. The finding of high seropositivity on horses is not surprising. A previous seroepidemiological study in the Gambia detected seropositivity as high as 81% on horses (Staeuber *et al.*, 1993). A recent report also indicated a subclinical condition of AHS on naturally infected cases (Weyer *et al.*, 2010). Lack of statistically significant difference in seropositivity of age and sex groups was a consistent finding among the current and previous studies.

The data obtained from the 178 respondent equine owners revealed that the disease was not considerably identified. There was also lack of sufficient information on the transmission of the disease. Respondents from Cheta district provided higher level of information indicating that the disease was more common in the district. The known reservoir of the disease, zebra, was not known to be present in both areas. Few respondents considered AHS as a contagious disease while other did not know how it transmits among the equine population. Local name of AHS was different in different areas. It is named as 'Derbetu' and 'Dibe Ferda' in Tikur Inchini (Oromia region) and 'Horyo' in Cheta (SNNPRS). Equine biting insects were known to be abundant in these areas and the suspected vectors according to the respondents are named as 'Bombisa' in Tikur region and 'Gemo' and 'Yubo' in Cheta. The respondents mentioned that AHS was commonly occurred as an epidemic during the months of September to November in Tikur and May to September in Cheta districts. However, all of the equids were reported to be kept in free environment where there was no protection from flies. Equids in both localities were used primarily for transportation of goods and people from place to place.

The retrospective data analysis for the past three years between January 2007 and December 2009 indicated that a total of 482 outbreaks were reported to APHRD in the MoARD. The results indicated that AHS was reported from six regions of the country, namely Afar, Amhara, Dire Dawa, Oromia, SNNPRS and Tigray. The overall highest frequency of outbreaks was observed in the duration from August to December in all the three years. This seasonality is in agreement with reports elsewhere indicating that the disease is more common after rainy season (Baylis *et al.*, 1999; Radostitis *et al.*, 2007).

6 CONCLUSION AND RECOMMENDATIONS

AHS is endemic and series problem of horses and mules in central, eastern and southern parts of Ethiopia. It causes significant economical impact where equine owners have insufficient information on nature of the disease and its transmission and the need for regular vaccination programmes. The cardiac form of the disease is the most common form of AHS in outbreaks of central, eastern and southern parts of Ethiopia although all forms of AHS observed. Multiple serotypes of AHSV identified, which are AHSV-4, AHSV-6, AHSV-8 and AHSV-9. Identification of AHSV-4, AHSV-6 and AHSV-8 is first report to the country and indicates increasing tendency of risk from AHS. Among the serotypes identified AHSV-9 is the major circulating virus in central and southern parts of Ethiopia. The comparisons of ASHVs made between the identified isolates to the reference isolates of the IAH, Pirbright will help as benchmark for further investigations and comparison among Ethiopian AHSV isolates and neighboring countries. Based on these concluding remarks, the following are recommended:

- The identification of multiple serotypes in the current study in a limited area warrants that country should be prepared for possible occurrence of devastating outbreaks which might result from the existent multiple pathogenic serotypes by producing vaccines which confer protection against all identified serotypes.
- Extensive awareness enhancement strategies should be designed and implemented for regular prophylactic vaccination of horses and mules against AHSVs and early detection of outbreaks.
- Further countrywide investigations of outbreaks and identification of other circulating serotypes is recommended in order to get complete picture of circulating AHSV serotypes and prevent future occurrences of outbreaks through development of polyvalent vaccines for all AHSVs.

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8 ANNEXES

Annex 1. Test procedure used for blocking ELISA

This is test procedure for blocking ELISA using commercial kit called Ingezim Compac Plus 14.AHS.K3, Spain. Washing solution, conjugate and substrate were reconstituted in the laboratory following manufacturer's instruction. In a well of polystyrene plate, 20µl of each sera sample was diluted in 80µl of the provided diluents. Then, each diluted serum was homogenized with new pipette and then 100µl/well of each positive and negative control solution was dispensed in duplicate wells. The plate was covered and incubated for 1 hour at 37^0C. The content was discharged and the plate was washed five times using 300µl/well of washing solution. Then, the plate was turned over on an absorbent cotton towel with gentle hitting until all droplets removed. In each well 100µl of the prepared conjugate was added and incubated for 30 minutes at 37^0C. This was washed five times as described above and 100µl/well of the prepared substrate solution was added using multichannel pipette and incubated for 10 minutes at room temperature. Finally, 100 µl/well of the stop solution was added and the optical density (OD) value reading was done with a spectrophotometer at 405nm wavelength.

Annex 2. Test procedure used for virus isolation

3ml of whole blood sample was mixed with equal volume of PBS in a sterile test tube and centrifuged at 2500 rpm for 5 minutes and the supernatant was decanted and the blood cells were washed twice with 7ml PBS and centrifuged in the same way and the supernatant decanted. Hemolysis of RBCs was done by diluting in 1:10 proportion of sterile distilled water at PH 7.4.

For culturing, 0.5ml of the lysate was inoculated into confluent monolayers of Vero cells grown in 25 square centimeters plastic tissue culture flask. After incubation for 1 hour at 37^0C of adsorption time, the inoculated suspension was discarded. Then, the culture was washed with 10ml sterile PBS and the cell culture flask was re-filled with 10ml of minimal essential medium (GMEM) supplemented with 5% fetal calf serum and antibiotics (penicillin

G 100 IU/ml and streptomycin 100 μg/ml) and incubated at 37^0C. The culture was observed daily for the development of CPE. Sub-culturing was done by harvesting 1ml of cell suspension after three consecutive freezing and thawing of the culture which showed 80% or more CPE or at two weeks postinoculation from others. When CPE was not observed in three blind passages, then the sample was considered negative for virus isolation.

Annex 3. Test procedure for RT-PCR

This is viral double-stranded RNA extraction procedure using the commercial RNeasy Mini Kit (Qiagen). For purification of viral RNA using spin technology from cell culture supernatants, 460 μl of the sample was added into a 2 ml collection tube. An equal volume of lysis buffer RLT was added and mixed by vortexing. Then, 460 μl of 70% ethanol was added and mixed to the homogenized lysate. At first 700 μl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube. The lid was gently closed and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. The remaining 680 μl sample was also centrifuged in the same RNeasy spin column. The flow-through was discarded and the spin column was washed with 700 μl of wash buffer RW1 and centrifuged as before. Second wash was made with 500 μl of another wash buffer RPE and centrifuged as above for 15 seconds. The later step was repeated with centrifuging for 2 minutes to dry the membrane. The RNeasy spin column was then place on a new 2 ml collection tube and the used one with its flow-through was discarded. The lid of the RNeasy spin column was gently closed and centrifuged for 1 minute at 10,000 rpm. Finally, the spin column was placed on a new 1.5 ml collection tube and 50 μl of Rnase free water was added directly to the spin column membrane. The lid was closed and centrifuged for 1 minute at 10000 rpm to elute the RNA. Then the product was labeled and stored at -20^0C until used for PCR amplification.

The RNA was reverse transcribed into cDNA in a reverse transcription (RT) reaction. For this application, AHSV dsRNA was first denatured by placing the tubes in a thermal cycler at 95^0C for 5 minutes in a 10 μl mix that contained 2 μl of sample RNA template, 7 μl of nuclease-free sterile water, primer 1, 20 pmol/μl (0.5 μl) and primer 2, 20 pmol/μl (0.5 μl). After brief centrifugation of the tubes to remove drops from the inside of the lid, they were immediately chilled on ice.

In a sterile 1.5 ml microcentrifuge tube RT-PCR reaction mixture was prepared in bulk for the number of samples to be assayed allowing for one extra sample. The per reaction mixture was composed of Nuclease-free sterile water (5 µl), PCR Buffer 5× with magnesium chloride (5 µl), dNTP mix 10 Mm (0.5 µl), Q solution 5x (5µl), Rnase inhibitor 20 U/µl (0.25 µl) and 1 µl Enzyme Mix (Omniscript and Sensiscript Reverse Transcriptases, HotStartTaq DNA polymerase). To each PCR tube containing the denatured RNA template, 15 µl of the RT-PCR mix was added and a positive reaction control (2 µl of the AHSV serotype 9 RNA prepared from the NVI vaccine) and a negative reaction control (2 µl of distilled water) were included.

All the tubes including the positive and negative controls were placed in an automated thermal cycler and reverse transcription–amplification was carried out in one-step with the following incubation program: one cycle at 55^0C for 30 minutes for cDNA synthesis, one cycle at 95^0C for 15 minutes, 40 cycles at 94^0C for 30 s, 58^0C for 30 s, 72^0C for 30 sec, and finally one cycle 72^0C at for 7 minutes and held at $+4^0$C. Then, 10 µl of each sample was mixed with 2 µl of 10x loading buffer and loaded in 1.5% agarose gel in TAE buffer containing 0.5 µg/ml of ethidium bromide and 6 µl of a standard 100 base-pair ladder (marker) was mixed with 2 µl of the 10x loading buffer and loaded on separate lanes on either side. Finally, the amplification products were analysed by electrophoresis at 120 volts for 45 minutes (Belen *et al.*, 2008) and the result from the gel was examined over a UV light source. In a positive sample, a discrete band was present and the size of the PCR product was calculated by reference to value for the standard markers used. Serotypes of the infecting AHSV were identified according to published band results for each serotype and the specificity of the primers used.

Annex 4. Outbreak investigation and questionnaire format

OUTBREAK INVESTIGATION OF AFRICAN HORSE SICKNESS (ETHIOPIA)																	
Code: ET / / / /									**GENERAL INFORMATION ON THE AREA**								
DATE OF REPORT TO NVI	d	d	m	m	y	y	y	y	**Geographical location**						Altitude (m.a.s.l.):		
									East			South			Annual Average Temp.:		
PERSON WHO DIAGNOSED THIS OUTBREAK:									Deg.	Min.	Sec.	Deg.	Min.	Sec.	Annual Average Rainfall:		
Name:				Tel											Annual Average Humidity:		
Farmer/owner				Gov't vet					Region:						Wereda:		
A.H.A.				Private vet					Zone:						PA/Kebele:		
Other									No of equids: Horses-			Mules-			Donkeys-		
NATURE OF DIAGNOSIS									**CONTACT DETAILS OF OWNER:**								
Suspicion	Clinical			Postmortem					Owner's Name:						Kebele:		
Laboratory	Other								Owner's Address:						Owner's tel. no:		
Laboratory where diagnosis was made:									Diagnostic test used:								

Sampling date	(dd / mm / yyyy)										(dd / mm / yyyy)							
Sample type:	whole blood (in heparin)				serum				**Fresh tissues**	lung		spleen		lymph node		other	
Method of transportation:																		

DETAILS OF OUTBREAK:	DESCRIPTION OF EXAMINED ANIMAL	SUSCEPTIBLE ANIMALS OWNED
No. of cases: Horses- Mules- Donkeys-	Species and breed:	No of horses:
No dead or killed: Horses- Mules- Donkeys-	Age in years:	No of mules:
Date of initial detection of current outbreak: (dd / mm / yyyy)	Sex:	No of donkeys:
	Type of work:	Dogs:

CLINICAL SIGNS SHOWN: **pulmonary form** **cardiac form** **mixed form** **mild form**

Descriptions:

VACCINATION HIISTORY: Unknown No Yes, date of last vaccination (dd / mm / yyyy):

EPIDEMIOLOGICAL COMMENTS	
Do you know a disease characterized by dullness, supraorbital edema, respiratory distress and frothy nasal discharge? No. Yes, local name?	
Have you seen the above mentioned disease on your equids with in the past two years? Yes No If yes, which species of equids? Horse Mule Donkey	
If yes, when was the date of commencement of the first sign? (dd / mm / yyyy)	
In which months of the year the disease occurs? Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec	
Which species are more affected? Rank them _____ Horses, _____ Mules, _____ Donkeys	
How long have you had this animal (sampled)?	How is the disease transmitted?
If less than 40 days, where was the animal come from?	Are there equine biting insects in your locality? Yes No
	Local name of vectors:
How far is the place in Km?	Are your stables insect proof? Yes No, it is open air. Other
Previous outbreak in the area? No Yes Unknown	Are there zebras in the area? Yes No
If yes, when was it last occurred (dd / mm / yyyy)?	Other comments:

Investigated by _____ Date: _____ Signature:

Annex 5. Result of identified AHSV serotypes from whole blood samples submitted to IAH, Pirbright, UK

Sample ref. no.	IAH dsRNA Virus collection no.	Sample	Species	Collection Area	Date of collection	Serotype	Result	Ct
ETH/AHS 01/10		EDTA blood	Horse	Bonga/Cheta	19/04/2010		neg	No Ct
ETH/AHS 02/10		EDTA blood	Horse	Ada'a/Godino	10/06/2010	9	pos	27.14
ETH/AHS 03/10		EDTA blood	Horse	Akaki	11/06/2010	9	pos	28.00
ETH/AHS 04/10		EDTA blood	Horse	Ada'a/Debre zeit	15/06/2010	9	pos	33.27
ETH/AHS 05/10		EDTA blood	Horse	Wonji	16/06/2010	9	pos	27.23
ETH/AHS 06/10		EDTA blood	Horse	Ada'a/Debre zeit	21/06/2010		neg	No Ct
ETH/AHS 07/10		EDTA blood	Horse	Ada'a/Debre zeit	21/06/2010	9	pos	37.46
ETH/AHS 08/10	ETH2010/19	EDTA blood	Horse	Ada'a/Yerer	21/06/2010	6	pos	29.56
ETH/AHS 09/10		EDTA blood	Horse	Ada'a/Filtino	21/06/2010	9	pos	29.28
ETH/AHS 10/10		EDTA blood	Horse	Ada'a/Hidie	21/06/2010	9	pos	25.21
ETH/AHS 11/10	ETH2010/01	EDTA blood	Horse	Ada'a/Hidie	21/06/2010	2	pos	19.71
ETH/AHS 12/10		EDTA blood	Horse	Ada'a/Debre zeit	21/06/2010	9	pos	27.09
ETH/AHS 13/10		EDTA blood	Horse	Ada'a/Dalota	21/06/2010	9	pos	24.76
ETH/AHS 14/10		EDTA blood	Horse	Akaki	21/06/2010	9	pos	26.64
ETH/AHS 15/10	ETH2010/13	EDTA blood	Horse	Akaki	21/06/2010	9	pos	26.87
ETH/AHS 16/10		EDTA blood	Horse	Akaki	21/06/2010		neg	No Ct
ETH/AHS 17/10		EDTA blood	Horse	Wonji	21/06/2010	9	pos	27.14
ETH/AHS 18/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct
ETH/AHS 19/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct

Continued

Sample ref. no.	IAH dsRNA Virus collection no.	Sample	Species	Collection Area	Date of collection	Serotype	Result	Ct
ETH/AHS 20/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct
ETH/AHS 21/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct
ETH/AHS 22/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct
ETH/AHS 23/10	ETH2010/03	EDTA blood	Horse	Wonji	21/06/2010	4	pos	29.90
ETH/AHS 24/10		EDTA blood	Horse	Wonji	21/06/2010		neg	No Ct
ETH/AHS 25/10		EDTA blood	Horse	Adama	22/06/2010		neg	No Ct
ETH/AHS 26/10		EDTA blood	Horse	Adama	22/06/2010		neg	No Ct
ETH/AHS 27/10		EDTA blood	Horse	Ada'a/Debre zeit	22/06/2010		neg	No Ct
ETH/AHS 28/10		EDTA blood	Horse	Ada'a/Debre zeit	03/07/2010	9	pos	30.08
ETH/AHS 29/10		EDTA blood	Horse	Ada'a/Debre zeit	23/06/2010		neg	No Ct
ETH/AHS 30/10		EDTA blood	Horse	Ada'a/Filtino	24/06/2010	9	pos	33.69
ETH/AHS 31/10	ETH2010/10	EDTA blood	Horse	Ada'a/Filtino	24/06/2010	8	pos	24.13
ETH/AHS 32/10		EDTA blood	Horse	Ada'a/Erobe	24/06/2010		neg	No Ct
ETH/AHS 33/10	ETH2010/04	EDTA blood	Horse	Ada'a/Gotia	24/06/2010	4	pos	27.91
ETH/AHS 34/10	ETH2010/05	EDTA blood	Horse	Ada'a/Debre zeit	24/06/2010	4	pos	28.30
ETH/AHS 35/10	ETH2010/06	EDTA blood	Horse	Ada'a/Debre zeit	24/06/2010	4	pos	30.29
ETH/AHS 36/10		EDTA blood	Horse	Ada'a/Debre zeit	24/06/2010		neg	No Ct
ETH/AHS 37/10	ETH2010/11	EDTA blood	Horse	Ada'a/Debre zeit	25/06/2010	8	pos	31.85
ETH/AHS 38/10		EDTA blood	Horse	Ada'a/Gotia	29/06/2010	9	pos	28.44
ETH/AHS 39/10		EDTA blood	Mule	Ada'a/Gichi	26/06/2010	9	pos	27.45

Continued

Sample ref. no.	IAH dsRNA Virus collection no.	Sample	Species	Collection Area	Date of collection	Serotype	Result	Ct
ETH/AHS 40/10		EDTA blood	Horse	Ada'a/Dembie	03/07/2010	9	pos	30.52
ETH/AHS 41/10		EDTA blood	Horse	Ada'a/Dalota	01/07/2010	9	pos	29.98
ETH/AHS 42/10		EDTA blood	Horse	Ada'a/Debre zeit	28/06/2010	9	pos	29.27
ETH/AHS 43/10		EDTA blood	Horse	Ada'a/Debre zeit	28/06/2010	9	pos	27.42
ETH/AHS 44/10		EDTA blood	Horse	Ada'a/Debre zeit	28/06/2010	9	pos	27.27
ETH/AHS 45/10		EDTA blood	Horse	Akaki	02/07/2010		neg	No Ct
ETH/AHS 46/10		EDTA blood	Horse	Akaki	02/07/2010		neg	No Ct
ETH/AHS 47/10		EDTA blood	Horse	Akaki	02/07/2010		neg	No Ct
ETH/AHS 48/10		EDTA blood	Horse	Akaki	02/07/2010	9	pos	28.92
ETH/AHS 49/10	ETH2010/12	EDTA blood	Horse	Awash Melkassa	29/06/2010	9	pos	27.46
ETH/AHS 50/10	ETH2010/07	EDTA blood	Horse	Awash Melkassa	29/06/2010	4	pos	29.46
ETH/AHS 51/10		EDTA blood	Horse	Ada'a/Debre zeit	08/07/2010	9	pos	32.38
ETH/AHS 52/10		EDTA blood	Horse	Ada'a/Debre zeit	08/07/2010		neg	No Ct
ETH/AHS 53/10		EDTA blood	Horse	Ada'a/Debre zeit	08/07/2010		neg	No Ct
ETH/AHS 54/10		EDTA blood	Horse	Ada'a/Debre zeit	12/07/2010	9	pos	28.01

Annex 7. Pictures of exemplary clinical cases observed during outbreaks and postmortem findings

Plate 1. Supraorbital fossae edema (cardiac form)

Plate 2. Generalized edema of the head region(cardiac form)

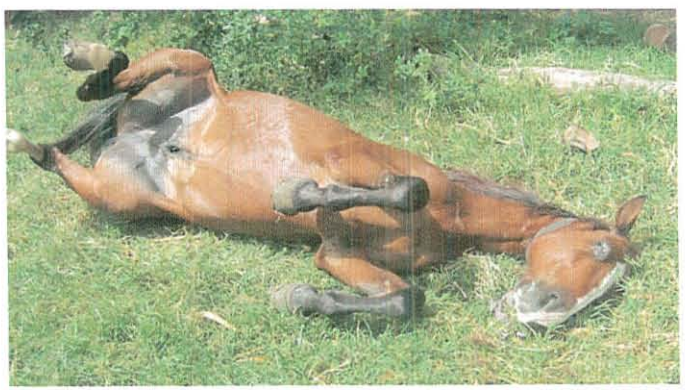
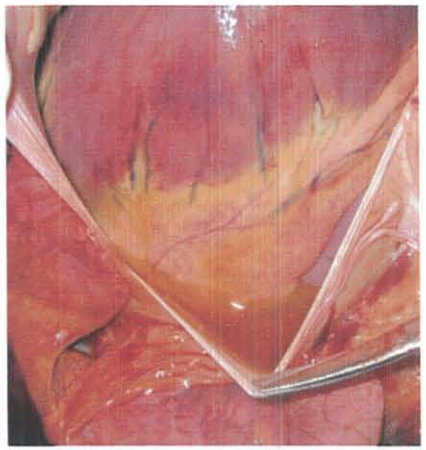


Plate 3. Colic at the terminal stage of cardiac form of AHS

Plate 4. Pettechiation of serosal surface of the tongue (cardiac form)



Plates 5. Hydropericardium and pettechiation of serosal surface of the heart

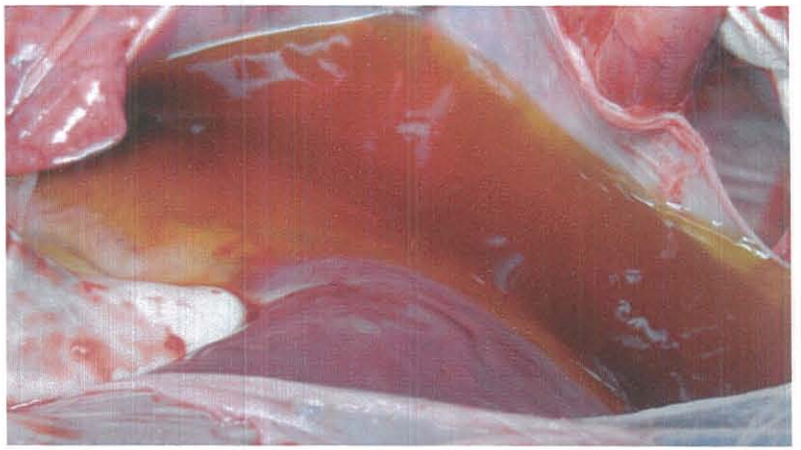


Plate 6. Hydropericardium and pettechiation of serosal surface of the heart

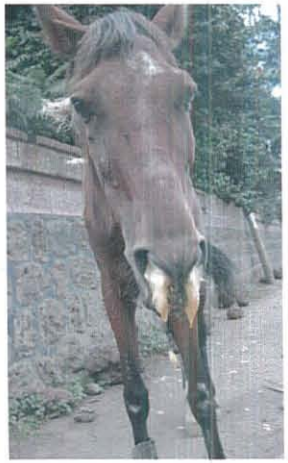


Plate 7. Bilateral yellowish and foamy discharges through nostrils (pulmonary form)

Plate 8. Bilateral yellowish and foamy discharges through nostrils (pulmonary form)

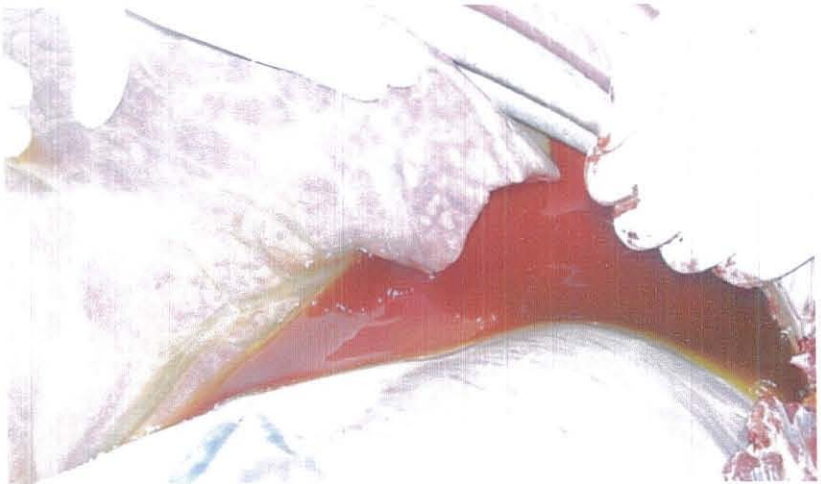


Plate 10. Hydrothorax and edema of the lung (pulmonary form)

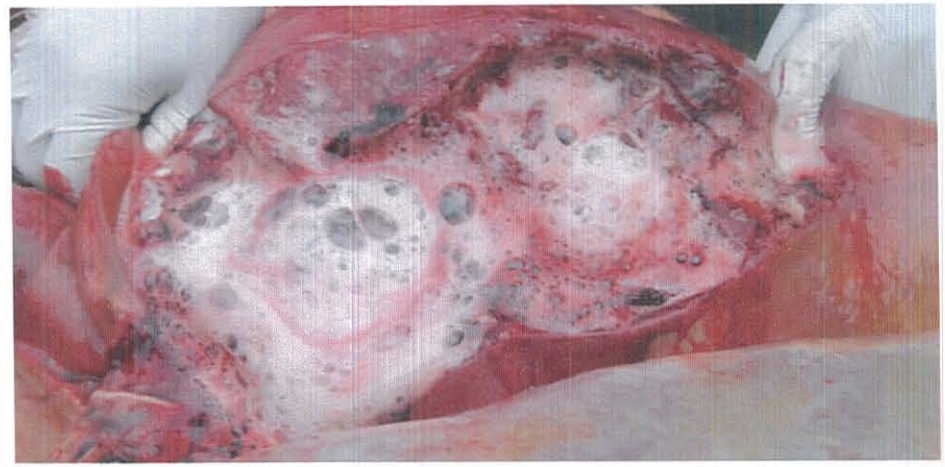


Plate 11. Foamy discharges after incising bronchioles of the lung (pulmonary form)

Plate 11. Mixed form of AHS. Note both edema of supraorbital fossae and bilateral yellowish discharge through nostrils.

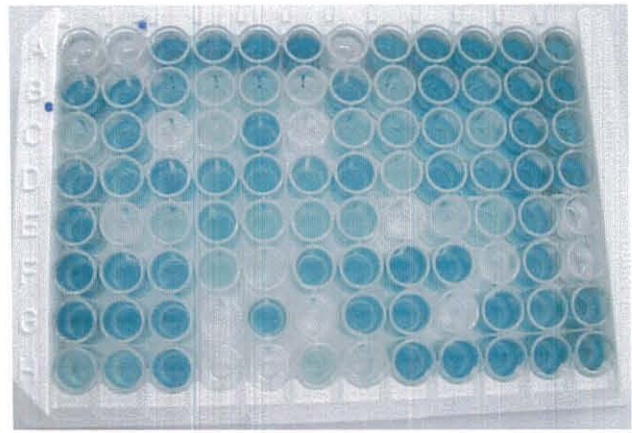


Plate 12. Blocking ELISA test result ready for a spectrophotometer reading. A1 and A2 are positive controls and B1andB2 are negative controls.

9 SIGNED STATEMENT OF DECLARATION

This thesis is my original work, has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name _____

Signature _____

Date of submission _____

This thesis has been submitted for examination with my approval as a University advisor

Name _____

Signature _____

Date _____