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ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

ISOLATION AND MOLECULAR DETECTION OF AFRICAN HORSE SICKNESS VIRUS FROM AHS OUTBREAK CASES IN HORSES IN SELECTED AREAS OF ETHIOPIA

BY

DEGU FHETANEGEST

AUGUST 2021
BISHOFTU, ETHIOPIA

ISOLATION AND MOLECULAR DETECTION OF AFRICAN HORSE SICKNESS VIRUS AHS OUTBREAK CASES IN HORSES IN SELECTED AREAS OF ETHIOPIA

A thesis submitted to the College of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Veterinary Microbiology

BY

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As MVSc research advisor, we here by certify that we have read and evaluated this thesis prepared under our guidance by entitled: “Isolation and Molecular Characterization of African Horse Sickness Virus AHS Outbreak Cases In Horses in selected areas of Ethiopia” we recommended that it can be submitted as fulfilling the MVSc Thesis requirement.

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AUGUST 2021

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As member of the Board of Examiners of the MVSc, Open Defense Examination, we certify that we have read and evaluated the thesis prepared by Degu Fhetanegest and examined the candidate. We recommended that it can be accepted as fulfilling the thesis requirement for the degree of Master Science in Veterinary Microbiology.

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ABBREVIATIONS

AHSV	African Horse Sickness Virus
BHK	Bovine hamster Kidney
CFT	Complement Fixation Test
CPE	Cytopathic Effect
CSA	Central Statistics Agency
ddNTPs	Dideoxynucleotides Triphasphates
DIVA	Differentiate Between Infected and Vaccinated
DMEM	Dulbco's Minimum Essential Medium
RNase	Ribonuclease
dsRNA	Double Stranded Ribonucleic Acid
EDTA	Ethylenediaminetetra-Acetic Acid
LAV	Live Attenuated Vaccine
MAb	Monoclonal Antibody
GMEM	Glasgow Minimum Essential Medium
NS	None Structural Protein
NS1	Non-Structural Virus Protein 1
NS2	Non-Structural Virus Protein 2
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
rRT-PCR	Real Time Polymerase Chain Reaction
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
SNT	Serum Neutralization Test
TAE	Tris, Acetic Acid and EDTA
TBE	Tris, Boric Acid and EDTA
TCID	Tissue Culture Infective Dose
VIB	Viral Inclusion Bodies
VNT	Virus Neutralization Test
VP	Virus Protein

ABSTRACT

Equines play an important role in the country's economy and are a lifeline for millions of people in rural and peri-urban areas of Ethiopia. However, the productivity and welfare of equids are constrained by numerous infectious diseases especially in developing nations like African Horse Sickness disease (AHS). A H S is one of the major infectious diseases that cause severe socio-economic losses to the equine population and the national economy in general. A cross-sectional study design was undertaken in equines to isolate and detect African horse sickness virus (AHSV) from November 2019 to May 2021 in selected and epidemic areas of Ethiopia. A Total of 30 whole bloods and 2 tissue specimens were collected aseptically from recently dead and clinically sick equids that manifested prominent signs of the disease and transported under cold chain to the National Veterinary Institute, Bishoftu, Ethiopia. A total of 32 samples were subjected to conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique targeting Viral protein 7 (VP7) genes to amplify fragments of segment 7 of all serotypes using sero-group specific primers. Only 7 (21.88%) samples were detected with a band size of 102 bp fragments on a 2% agarose gel elctrophoresis. For serotyping, seven universal PCR positive samples were detected again targeting to a gene encoding viral protein 2 (VP2) using serotype-specific primers. Serotype 9 with a band size of 228 bp was identified from tissue samples. Only tissue samples were grown on Vero cells and showed cytopathic effects characterized by aggregation, rounding and detaching of cells on cell line. In conclusion, African Horse Sickness caused by serotype 9 severely affects equines results in death of horses. Strong strategic control of disease through vaccination should be done and further assessment to determine the potential of outbreaks and genotypic characterization of virus from the outbreaks and insects needs further study.

Key words: *AHS, Horse, Molecular Detection, RT-PCR, Serotype - 9*

1. INTRODUCTION

The livestock sector has been contributed a considerable amount of the country's economy and continues to promise to rally around the country's economic development (CSA, 2020). Ethiopia hosts the largest equine population following China in the world. The country's current total population of equines is 12.456 million. The country's estimated population of horse, donkeys and mules are 2.11 million, 8.98 million and 0.38 million, respectively (CSA, 2020). These animals play a vital role to the country's economy and provide a lifeline for millions of people living in rural and pri-urban areas (Aklilu and Stringer, 2014; Admassu and Shiferaw, 2011).

Horse, Donkey, Mule and camels are among draught or working animals that provide power for small scale farming and crop threshing virtually throughout the country. They are also play significant role in mode of transportation such as transport small scale farmers, families and agricultural products to the market and bring back their domestic necessities (CSA, 2018; Zeleke, 2017). However, the productivity and welfare of these equids are constrained by numerous infectious diseases especially in developing nations (Stringer, 2014). Among the infectious disorders that bring serious socio-economic consequences of the equine populations are African horse sickness and Epizootic lymphangitis disease. In general, African horse sickness (AHS) and epizootic lymphangitis are major infectious diseases that cause severe socio-economic losses to the equine population and the national economy (Admasu and Shiferaw, 2011). So understanding the importance and causes of these devastating equine diseases is crucial in order to prevent and control effectively.

African horse sickness is an acute or sub-acute, non-contagious, arthropod-borne viral disease, characterized by severe pyrexia, widespread haemorrhages and oedematous exudations (Stoltz *et al*., 1996). It is caused by the African horse sickness virus which is a virus species within the genus *Orbivirus* belonging to the *Reoviridae* family. AHSV is a non-enveloped virus with a linear double-stranded RNA genome of ten segments encoding for seven structural (four major and three minor) and five non-structural proteins (Roy *et al*., 1994). Nine antigenically distinct serotypes of African horse sickness virus (AHSV) have been identified by virus neutralization (OIE, 2017; Meiswinkel and Paweska, 1999). The disease's effect can be devastating with fatality rates exceeding 90% and it is endemic in Sub- Saharan Africa and East Africa (Mourits, 2012). AHS is an OIE listed and

regulated disease due to its severity, ability to expand rapidly out of its endemic areas, and its importance for the worldwide trade of animals (Mourits, 2012; OIE, 2020). Nine antigenically distinct serotypes of AHSV have been identified by virus neutralization test across the world (OIE, 2020; Meiswinkel and Paweska, 1999).

Although AHS disease is certainly originated from Africa, the first recorded epidemic of AHS was in 1327 in Yemen (Henning, 1956). The virus could have been introduced in to Ethiopian equines by wind- borne infected midges (Culicoides) from endemic regions of Africa (Quinn *et al*, 2002). In South Africa, the first outbreak was occurred in 1719 results in the deathof 1700 horses following sixty years after introduction of horses (Grobbelaar, 2007).

African horse sickness affects primarily equines. Horses are the most susceptible to the disease (mortality of 70%-95%) followed by mules (mortality of 50%-70%) while infections of donkeys and zebras are mostly subclinical. Generally, horses of all breeds are equally susceptible to AHS, but variation in susceptibility to the same virus in individual horses has been reported (Maureen, 2014; Coetze and Guthrie, 2004).The disease is transmited biologically by *Culicoides* species of which *Culicoides imicola* and *Bolitinos* have been shown to play an important rolein Africa (Mellor and Mertens, 2008).

A number of distinct serotypes of AHSV were detected in Ethiopia as different epidemiological survey indicated. Prior to 2005, a number of confirmed outbreaks have occurred in 1999, 2004, and 2008 and the predominant serotypes were AHSV-6 and AHSV-9 and AHSV-2 isolated in 2008. In 2005 (serotype 6 and 9), 2010 (serotype 2, 4, 6, 8 and 9), 2011(serotype 9) were identified (Aklilu *et al*., 2014; Ayelet *et al*., 2013; Zeleke *et al*., 2005). Among these serotypes, serotype 9 was the most abundant and plays a significant role in causing AHS disease outbreaks as different epidemiological survey reported in a various period of time (Aklilu *et al*., 2014; Leforban *et al*., 1983).

African horse sickness disease is the most challenging equine disease in African mainly in Ethiopia since it is endemic and several outbreaks of the disease recognized in different geographical location of the country in different periods (Guthrie, 2018; Long and Guthrie, 2014). In Ethiopia, which has 42% of the African equid population resides, vital for transportation for human and goods. The disease is leading causes of productivity and welfare constraints of equine which results in significant socio-economic impacts to the country. Morbidity and mortality within

working equids may constrain the draft power in low-income countries, thereby affecting food security, poverty alleviation, and gender equality (Carpenter, 2017). Serological and virological study evidence has been showed that the presence of the disease in many African countries. Endemic occurrence of the disease in different geographical location of the country reported from various types of studies following the first documented evidence of AHS disease in Ethiopia (Aklilu *et al*., 2014; Kasa, 2006; Zeleke *et al*., 2005; Leforban *et al*., 1983).

Based on previous AHS outbreak data from Ministry of Agriculture (MoA); a total of 961 outbreaks, 21, 270 cases, and 4073 deaths were recorded in different regions of the country between January 2007 and December 2011(Guthrie, 2018; Aklilu *et al*., 2014, Ayelet *et al.,* 2013). In spite of overwhelming death of horses caused by AHS infection, there are not well documented reports about the current circulating serotypes as well as its epidemiological status the disease in most regions of Ethiopia. Moreover, there is huge gap in identifying and characterizing the epidemic serotypes which would be important for the rapid deployment of an appropriate vaccine strain. Therefore, the objectives of this study were:

- To isolate and detect the presence of AHSV in selected areas of Ethiopia through virology and molecular methods.
- To serotype the circulating AHSV from field outbreaks

2. LITERATURE REVIIEW

2.1. Etiology

2.1.1. Taxonomic classification and Virion properties

African horse sickness virus belongs to the family *Reoviridae*, genus *Orbivirus* and shares many morphological and structural characteristics with other members of this genus, *Bluetongue virus* (BTV) and equine *Encephalosis virus* (EEV) (MacLachlan and Dubovi, 2017; Knowles *et al*., 2011). This family consists of non-enveloped multilayered viruses with a double-stranded RNA genome consisting of 9 to 12 genome segments. The *Orbivirus* genus of the *Reoviridae* family contains African horse sickness virus (AHSV), bluetongue virus, and epizootic hemorrhagic disease virus, which cause notifiable diseases and are spread by biting *Culicoides* species (Figure 1) (Attoui et al., 2011). AHSV is subdivided into nine serotypes (named AHSV-1 to AHSV-9). Cross-reactivity of the homologous antiserum to other subtypes in the Virus neutralization (VN) assay (cross-neutralization between types 1 and 2, 3 and 7, 5 and 8, and 6 and 9) indicates a partial antigenic relationship between these serotypes (Coetzer and Guthrie, 2004).

The genome of AHSV is composed of 10 double stranded RNA segments, enclosed within the core particle (Mellor & Mertens, 2008; Roy *et al*., 1994).The 10 double-stranded (ds) RNA segments are designated by its size and increasing electrophoretic mobility in 1% agarose gels and by molecular weight as L1-L3 (large segments), M4-M6 (medium segments), and S7-S10 (small segments). Both the seven structural proteins (VP1 to 7) and four non-structural proteins (NS1, NS2, NS3/A and NS4) are encoded by these 10 segments (MacLachlan and Dubovi, 2017). The non-structural proteins are encoded by the segments M5, S8 and S10. The two smallest proteins (NS3 and NS3A) are synthesized from the S10 RNA segment, probably from different in frame translation initiation codons (Roy et *al*., 1994).

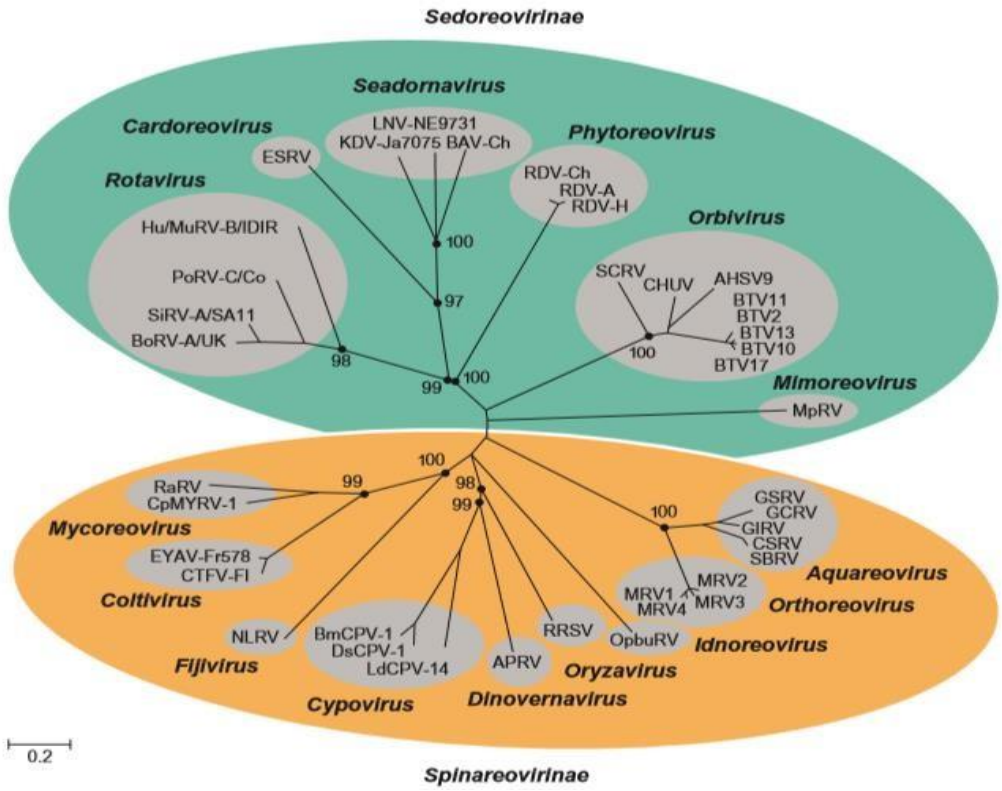


Figure 1: Phylogenetic tree (neighbor joining tree) of the family Reoviridae based on the amino acid sequence of the RNA-dependent RNA polymerase (RdRP)

Source: MacLachlan and Dubovi (2017); Attoui et *al*., (2011), Knowles *et al*., (2011).

2.1.2. Genomic and protein Structure

Virions are non-enveloped particles with a diameter of around 70 nm and a two-layered icosahedral capsid made up of 32 capsomeres. The genome of virus is enclosed with in the core particle consisting of two major proteins VP3 and VP7. These are highly conserved among all AHSV serotypes and three minor proteins, VP1, VP4, and VP6 forms the core of the virion (Ngoveni *et al*., 2019; Bremer *et al*., 1990). Two major structural proteins, VP2 and VP5 forms the outer capsid which involved in the attachment of viral particles to target cells and virus replication and hence are often associated with virulence and pathogenesis of AHSV (Hassan and Roy, 1999; Zhang *et al*., 2010). These proteins are mainly responsible for serotype antigenic characteristics since neutralizing

epitopes are mainly located in VP2 (Figure 2) (Dennis *et al*., 2019; Dennis *et al*., 2018; Burrage *et al*., 1993).

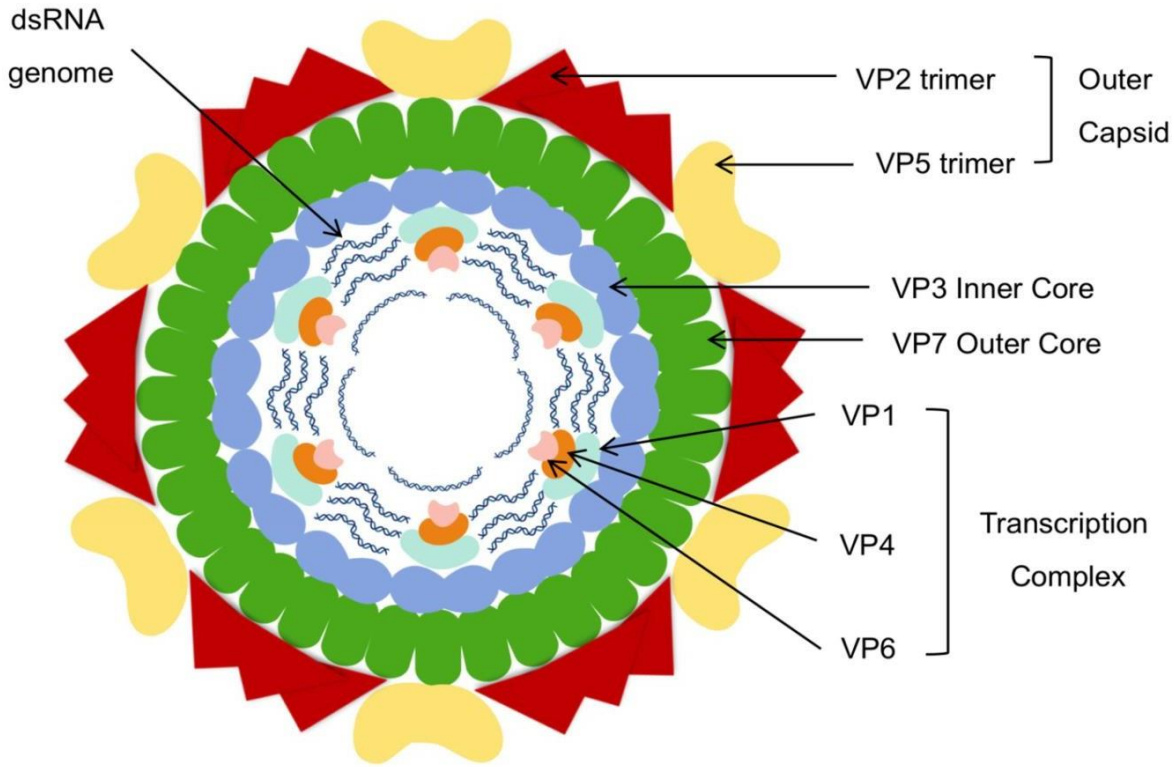


Figure 2: Schematic representation of the AHSV virion.

Source: Dennis *et al*.(2018); Lulla *et al*. (2016).

The minor proteins have different enzymatic activities such as RNA polymerase (VP1), RNA capping (VP4) and helicase activity (VP6) that all support the role of the core proteins in viral replication and transcription (van Schalkwyk *et al*., 2019, Burrage *et al*., 1993).

Five non-structural proteins, NS1, NS2, NS3, NS3a (lacking 13 N-terminal amino acids), and NS4 are synthesized in virus infected cells. The non-structural proteins are involved in virus replication (NS1), morphogenesis or assembly (NS2), and transportation or exit of from infected cells (NSP3/3A) (Foster, 2018; Roy *et al*., 1994; Iwata *et al*., 1992). The function of NSP4 has yet to be resolved but it can bind dsDNA and thought to play a similar role to the same protein found in Blue Tongue Viruse (BTV) of modulating host immunity. Viral particle release is mediated by NS3 which is the only AHSV glycosylated membrane protein. This, unlike BTV

NS3/NS3a, which is highly conserved, is the second most variable protein among different serotypes. NS3 and NS3A are encoded from two in-phase overlapping reading frames from the smallest of the ten genome segments (Table 1). In insect cells, Baculovirus expressed AHSV NS3 is membrane-associated and cytotoxic in insect cells. It's been suggested that it plays a role in virulence and influences the timing of virus release from infected cells (Iwata *et al*., 1992).

Table 1: Summary of the AHSV genome segments arrangement, the proteins they encode, molecular mass and size, location and function of each protein

RNA Segment (no. bp)	**Protein**	**Molecular mass (kDl)**	**Location**	**Function**
Seg-1 (3954)	VP1	149	Core	NA-dependant RNA polymerase
Seg-2 (2926)	VP2	111	Outer capsid	Serotype-specific antigen, neutralization, cell attachment, involved in determination of virulence
Seg-3 (2770)	VP3	103	Core	Interacts with internal minor proteins, controls overall size and organization of capsid structure
Seg-4 (1981)	VP4	76	Core	Capping enzyme, guanyl transferase, methyltransferase
Seg-5 (1769)	NS1	59	Infected cell	Not known
Seg-6 (1638)	VP5	64	Outer capsid	Helps determine virus serotype, can mediate cell fusion and has a role in cell entry
Seg-7 (1156)	VP7	38	Core	Involved in cell entry and core particle infectivity
Seg-8 (1124)	NS2	40	Infected cell	Viral inclusion body matrix protein, binds ssRNA, NTPase Activity
Seg-9 (1046)	VP6 & NS4	35/23	Core/infected cell	Binds ssRNA and dsRNA, helicase, NTPas
Seg-10 (822)	NS3 & NS3A	25/24	Infected cell	Membrane proteins involved in viral cell exit, may be involved in determination of virulence

Source: Belhouchet *et al*., (2011); Nieuwoudt, (2010); Maan *et al*., (2007); *Roy et al.*, (1994b).

2.1.3. Viral Entry and Replication

The outer capsid protein VP2, the host cellular surface receptor, and the capsid protein VP5, which has a characteristic coiled-coil motif typical of membrane fusion proteins, are thought to be responsible for AHSV host infection (Zhang *et al*., 2010).

The virus enters the cell via VP2 binding to sialic acid receptors and either clathrin-mediated endocytosis or phagocytosis. The virion is internalized into a vesicle and converted into a core particle. The acidic pH in the endosomes causes the core proteins to be released into the cytoplasm of the host cell and becomes transcriptionally active (Dennis *et al*., 2019; Mellor & Mertens, 2008). The host cell machinery is used for viral protein transcription and translation, while the VIBs act as assembly sites for the progeny virions. By NS3 interaction with calpactin, assembled core particles are then transported from the Viral Inclusion Bodies (VIB) onto exocytotic vesicles. To produce mature virions, the outer capsid proteins VP5 and VP2 are acquired during this phase. Particles are released from the cell by NS3-mediated budding or by lysis of the host cell (figure 3) (Patel and Roy, 2014).

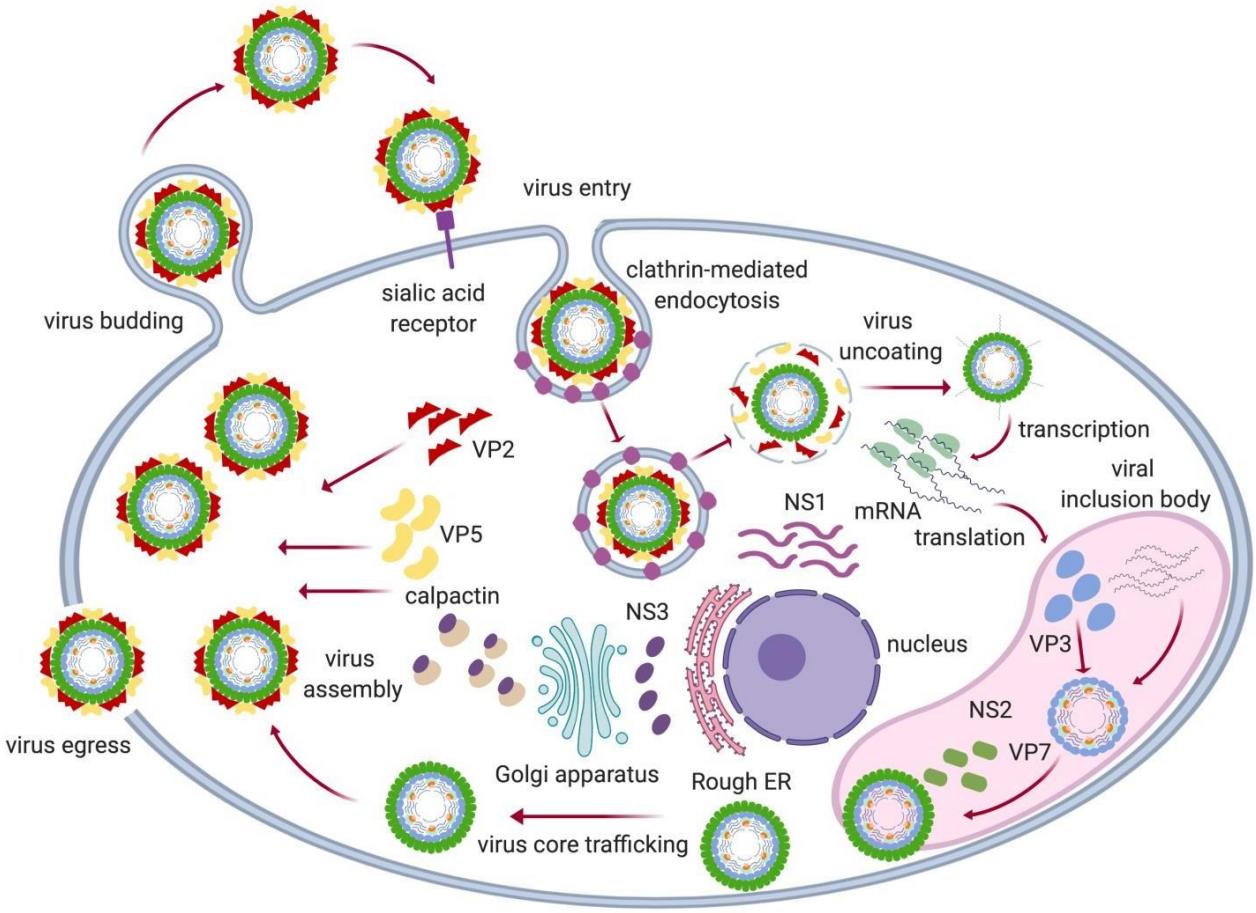


Figure 3: Diagrammatic representation of the replication cycle of AHSV

Source: Patel and Roy (2014)

2.2. Epidemiology

Host range of African horse sickness is restricted to equine groups. Equids are by far the most important vertebrate hosts of AHSV and the horse is the species most susceptible to disease, with mules and European donkeys somewhat less so (OIE, 2017; Coetzer and Guthrie, 2004). African donkeys are fairly resistant to clinical AHS, while zebra are usually only affected sub clinically. Occasionally, dogs or wild carnivores may become infected with AHSV by ingesting virus-contaminated equid meat and can die from the disease (Coetzer& Guthrie, 2004).

AHS is not contagious, it is transmitted biologically by *Culicoides spp*., of which *C. imicola* and *C. bolitinos* have been shown to play an important role in Africa (Coetzer and Guthrie, 2004).

Approximately 30 species of midges belonging to the genus *Culicoides* have been associated with AHSV transmission and replication (Figure 4) (Nagy and Simon, 1997). Until 2007, AHSV-9 was the main serotype circulating in the equine populations of Central Africa (Figure 4) (Mellor and Hamblin, 2004).

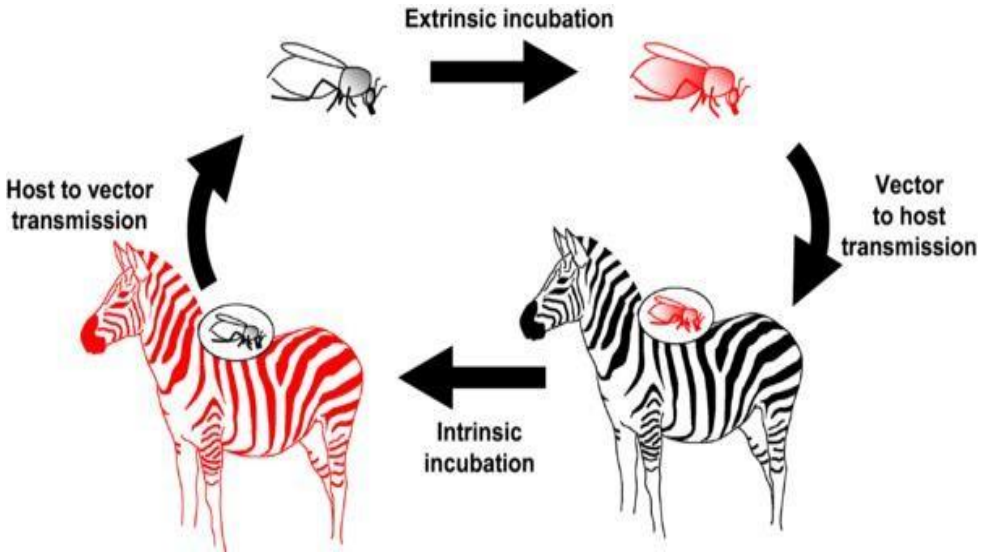


Figure 4: llustrates the biological transmission of AHSV by Culicoides vector.

Source: Wilson *et al*., (2009).

AHSV is considered to be endemic only in sub-Saharan Africa, with outbreaks involving all nine serotypes of AHSV occurring in South Africa (Von Teichman, 2010; Mellor & Hamblin, 2004). Ethiopia has been severely attacked by this disease for long period of time (Guthrie *et al*., 2018).

2.3. Pathogenesis

AHS characterized by a generalized disease of blood and lymphatic vessels, and clinical signs and lesions are related to endothelial damage and increased permeability, varying in severity with the infecting AHSV strain and serotype, infective dose and the host susceptibility (Coetzer and Guthrie, 2004; Laegreid *et al*., 1993). The factors and molecular basis that determine the virulence and pathogenesis characteristics of AHSV are not well known, although it is suspected that they lie with the proteins VP2, VP5 and NS3 and are multifaceted (Huismans *et al*., 2004).

The virus multiplies in the endothelium of lymph capillary vessels and regional lymph nodes after being bitten by an infected Culicoides, resulting in primary viraemia. The virus subsequently spreads via the bloodstream. The virus replicates secondarily in endothelial and mononuclear cells after the virus enters in to the blood and dissemination to capillary vessels of many organs then occurs, mainly to the lungs, large intestine, heart and lymphoid organs, causing a secondary viraemia (Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004).

In susceptible horses, viraemia may last between 4 and 8 days, and rarely is longer than 21 days. A titre of up to 10^5 $TCID_{50}$ of virus/mL may be observed. The onset of viraemia is usually accompanied by fever, which persists until viraemia disappears. In blood, AHS virus is closely associated with the erythrocytes, with very few viruses in plasma (Laegreid *et al*., 1993). The underlying pathology of AHS in the target organs is vascular endothelial damage with subsequent effusion, cardiovascular compromise and haemorrhage (Skowronek *et al*., 1995).

As with bluetongue, the precise mechanisms by which the virus induces the devastating vascular injury that characterizes African horse sickness are unknown, but it is highly likely that pro-inflammatory and vasoactive mediators released from virus-infected cells (dendritic cells, macrophages, and endothelial cells) are important (MacLachlan and Dubovi, 2017).

2.4. Clinical Signs

According to Dannis et al., (2019), the virulence of the virus, the immune condition and susceptibility of the animal, are the key factors that influence the severity and duration of the in susceptible horses. Host genetics must play a role, as evidenced by the susceptibility of both horses and zebra to AHSV, yet only horses contract AHS disease in the host can be classified according to the extent and severity of clinical signs into either acute (pulmonary), sub-acute (cardiac), mixed or febrile forms (Coetzer *et al*., 1994).

The pulmonary form of AHS known as "dunkop (thin head) form," is the most severe form, with fatality rates exceeding 95%. It is an acute febrile disease characterized by mild depression, sweating, spasmodic coughing, anorexia, and respiratory distress, with frothy nasal discharge in the terminal stages (Mellor and Hamblin, 2004).

The cardiac or sub-acute form of disease has an incubation period of about 7–14 days and the first clinical sign is fever. This is followed by edema, first of the supraorbital fossae and surrounding ocular tissues (which may also exhibit hemorrhage), then extending to other areas of the head, neck, and chest (Scacchia *et al*., 2015). The mortality rate in horses from this form of disease may be as high as 50% and death usually occurs within 4–8 days of the onset of fever (Mellor and Mertens, 2008).

The next most severe is the mixed form of AHS which is a combination of the cardiac and pulmonary forms with mortality rates in horses as high as 80%. The most common form, with a 70% mortality rate, is a mix of the pulmonary and cardiac forms (Dennis *et al*., 2019; Mellor and Mertens, 2008).The mildest form of AHS is generally not fatal and is accompanied by a low-grade fever, often more pronounced in the afternoon, anorexia, depression and congestion of the mucous membranes (Scacchia *et al*., 2009).

2.5. Diagnosis

The differential diagnosis includes equine viral arteritis, equine infectious anemia, *Hendra virus* infection, purpura hemorrhagica and equine piroplasmosis. In Africa, *equine encephalosis virus*, another *Orbivirus* transmitted by *Culicoides*, causes a syndrome resembling horse sickness fever. Toxins, anthrax and other causes of sudden death, as well as diseases that result in severe respiratory distress, should also be considered (Zientara *et al.*, 2015; Quinn, 2002).

2.5.1. Clinical Diagnosis

Clinical diagnosis of the pulmonary and cardiac forms is not difficult, because of the spectacularly severe nature of the disease and characteristic edema of the supraorbital fossae. Similarly, necropsy findings the severe pulmonary oedema, and pericardial and pleural effusion provides clinically to suspect the disease, especially in enzootic areas and in the appropriate season. Additionally, a history of prevalence or exposure to competent vectors or of travel from an enzootic area can be important factor (MacLachlan and Dubovi, 2017).

2.5.2. Virus isolation

Sample Collection and Submissions

In live animals, blood samples collected into anticoagulant should be taken for virus isolation. Detection will be effective if these samples are collected early during the febrile stage. Necropsy samples or tissue samples for virus isolation (or for antigen detection by ELISA or RT-PCR-based assays) samples should include spleen is best, followed by lung, liver, heart, and lymph nodes (Madoff and Woodall, 2005; OIE, 2020). The samples for virus isolation should be stored and transported at 4°C (39°F). For serology Serum should also be collected preferably, paired samples should be taken 14–28 days apart, and are particularly important in areas where the disease is endemic (Zientara *et al*., 2015).

The samples of choice for diagnosis include unclotted whole blood collected from sick animals during the early febrile stage of the disease, as well as small pieces (2–4 g) of spleen, lung, and lymph nodes from animals that recently died of African horse sickness. Prior to processing, samples should be kept at 4°C during transportation and short-term storage. (OIE, 2017) and then stored at -70^{o}C until processed.

Cell culture

Direct isolation of AHSV has been successfully 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. Blood samples collected in an appropriate anticoagulant can be used undiluted as the inoculum. After 15–60 minutes of adsorption at ambient temperature or at 37°C, the cell cultures are washed and maintenance medium is added. Alternatively and more commonly, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung, etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics(OIE, 2019).

2.5.3. Serological diagnosis

Indirect and competitive blocking ELISAs using either soluble AHSV antigen or a recombinant protein VP7 have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (Maree and Paweska, 2005, Laegreid, Skowronek *et al*., 1993). The complement fixation test (CFT) has been widely used, but some sera are anti-complementary, particularly donkey and zebra sera (Chuma *et al*., 1992).

Blocking Enzyme-Linked Immunosorbent Assay (Blocking ELISA)

The competitive blocking ELISA technique detects specific antibodies against AHSV, present in any equine species. VP7 is the main antigenic protein within the molecular structure of AHSV and it is conserved across the nine AHSV serotypes. A monoclonal antibody (MAb) directed against VP7 is used in this test, allowing high sensitivity and specificity. Moreover, other species of Equidae (donkeys, zebra, etc.) can be tested thus preventing the problem of specificity experienced occasionally using the indirect ELISAs. VP7 recombinant antigen is non-infectious, which provides a high level of security (OIE, 2019; European Commission, 2002).

The principle of this test is to block the specific reaction between the recombinant VP7 protein absorbed on an ELISA plate and a conjugated MAb against VP7. AHSV antibodies present in a suspect serum sample will block this reaction. A decrease in the amount of colour is evidence of the presence of AHSV antibodies in the serum sample. The competitive blocking ELISA is commercially available (OIE, 2017).

Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

The recombinant VP7 protein has been used as antigen2 for AHSV antibody determination with a high degree of sensitivity and specificity. Other advantages of these antigens are its stability and its lack of infectivity. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with horse, mules and donkeys. The method uses protein “G” as conjugate that also reacts with zebra serum (OIE, 2019; Maree and Paweska, 2005).

Complement Fixation Test (CFT)

The CF test has been used extensively in the past, but currently its use is decreasing and has been replaced in many laboratories by ELISA as a screening technique. This progressive replacement is because of the higher sensitivity and degree of standardisation of ELISA as well as a significant number of sera with anti-complementary activity. Nevertheless the CF test is a useful tool in endemic areas for the demonstration and titration of group-specific IgM antibodies against AHSV notably following a recent infection or vaccination (OIE 2019).

Virus Neutralization (VN) test

Virus neutralization (VN) test has been used The ‘‘gold standard’’ method for the determination of AHSV serotype for long pewriods of time. In VN detection is a test where the specificity of reactions between the virus and a panel of reference antisera, representing each of the known serotypes, is tested in tissue cultures for antigen typing. For serotyping VN test can be used to detect and measure the presence and magnitude of functional serotype specific antibody response for an individual to a known pre titred virus (reference Ag) (Mellor and Hamblin, 2004).

The virus neutralization test is important because it can be used to help determine the prevalence of a disease (infectious virus), the geographic dissemination of its virus, the titer (quantification) of antibody in an antiserum or the principles underlying the immunologic factors of an infection. One can with known virus demonstrate its specific antibody, and one can with known antibody reveal the identity of a virus. The test is simple in application when an appreciable amount of antibody is present; the antibody is detected by adding undiluted fluid, such as blood serum, which contains suspected antibody to suspensions which contain a known amount of virus (Gauger and Vincent. 2014).

Before the neutralization test is carried out, the known components that are to be used must be standardized. To identify a virus isolate, a known pre-titred antiserum is used. Conversely, to measure the antibody response of an individual to a virus, a known pre-titred virus is used (Gauger and Vincent, 2014; Specter *et al*., 2009). To titrate a known virus, serial tenfold dilutions of the isolate is prepared and inoculated into a susceptible host system such as cell culture or animal. The virus endpoint titre is the reciprocal of the highest dilution of virus that infects 50% of the host system e.g. 50% of cell cultures develop CPE, or 50% of animals develop disease. This endpoint

dilution contains one 50% tissue culture infecting dose ($TCID_{50}$) or one 50% lethal dose (LD_{50}) of AHS virus per unit volume. The concentration of virus generally used in the neutralization test is 100 $TCID_{50}$ or 100 LD_{50} per unit volume (Specter *et al*., 2009).

Even though VN test is The ‘‘gold standard’’ method for the determination of AHSV serotype, these antigen typing assays are labour intensive, time consuming, require prior virus isolation and can sometimes give inconclusive results. They are also dependent on availability of reference virus strains (as controls) and reference antisera which are highly characterized and may therefore be difficult to obtain. These assays may also require disease-secure laboratory facilities for safe handling the live virus (Bachanek-Bankowska *et al*., 2014). Serotype-specific antibody can be detected using the VN test. The VN test may have additional value in epidemiological surveillance and transmission studies, mainly in endemic areas where multiple serotypes are likely to be present (OIE 2019; OIE, 2017).

2.5.4. Molecular diagnostic technique

Molecular methods for determination of AHSV type, have been developed based on detection of Seg-2, including both conventional RT-PCR and probe-hybridisation methods (Bachanek-Bankowska *et al*., 2014).

Reverse- transcription RT-PCR is a highly sensitive technique that provides a rapid identification of AHS viral nucleic acid in blood and other tissues of infected animals. This technique has greatly improved the laboratory diagnosis of AHS by increasing the sensitivity of detection and shortening the time required for the diagnosis. The RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian cells. Therefore, positive results do not necessarily indicate the presence of infectious virus (OIE, 2019).

Several agarose gel-based RT-PCR assays for the specific detection of AHSV RNA have been described targeted at viral segments 3, 7 or 8 (Aradaib, 2009). The most widely used method employs primers corresponding to the 5‟ end (nucleotides 1–21) and 3‟ end (nucleotides 1160–1179) of RNA segment 7 (coding for VP7) amplifying the complete viral segment (Aradaib 2009; Zientara *et al*., 1994).

Real-Time RT- PCR

Real-time RT-PCR methods for the highly sensitive and specific detection of AHSV RNA have been developed based on the use of a pair of primers and a labelled probe from conserved sequences of viral segments 3, 5 or 7. A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for NS1 and NS2 respectively) (Quan *et al*., 2010; Agaero *et al*., 2008; Zientara *et al*., 1994).

Although both gel-based and real-time RT-PCR procedures can detect reference strains from the nine virus serotypes, real-time RT-PCR provides advantages over agarose gel-based RT-PCR methods, with its faster analysis time, higher sensitivity, and suitability for high-throughput automation. Nevertheless, gel-based RT-PCR methods, particularly those amplifying long RNA fragments can be very useful in the further genetic characterization of the virus by sequencing of the amplicons. In addition, it may be beneficial in laboratories without the capacity to perform real-time RT-PCR (OIE, 2019; Zientara *et al*., 2015).

Real-time RT-PCR based methods represent fast, robust and reliable tools for the detection and identification (typing) of AHSV. They provide a basis for the design and timely implementation of control measures for AHSV, including vaccination programs (Bachanek-Bankowska *et al*., 2014; Guthrie *et al*., 2013).

2.6. Prevention and Control

There is no cure for AHS and no specific treatment aside from rest and good animal husbandry. Various interventions, such as non-steroidal anti-inflammatory drugs for alleviating pain and reducing fever, antimicrobials to fight secondary bacterial infection or corticosteroids to help stabilize cell membranes and preserve vascular membrane integrity, have been employed, but all these treatments are supportive rather than curative (Dennis *et al*., 2019, Chiam *et al*., 2009). Effective quarantine and movement controls are essential to prevent the spread of virus by animals. All equidae should be stabled in insect-proof housing with a minimum, stabling from dusk to dawn, the period when *Culicoides* are most active, is recommended (AUSVETPLAN, 1996).

2.6.1. Vaccination

Live Attenuated Vaccine

In endemic areas, vaccination is strongly recommended for susceptible Equidae. Additionally, areas around the affected area should be vaccinated, as well, to produce a surrounding protection zone (Dennis *et al*., 2019). Vaccination remains the single most effective weapon in combating AHS, as there is no treatment for the disease apart from good animal husbandry. However, the only commercially available vaccine is a live attenuated version of the virus (LAV) (Dennis *et al*., 2018).

Although the LAV is currently the best option in the fight against AHS, its use has raised concerns with regard to reversion to virulence, gene segment re-assortment between outbreak and vaccine strains (Weyer *et al*., 2016),Teratogenicity (congenital abnormalities) and the absence of DIVA, which is the ability to differentiate between infected and vaccinated animals. Most importantly, the LAV is not licensed for use outside of the African subcontinent (Dannis *et al*., 2019).

Inactivated vaccines

Inactivated virus vaccines for AHS do not revert to virulence, do not cause a significant viraemia in inoculated animals, and do not re-assort with wild-type *Orbivirus* strains in the field. With inactivated vaccines, it may be possible to differentiate antibody elicited by the vaccine from that resulting from infection with an active virus, which would allow free international movement of equines (AUSVETPLAN, 1996).

Recombinant vaccines

Work on vaccines incorporating the virus proteins VP2, VP3, VP5, and VP7 is being done. VP7 is thought to be a group-specific antigen (AUSVETPLAN, 1996). Due to raised international awareness and local dissatisfaction with the current vaccine, AHSV research has focused in recent years on the development of recombinant vaccines based on selected antigenic AHSV proteins, particularly the outer capsid proteins VP2 and VP5. Baculovirus expression systems (Kanai *et al*., 2014) and poxvirus vectors (Alberca *et al*., 2014) have been used to produce vaccines that induce protective immunity against various AHSV antigens as cited by (Genis, 2019; Dennis *et al*., 2018).

2.6.2. Vector Control and Protection

If possible, all Equidae should be stabled in insect-proof housing. At a minimum, stabling from dusk to dawn, the period when *Culicoides* are most active, is recommended (Meiswinkel *et al*., 2000; Mellor and Hamblin, 2004). Possible methods for the control of Culicoides population include: treating livestock with insecticides, repellents or systemic anti-parasitic drug (e.g. avermectins), treating larval breeding sites or adult resting areas with insecticides, treating animal housing and/or transport with insecticides, removal or reduction of larval breeding sites on farm holdings (Carpenter *et al*., 2017).

2.7. Disease Outbreaks, Distribution and Epidemiological Status in Ethiopia

Due to the presence of large equine population and favorable agro-climate conditions for vector borne disease, African horse sickness virus (AHS) has long affected Ethiopian equids and many outbreaks of the disease has been reported. An epidemiological survey conducted between 1977 and 1981 indicated that the majority of these outbreaks were caused by serotype 9 (AHSV-9) (Leforban *et al*., 1983). Zeleke *et al*., (2005) reported many outbreaks of AHS in different regions of Ethiopia.

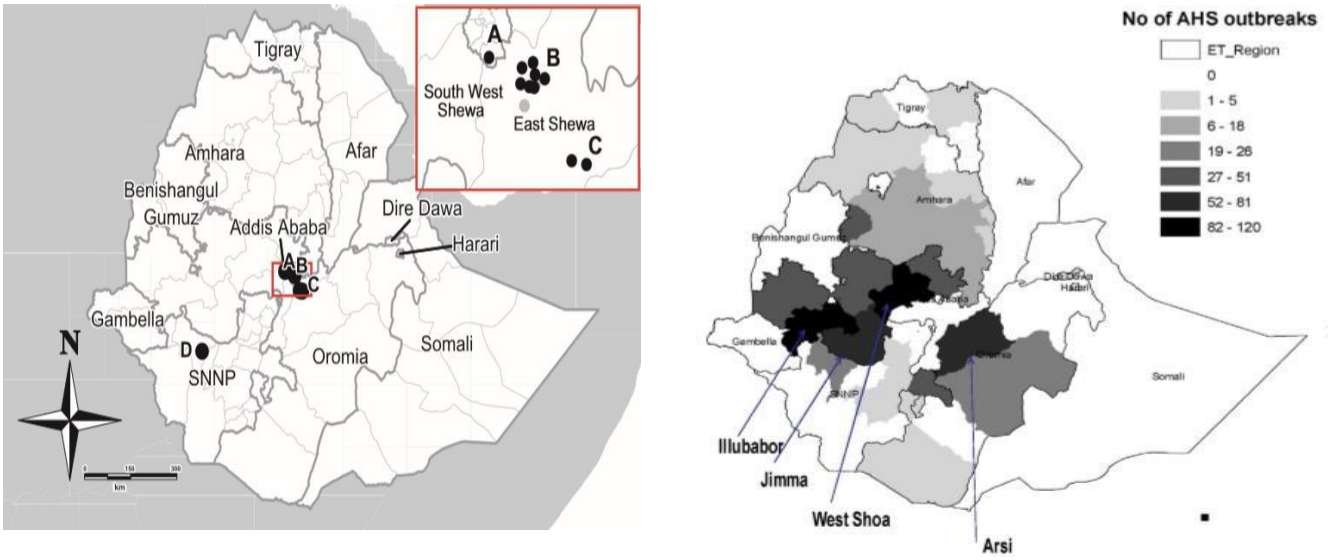
The virus could have been introduced in to Ethiopian equines by wind- borne infected midges (*Culicoides*) from endemic regions of Africa (Quinn and Leonard, 2002). A study conducted by Zeleke *et al*, (2005) investigated both serotype 6 and 9 in different regions; however, the majority of the outbreak is caused by serotype 9. As stated by Gebreegziabher (2008), nearly 2200 equids have died in an outbreak of AHS across 15 villages 4000 cases have been reported in the Southwestern part of a country. A sero-epidemiological survey using competitive ELISA in different agro-ecological zones of central Ethiopia indicated a prevalence rate of 10.4%, 29.7% and 10.3% in horses, donkeys and mules, respectively (Kassa, 2006).

During 2007–2010, 737 AHS outbreaks were occurred in six regions of the country, namely Afar, Amhara, Dire Dawa, Oromia, SNNPR and Tigray.Most of these outbreaks were observed in Oromia (573), followed by Amhara (100) and SNNPR (53). Outbreaks occurred mainly in August to December, with greatest numbers in October (133) and November (143) and least in February (15) (Aklilu *et al*., 2014). Similarly the retrospective data analysis for 2011 indicated that a total of 208

outbreaks, 3036 cases and 1167 deaths were reported to Ministry of Agriculture (MOA), Ethiopia. The highest outbreaks were reported from Oromia with 151 outbreaks and followed by SNNPR and Amharawith 33 and 22 numbers of outbreaks respectively (Figure 5) (Ayelet *et al*., 2013).

Aklilu *et al*., (2014) reported the presence of multiple AHS virus serotypes (AHSV serotype 2, 4, 6, 8 and 9) were detected by molecular methods (type-specific real-time RT-PCR assays) in 2010. AHSV Serotype 9 was the only serotype identified from outbreaks in 2011 (Ayelet *et al*., 2013).

In Ethiopia, control measures mainly based on the use of emergency vaccination. Despite vaccination is ongoing, Ethiopia has been facing a great challenge now a days due to the increasing diversity of vectors, occurrence of virulent strains (result of genetic re-assortment) and revert to virulence with live vaccines. Currently a trivalent vaccine composed of three serotypes (2, 4, 9) is incorporated for increasing the cross protective immunity and the vaccine has been distributed in susceptible regions and given annually (National Veterinary Institute, 2019).



A B

Figure 5: Map (A) shows location of AHSV isolates derived from samples collected in Ethiopia in 2010. AHSV isolates (2, 4, 6, 8, 9) indicated with black dots were derived from locations in four clusters (Clusters A, B and C and D). Grey dots indicate locations of samples identified as positive by real-time RT-PCR from which isolates were not detected. Map (B) shows the number of outbreaks per zones in Ethiopia 2011 and these figures indicated that AHS outbreaks and isolated serotypes mainly occurs in the mid and highland part of the country which is known for its horse population (Aklilu *et al*., 2014; Ayelet *et al*., 2013).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted in purposively selected study areas of Ethiopia from November 2019 to May 2021. The selected study areas were Liben Chukuala (Adulalla), Moretna Juru (Enewri), Negelle Arsi, Goro, Yabello, Kimbibit (Sheno) and Bacho (Jimma) (Figure 6). Negelle Arsi district is located in west Arsi Zone of Oromia Regional State at about 225 km from Addis Ababa. The district located 7049'00'N latitude and 380 48' 09'' E longitudes with altitude of 1500 to 2300 meter above sea level m.a.s.l. The mean annual minimum and maximum temperatures of the district are 6.8°C and 27.2°C, respectively. The annual rainfall ranges between 500-1150 mm (CSA, 2019).

Jimma town is located 352 km southeast of Addis Ababa in Oromia regional state of administration. The town is located 7°41' N latitude and 36°50' E longitude with an elevation ranging 880-3360 m.a.s.l. the town has mean annual maximum and minimum temperature of 30°C and 14°C, respectively. The annual rainfall ranges from 1138 to 1690 mm. The equine population of the area is 2463 (CSA, 2020).

Yabello town, is located 570 km south of Addis Ababa in Oromia regional state of administration and geographically it is located 4°53′N 38°5′E and an elevation ranging from 350 to 1857 m.a.s.l. The annual mean daily temperature ranges from 19 to 24°c and average annual rainfall ranges from 300 to 700 mm. The total animal population of the Yabelo district are; donkey 4,827, horse 371, mule 373 (CSA, 2020).

Moretna Jurru is located 195 km North East of Addis Ababa in Amhara Regional State. The area is located with 39° 19' 24'' E and 10° 6'N with an altitude ranges from 1,500 to 2694 m.a.s.l and receives an annual rainfall of 850 mm while the temperature varies from 5.2°C to 28.8°C (CSA, 2019).

Goro district is located 490km from Addis Ababa in Oromia regional state of administration, it is located 41° 49' E, and 9° 41' N with altitude ranges of 760m to 2800 m. a. s. l. and a mean temperature of 27° (Kedir, 2019). The total equine population were found to be 41, 748 (5626

Horses, 4046 mules and 32076 Donkeys) (CSA, 2020).Sheno town is located in Oromiya Regional state. The area is situated at an altitude of 2,630 – 3,020 m.a.s.l and an average maximum and minimum temperature of 24°C and 8.5°C (CSA, 2020).

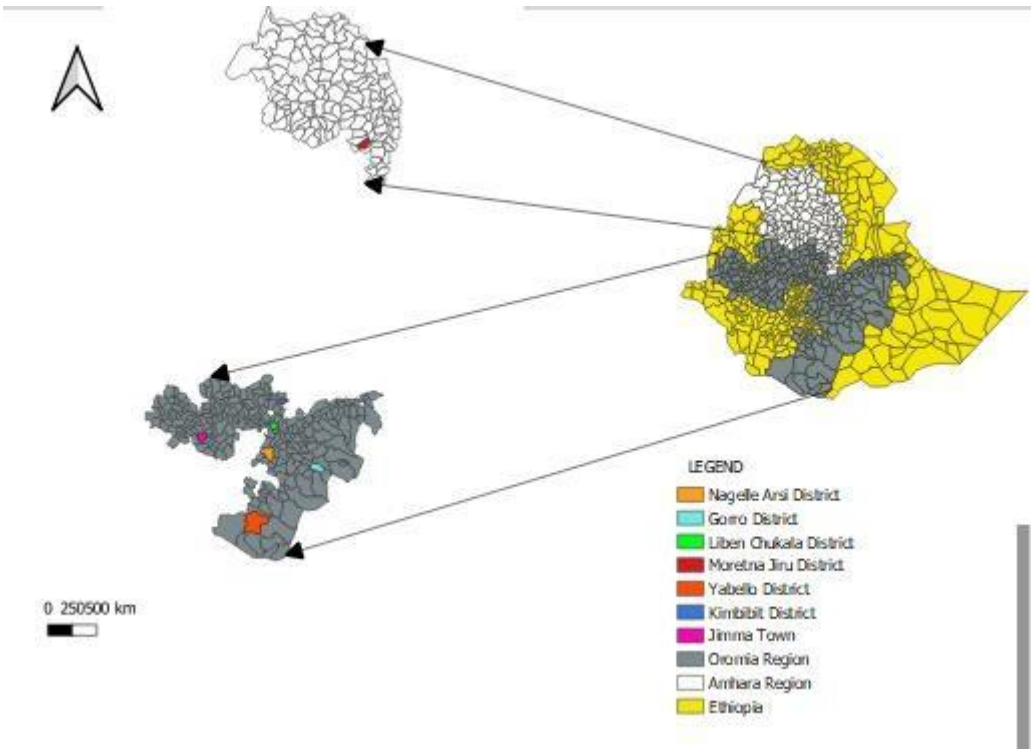


Figure 6: Map of Ethiopia showing the study areas where samples were collected.
Mapped using QGIS software version 3.14.

3.2. Study Design and Study Population

Outbreak based cross-sectional study design was carried out in equines found in purposively selected study areas of Oromia and Amhara region of the country. The study areas were purposively selected based equine population and active outbreaks of African horse sickness. The study populations were all working equines (horse, donkeys and mules) found in selected study area. The number of samples collected in the study areas were determined depending on the frequency of outbreaks found. Recently dead and sick equines that had experienced typical clinical signs of AHS

disease were sampled regardless of age group, sex, body condition and breed to isolate and molecularly detect of AHSV. During sample collection, none of the mules was found to fulfill the selection criteria hence couldn't be sampled. The age of equids were categorized into three groups for convenience purposes: ≤3 years (yearlings and young equine), 4-10 years (adult), and above 10 years (old) (table 5).

3.3. Sample collection, transportation and storage

Representative tissue (lung and spleen) and whole blood samples were collected aseptically from recently dead and sick equines showing typical clinical signs from suspected AHS disease outbreaks. A total of 30 whole blood and 2 tissue specimens (lung and spleen) were collected from clinically sick and recently dead equines. The equines that show clinical signs were restrained safely to take whole blood specimens. The site of jugular vein was located first and then the skin at the site of venipuncture shaved, swabbed with 70% alcohol and allowed to dry. About 10ml of blood specimen per individual animal was collected from jugular vein using anticoagulant ethylene diamine tetra- acetic acid (EDTA) which was coated with vacutainer tube. Tissue specimens (spleen and lung) were collected from recently dead horses and placed in sterile bottle containing viral transport media (VTM). The VTM was composed of an equal amount of glycerol and phosphate buffer saline (PBS) at pH 7.2-7.6 with antibiotics and antifungalat a pH range of 7.2-7.6. Then the collected specimens were labled properly, placed in ice plastic bag and transported to NVI, Bishoftu, Ethiopia. The specimens were stored at -80^{o}C until processed (OIE, 2019). Sample collection, transportation and storage were undertaken following standard techniques recommended by OIE (2019).

3.4. Molecular detection of African Horse Sickness Virus

Euipments, wares and reagents was prepared and sterilized according to the standard operating procedures (SOP) prepared by the AU-PANVAC, Ethiopia. Personal Protective Equipment (gown, RNase free glove, mouth mask, medical helmet), Equipments (Biosafety cabinet class II, 1.5 Micro centrifuge, Vortex, mini centrifuge, spinner centrifuge, pestle and mortar, petridish, tissue forceps and scissors, centrifuge tube, balance, conical flask, measuring cylinder, microwave, gel electrophoresis, power supply, gel documentation system) and consumables includes (micro-

pipettes, Micro centrifuge tube, Collection tube, PCR tube strips (0.2 ml), Filter tips (eppendorf)), reagents (Qiagen RNA extraction kits, Positive and Negative reference sample, Ethanol, normal saline, Qiagen one step RT-PCR kit, template RNA, agarose gel, electrophoresis buffer (1x TAE), ethidium bromide (gel red), gel loading buffer, DNA molecular weight marker and recording materials (ink, paper, plaster, etc.) were prepared.

3.4.1. Sample processing

Tissue samples were thawed or equilibrated at room temperature before processing. Approximately 1gm of tissue samples were taken and placed in sterile Petridish. The tissue specimens were cut into pieces using sterile scissors, grind by pestles and mortar and finally washed three times by PBS solution. Following grinding of tissue, 9ml of PBS was mixed with disrupted tissue and the tissue suspension was decanted into micro-centrifuge tube. Then the micro-centrifuge tube containing the suspension was centrifuged at 3500 revolution per minute (rpm) for three minutes. The supernatant was carefully harvested using pipetting, transferred into a new micro-centrifuge tube, and stored at -70°C until it continued for further procedures.

3.4.2. RNA Extraction

AHS virus extraction was performed from 100µl of whole blood and tissue suspension using QIAamp viral RNeas Mini kit (Qiagen, USA) according to the manufacturer instructions. 100µl of tissue suspension was mixed with 400µl of lysis buffer in 1.5ml micro-centrifuge tube then mixed thoroughly by vortexing for 15 seconds. The homogenized solution was centrifuged for three minutes at full speed and the supernatant was harvested by pipetting and transferred into a new micro-centrifuge tube. Then 500ul volume of 70% ethanol was added to the mixture and properly vortexed for 15 seconds to mix with solution. 700ul of the mixture from previous steps were allocated into 2ml RNeasy spin column collection tube for binding of nucleic acid and centrifuged for 15 seconds at 10,000rpm. Then the supernatant mixture discarded. The remaining mixed solution was added to the same RNeasy spin column tubes and centrifuged at 10,000rpm for seconds. The flow through solution was discarded. 700ul of buffer RW1 washing was added into the RNeasy spin column and centrifuged at 10,000rpm for 15 seconds to remove unwanted protein and DNA. The filtrate solution and collection tubes discarded. RNeasy spin column was allocated into a new collection tube and 500ul of RPE washing buffer was added to the spin column tube. The tubes were centrifuged at 10,000rpm for 15 seconds. The RNeasy spin column was placed in a new collection

tube and 500ul of RPE buffer was added in the spin column and then centrifuged for 2minutes at 10,000 rpm for drying of the column. Following discarding the old collection tube with its filtrate, RNeasy spini column was placed into a new 2ml spin column and centrifuged at 15,000rpm for 1minute again to dry the spin column and to get concentrated RNA. The spin column transferred into a new labeled 1.5ml micro centrifuge tube and 60ul of RNase free water was added into spin column. Finally, the tube was centrifuged at 10,000rpm for 1minute to elute RNA. Then the extracted viral RNA was kept at +4°C for further investigation and the eluted ribonucleic acid yield was used for PCR amplification.

3.4.3. Conventional Polymerase chain reaction assay

Molecular detection of AHSV genomic RNA was performed using conventional Reverse Transcriptase polymerase chain reaction (RT-PCR) assay at molecular biology laboratory, African Union - Pan African Veterinary Vaccine Center (AU – PANVAC), Bishoftu, Ethiopia. One step RT-PCR was conducted targeting to amplify 102 bp fragment of viral protein 7 (VP7) encoding genes located in segment seven (7) of all nine AHSV serotypes using forward and reverse primers (Table 2). The VP7 encoded by gene segment 7, is highly conserved among different AHSV serotypes and the basis for several nucleic acid diagnostic assays (Agüer et al., 2008; Weyer et al., 2015). Both the forward and reverse primers were designed from segment 3 of AHS-3 and 4; respectively (Fernandez-Pinero et al., 2009).

RT-PCR was performed targeting to amplify the genes encoding VP2 protein located in segment two for identification of AHSV serotypes using forward and reverse primers (Sailleau *et al*., 2000) (Table 2). The primers are specific for each of the nine AHS serotypes.

Table 2: Serogroup specific (universal) and serotype specific forward and reverse primers used in RT-PCR assay

Serotype	Primer	Sequence (5'-3')	Location (bp)	Size of amplicon
Universal	Forward	GGCTCCAACACTCACAAGATGT	1 – 20	102 bp
	Reverse	GGCGGATTAATAGGCTGCATA	1179 – 1159	
Serotype 2	Forward	AATTGTGATGTTATTGTTAC	76 – 95	152 bp
	Reverse	GGCGAAAAGTACATTTCACC	394 – 371	
Serotype 4	Forward	GTTTAATTCACCATGGCGTC	1 – 20	352 bp
	Reverse	TACATCATTCCAAATCTCAGC	666 – 646	
Serotype 9	Forward	GTTTAATTCACCATGGCGTC	1 – 20	228 and 282bp
	Reverse	CGTTTGTTTGTACATAAAA	2778 – 2758	

Source: Fernandez-Pinero *et al*., (200); Sailleau *et al*., (2000)

Master Mix preparation and PCR amplification

The PCR master mix was prepared in 1.5 ml micro-centrifuge tube and proportionally scaled up for the total number of reactions (Table 3) following one step RT – PCR kit manufacturer instructions (Qiagen, USA).

A total of 720 µl PCR master mixes without a template RNA for 36 reactions were added into 1.5 ml micro-centrifuge tube and mixed thoroughly by vortexing, and then 20 µl of mixed solution was distributed in to each PCR strip tube. Finally, 5 µl of each extracted template RNA was added to the corresponding tube (Tabel 3). The PCR plate closed and placed in a conventional thermal cycler programmed with the following profile (Cycle): RT- PCR steps 50°C – 30 minutes denaturation step: 95°C – 15 minutes, 40 cycles (denaturation: 95°C – 1minute, Annealing step: 58°C – 1 minute. extension step: 72°C – 2 minutes), final extension: 70°C – 5 minutes. Amplified fragmented size: the total gene amplified was 102 bp.

Table 3: Reagents and primers used for master mix preparation using universal primers.

Reagents	Volume for each reaction (25 µl)	25 µl for 36 reactions
Nuclease free water	4 µl	144 µl
One step RT- PCR buffer (5X)	5 µl	180 µl
Q – solution (5X)	5 µl	180 µl
dNTPs mix 10 mM each	1 µl	36 µl
Primer F- 5 µM	2 µl	72 µl
Primer R - 5 µM	2 µl	72 µl
One step enzyme mix	1 µl	36 µl
Volume w/o RNA	20 µl	720 µl
Template RNA	5 µl	5 µl each
Volume final for each	25 µl	25 µl each

A total of 308 µl PCR master mixes without a template RNA for 11 reactions were added into 1.5 ml micro-centrifuge tube and mixed thoroughly by vortexing, and then 28 µl of mixed solution was distributed in to each PCR strip tube. Finally 7µl of each extracted template RNA was added to the corresponding tube (Table 4). The PCR plate closed and placed in a conventional thermal cycler programmed with the following profile (Cycle): RT-steps 50°C–30 minutes denaturation step: 95°C – 15 minutes, 40 cycles (denaturation: 95°C – 1minute, annealing step: 48°C – 1 minute. extension step: 70°C – 2 minutes), final extension: 70°C – 8 minutes.

Table 4: Reagents and primers used for master mix preparation using serotype specific primers.

Reagents	25 µl mix for 1 reaction	25 µl for 11 reactions
Nuclease free water	4 µl	44 µl
One step RT- PCR buffer (5X)	5 µl	55 µl
Q – solution (5X)	5 µl	55 µl
dNTPs mix 10 mM each	1 µl	11 µl
Serotype 2-P95-F 5pm/µl	2 µl	22 µl
Serotype 2-2N228-R 5pm/µl	2 µl	22 µl
Serotype 4-P20-F 5pm/µl	2 µl	22 µl
Serotype 4-4N353-R 5pm/µl	2 µl	22 µl
Serotype 9- P20-F 5pm/µl	2 µl	22 µl
Serotype 9- 9N228-R 5pm/µl	2 µl	22 µl
One step enzyme mix	1 µl	11 µl
Volume without RNA	28 µl	308 µl
Template RNA	7 µl	7 µl each
Volume final for each	35 µl	35 µl each

Gel preparation and electrophoresis process

Two hundred ml of 5x TBE buffer was measured and poured in to conical flask which contains 800 ml in order to prepare 1x TBE buffer. One gram of 2% agarose was weighed and mixed with 200 ml of 1x TBE-buffer in to the flask. Then the Agarose solution was allowed to melt using microwave for 2 minutes at highest setting. After the solution left on the bench for 5 minutes, 4 µl of Gel red (4 mg/ml) was added in to 200 ml agarose (2%) and mixed by swirling. The agarose gel slowly poured in to the prepared tank and the gel left to settle for at least 15 minutes until it was solidified. After transferring the agarose in to the gel tank, the comb was gently removed. 5 µl of each amplified complementary DNA (cDNA) was transferred to a fresh parafilm and mixed with 2 µl loading buffer. After loading the first well with DNA marker, 32 specimens and 4 positive and negative controls were loaded onto each well and finished with a final lane of marker.Then the power supply was switched on at 120 voltages and the gel electrophoresis was run for 1:20 hours. Following

achieving its adjusted period of time, the gel was taken into gel documentation system (gel box) and the picture was taken to interpret the result.

3.5. Isolation and propagation of AHSV in Vero cells

3.5.1. Preparation of Vero cell monolayer

African horse sickness virus isolation was done by using African Green Monkey kidney Cell line (Vero). The Glascow Minimium Essential Medium (GMEM) solution was supplemented with 10% calf serum, 10% tryptose phosphate Broth (TPB) (Oxoid, England), 1% gentamycin solution prior to used. Vero cell line at passage 33 was grown in 75 cm^2 plastic tissue culture flask (Roux flask) having confluent monolayer (90%) observed under inverted microscope. These cell lines were processed for harvesting and transferring to new flasks. The growth medium overlaying the cell monolayer was poured off in a sterile beaker under sterile conditions. After rinsing and washing three times with 10 ml sterile PBS, the monolayer was covered with 5 ml of sterile 0.25% trypsin and incubated at 37ºC for about 3 - 5 minutes. The monolayer was observed under an inverted microscope for rounding and detachment of cells. After trypsin was removed, the cells detached from the flasks was collected and mixed to form homogenous cell suspension. Equal volume of the cell suspension added to each of the three tissue culture 25cm^2 flasks already containing growth medium with 10% fetal calf serum and incubated at 37ºC.

When a complete monolayer Vero cell (above 80%) was obtained, the processed supernatants kept at -80ºC were thawed at room temprature. The exhausted medium was discarded from the tissue culture flask and 1ml filtered supernatant was inoculated onto a monolayer of Vero cells in two 25 cm^2 tissue culture flasks and the third flask was filled with media only and kept as negative control flask, Following inoculation, the flasks were incubated at 37°C for 1 hour for absorption. Then 9ml Glasgow minimum essential medium (GMEM, Sigma-Aldrich) containing 0.1% gentamicin and 2% fetal calf serum (Sigma-Aldrich) was added to the infected cells of the flask. Then the presence of cytopathic effect (CPE) was observed daily for ten days with GMEM using inverted microscope.

3.6. Data management and Analysis

Data generated from laboratory investigations were recorded using Microsoft Excel spreadsheets and analyzed using STATA version 14 for Windows (Stata Corp. College Station, TX, USA. The association of potential risk factors (origin, age, sex, species, and season) with that of AHS isolates identified was computed by Pearson's chi-square and fisher's Exact test.

3.7. Ethical approval

The Animal Research Ethical Review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu Campus, Ethiopia Provided with ethical clearance for the study with Reference number of **(VM/ERC/33/06/13/2021)** which is in accordance with the International Guidelines for Animal Welfare and Ethics. Sampling from animals was carried out according to the experimental practice and standard approved by this committee.

4. RESULTS

4.1. Field Clinical and Necropsy findings

Equines showing clinical signs of AHS disease were examined and representative whole blood and tissue specimens were collected. Clinical examinations were undertaken on AHS disease suspected equines from detected in Oromia and Amhara regional states during the study period. The major clinical findings observed in affected hosts were fever, dyspnea, frothy fluid discharges, sweating, depression, and swelling of the head region (Figure 7). Post-mortem examination was conducted on two recently (less than six hours) dead horses suspected of AHS disease in Adulala town and the gross lesion were indicative of AHS disease. The post-mortem findings revealed that hyperemic and edematous lesion in lungs (pulmonary edema), frothy discharges in the upper respiratory hydrothorax, and petechial hemorrhages on serosal tracts and mucosal surfaces of spleen, heart, and intestine (Figure 7-D).

Figure 6: Clinically signs and post-mortem gross lesions of AHS suspected horses. Clinically diseased horses suspected of AHS disease characterized by swelling head (A) and neck region and hemorrhages and oedema in conjunctival tissue (B), a horse dead of per acute AHS disease with frothy discharge (C) and edema, swelling and petechial hemorrhage in the lung (D).

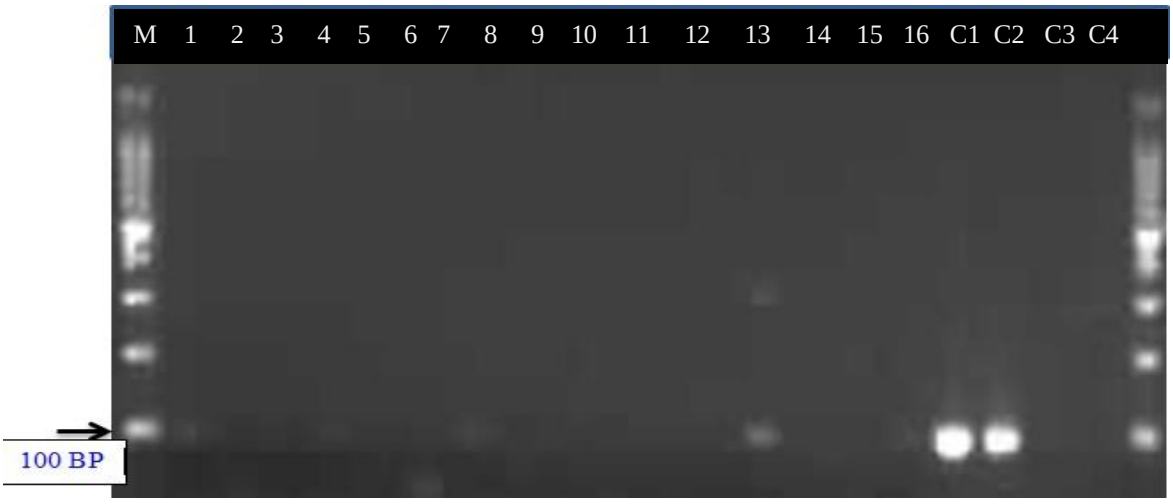
4.2. Molecular Detection Results and Associated Risk Factors

A total of 32 whole blood and tissue specimens were collected from selected study areas and detected using a standard RT-PCR technique that targeted the virus's VP7 encoding gene of segment 7 by employing universal primers. Among these specimens, 30 specimens were whole blood and the remaining two specimens were tissue. 16.7% (5/30) of whole blood and 100% (2/2) tissue specimens were detected as positive using standard RT-PCR test by observing the amplified band from gel electrophoresis. Samples that generated a strong PCR product band of the expected size (102bp) were considered to contain AHSV RNA and used for further processing (serotyping and virus isolation). The overall genetic detection rate of AHSV in the study areas were found to be 21.87% (7/32) as indicated in Table 5.

Molecular detection rate of AHS was recorded higher in donkeys 25 %) (2/8) than in horses 20.8% (4/24) even though not statistically significant variation (p=0.85) with in species difference. In the comparison of AHS infection by sex difference, the proportion of female infected 30% (3/10) was not significantly higher than male 18.2% (4/22). In this study, significantly higher proportion of older age equids 6/8(75%) was found positive for AHS followed by adults between the age of 4 and 10 years 6.67% (1/15) and none of young equines under the age of 4 years detected positive for AHS disease.

The variety forms of AHS signs also compared with the molecular result and non- significant association (P = 0.299) was observed with high number of AHS virus was detected from sub-clinical (2/6 = 33.3%) and pulmonary form (1/3 = 33.3%) followed by mixed form 26.67% (4/15). AHS virus was not detected from cardiac forms of signs. Similarly, the detection rate of AHS was compared between the clinical case reported areas and the result reveals that higher rate of the disease (100%) was recorded in Adullala, followed by in Sheno (50%), Arsi Negele (25%) and Jima (20%). In the present study AHS was not detected in Moretina Juru of Amhara region, Bale Goro and Yabello of Oromiya region (Table 5). Relatively higher rate of AHS (33.3%) was detected in Equids that shown the clinical sign of respiratory problem (pulmonary) and similar problem to subclinical form of AHS. Equine that show mixed form of the disease were detected positive at 26.67%.

Panel A



Panel B

Figure 7: Electrophoretic analysis of PCR products generated in AHSV group-specific RT-PCR assays using Segment-7 specific primers. PCR amplicons of 102 bp were generated from AHSV Seg-7 specific RT-PCR of dsRNA extracted from equines whole blood, lung and spleen and PCR control. Lane 1, 4, 7, 13 and 20 showed a weak band (blood samples extracts), lane 31 and 32 generated strong band (tissue samples). C1 and C3 are extraction and PCR positive controls respectively, negative controls are indicated by C3 and C4. All the remaining lanes are confirmed negative results, cDNA molecular weight markers (M) with 100 bp are shown at both ends of the panel.

The comparison of detection rate of AHS based on season of the year (month) at which active case of the problem reported and sample collected, highest rate (36.4%) was recorded at November followed by December (22.2%) and January (11.1%). The detection rate of the disease was null at February. However, the most of the above variables, except age, reveal no statistically significant difference with ($p>0.05$). Several factors such sample size, sample collection time (epidemic cycle) and diagnostic techniques employed may affect the association between season and species of animal.

A non-significant variation was observed among season and higher detection incidence of the virus was demonstrated in November as compared to December and January (Table 5). AHSV was not identified in February.

Table 5: Summary result of Conventional RT- PCR test with respect to different risk factors

Variables	No. of Equids	AHS positive (%)	corrected X^2/Fishers Exact	P-value
Species				
Donkey	8	2 (25%)	0.061	0.805
Horse	24	4 (20.8%)		
Sex				
Female	10	3 (30%)	0.562	0.454
Male	22	4 (18.2%)		
Age group (years)				
≤4	9	0 (0)		
4-10	15	1 (6.67%)	17.762	0.00
>10	8	6(75%)		
District				
Jima	10	2(20%)		
Arsi Negele	8	2 (25%)	0.139	
Moretina Juru	5	0 (0)		
Bale Gorro	3	0 (0)		
Adulaala	2	2(100)		
Kembibit(sheno)	2	1 (50)		
Yabello	2	0 (0)		
Forms of disease sign				
Mixed	15	4 (26.67%)		
Cardiac	8	0 (0)	0.299	
Subclinical	6	2 (33.3%)		
Pulmonary	3	1 (33.3%)		
Season				
November	11	4 (36.36%)		
December	9	2 (22.22%)	0.721	
January	9	1 (11.11%)		
February	3	0 (0)		

4.3. AHSV Serotyping

AHSV positive specimens (7) using universal primers were subjected to RT-PCR assay to detect AHSV serotypes 2, 4 and 9 by targeting the VP2 encoding gene of segment 2 using serotype specific forward and revers primers to amplify specific fragments of each serotype. The test result reveal two (2) strong positive bands with the amplicon size of approximately 228 bp of AHSV serotype 9 (AHSV9). It is confirmed that AHSV serotype 9 was detected only from tissue samples (lung and spleen). The remaining five (5) RNAs detected positive for AHSV are among the serotypes of AHSV not subjected to be amplified due to temporary absence of serotype specific primers for each serogroup i.e it can be serotype 1,3,5,6, or 7. But it is confirmed that the RNAs are not genes of serotype 2 or 4. Because no visible band was detected indicating no amplification has occurred through the use of serotype 2 and 4 primers (Figure 8).

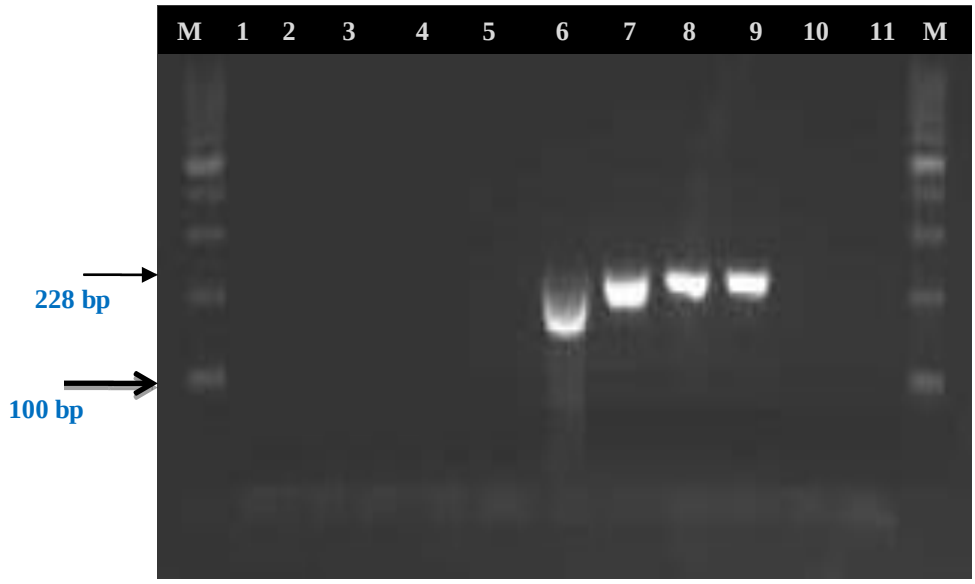


Figure 8: Electrophoretic analysis of PCR products generated in AHSV conventional RT-PCR assays using serotype specific primers designed and taken from seg-2 (VP2) to serotypes 2, 4 and 9 AHSV. Lane 1, 2, 3, 4 and 5 represents extracted specimens from whole blood which were not detected in this reaction and lane 6 and 7 were tissue samples identified as serotype 9 (AHSV - 9) with an appropriate band size of 228 bp. M is molecular ladder, both extraction and PCR positive controls were indicated by Lane 8 and 9 respectively; Lane 10 and 11 represented negative controls.

4.4. Cytopathic effect on Vero cell lines

In cell culture, the cytopathic effect (CPE) of AHS virus is measured by refractivity and detachment of cells. Seven samples that were detected positive for AHSV were inoculated to the prepared monolayer Vero cell line. Virus was isolated from tissues (lung and spleen) and these isolates were grown and showed the CPE after 7 days after second blind passage. Following three blind passages, a clear CPE was observed and characterized by aggregation of dead cells, detaching from the surface and destruction of monolayers were in line with the reports made by Were (2011) and Elganay (2003). Three serial passages were completed on all specimens before the isolation attempt was considered negative. Positive samples in cell culture were confirmed by using conventional RT-PCR AHSV genome using the universal primers. Cultures showing no CPE after three passages were considered as negative for AHSV.

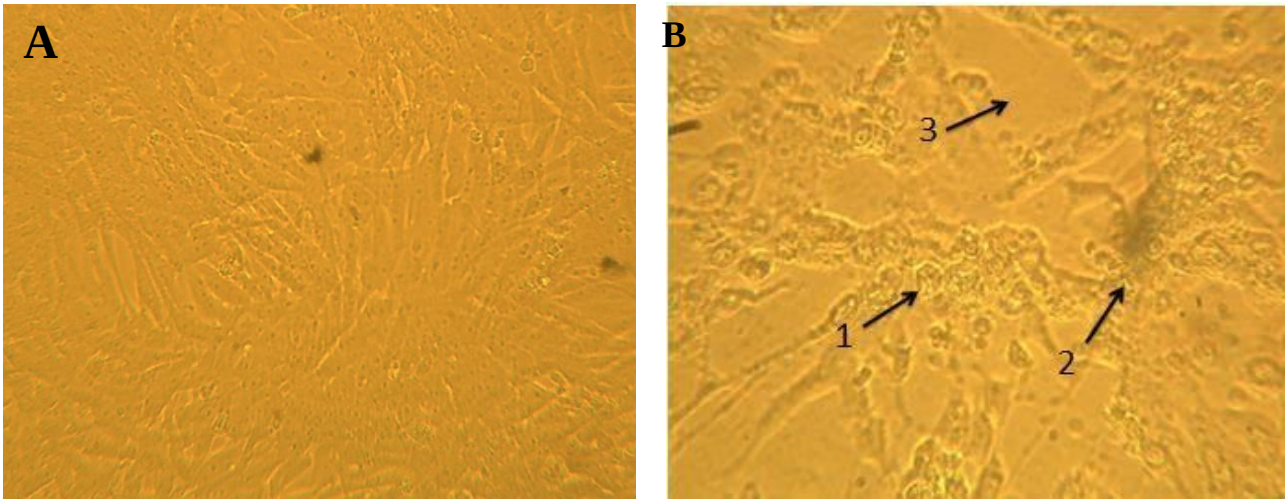


Figure 9: CPE observed on a monolayer Vero cells infected with AHSV field isolates. (A) Indicated a control monolayer Vero cell and (B) CPE was observed on Vero cells infected with AHSV serotype 9-field isolate which was detected from tissues (spleen and lung) of horses (the arrow indicates refractivity lesions (1), aggregation of dead cells (2) and detachment or destruction of monolayers (3)).

4.5. Constraints and limitations of the research

Generally the major constraints of this finding were; difficulties in sample collection due to inaccessible sample storage (refrigerators) in field outbreaks (districts), dispersed outbreaks areas i.e. most outbreaks occur in remote areas which made difficult to access active (viremic) cases, negligence of outbreak surveillance in regional laboratories, inaccessible to diagnostic kits. Limitations of research, in accessibility of primers for each serotype and virus isolation from vectors to know molecular relationships and genetic variations between vector isolates with outbreak strains were not succeed.

5. DISCUSSION

African horse sickness disease is the most economically devastating and non-contagious disease of equines. Typical clinical indications of high fever, dyspnea, severe respiratory distress, hyperemic conjunctiva and swelling of the head region suggestive of AHSV infection observed in affected equines, according to the present study. Different clinical forms of the disease were also observed during the present study period such as per acute (pulmonary) form, subacute (cardiac) form, and acute forms and mixed forms. The results of postmortem and clinical examinations in the current study was similar to the earlier reports of Coetzer and Guthrie (2005), Gomez-Villamandos *et al*. (1999), Bunpapong *et al*. (2020) and Fener's (2017).

The study was designed to isolate and investigate the circulating AHSV serotypes in equine populations using the OIE recommended diagnostic technique such as cell culture and conventional RT-PCR, respectively (OIE, 2019). Isolation of the infectious field virus isolates were conducted using Vero cell culture; and the present finding also in agreement with Ayelet et al (2014) that they also isolated using Vero cell line. Molecular detection of the viral genome using conventional RT-PCR test revealed that 21.86% of the overall samples confirmed as positive for AHS virus. Demonstration of AHSV using RT-PCR test is rapid, sensitive and specific compared to virus isolation diagnostic method. The molecular detection rate of the present finding is much lower than the previous study (Aklilu *et al*., 2014; Ayele *et al*., 2014). An increase in the improvement of government vaccination and disease control strategy with accessibility of trivalent vaccines (NVI, 2019) and seasons of sampling (Baylis *et al*., 1999; Leforban *et al*., 1983) influence a lower infection rate in the current finding.

The PCR analysis successfully amplified the virus VP7 encoding gene and resulted in a single major band of approximately 102 bp fragments of dsDNA on agarose gels (Fernandez-Pinero *et al*., 2009) which revealed that the equines were infected with AHS virus. This PCR finding was in agreement with the previous report of Aklilu *et al*., (2014) and Ayelet *et al*., (2014) and the same PCR product band size obtained.

According to the current findings, despite the fact that the majority of clinical findings in affected equines were suggestive of AHS, no amplified products of cDNA were detected in reactions with the other 25 blood samples. The level of viremia might be low that the detection of AHSV genome was negative. The presence of large levels of RNases protein, very low (insufficient amount of virus particles), and various PCR inhibitors might influence the negative outcome (Marschat *et al*., 1994).

With the exception of age, there was no statistically significant difference in the presence of AHS based on all considered risk factors. The study revealed a significant variation on infection with AHSV among the three age groups. High infection rate in old age group mainly due to excessive administration of vaccine over many years lead to a state of immunological unresponsiveness or hypersensitivity (Gordon *et al*., 2013). This finding was in line with studies conducted in Sudan (Ahmed *et al*., 2020), but it contradicted with the previous study of Burrage and Laegreid (1994) in which host factors such as age, sex and body condition have no influence on the susceptibility to AHS in naïve horses. In the present study, the infection rate of AHSV was not determined in sex groups of equidae that was in line with Burrage and Laegreid (1994).

The present finding indicated that the association between type of Equids and season (months) with AHS Viral infection were not statistically significant. This finding was not in agreement with Coetzer and Guthrie (2004) and Rodríguez *et al*., (2020) in which the highest incidence of the disease reported in late summer and early autumn. They also stated that Horses were the most susceptible species to AHS with a mortality rate of 50–95%. Sample size, study design and sampling time (epidemic cycle) may affect the association between occurrence of AHS with in species and season in these study areas (Coetzer and Guthrie, 2004, Rodríguez *et al*., 2020).

The occurrence of AHS virus across the districts also revealed that the disease is endemic in different agro-ecological conditions, which was supported by Kasa (2006) and Leforban *et al*. (1983). The current molecular serotyping investigation confirmed that AHSV serotype 9 was detected in tissues samples with a PCR product size of 228 bp (Saillaeo *et al*., 2000). The present finding showed strong agreement with previous studies; stating that AHSV serotype 9 is the most

prevalent serotype in Ethiopia (Ayelet *et al*., 2014; Zeleke *et al*, 2005) and the majority of outbreaks were caused by AHS serotype 9 (Aklilu *et al*., 2014; Kasa *et al*., 2006).

The virus isolation results in the current study indicated that only two samples (tissues) showed CPE results on vero cell line. All the samples from blood found to be negative to the cell culture and a similar finding was also reported by Kasa (2006). A negative result of CPE from blood samples might be due the stage of the disease course during sampling, time and storage conditions and the presence of inactivated or low live virus load to induce multiplication in the cell line (OIE, 2019). Weyer (2011) and Elganay (2003) in which tissue samples were able to show CPE characteristics as compared to the blood samples reported similar findings. The present and the previous findings showed that tissues are more preferable for virus isolation (Wohlsein*et al*., 1998; Marschat *et al*., 1994) and this is more likely because of the existence of high virus load in the clinical tissue samples

6. CONCLUSION AND RECOMMENDATIONS

AHS disease is one of the leading constraints for the productivity and welfare of equines, which results in significant socio-economic impacts to the country. The present study reveals relatively high prevalence circulating AHSV in different study areas. The present study also revealed that AHSV serotype 9 was confirmed using molecular and virological technique, and known to circulate in Liben Zikuala districts and cause the most severe respiratory and circulatory impairment, which results in death of horses.

Based on this finding, the following recommendations were forwarded:

- ✓ Strategic vaccination is required to prevent and control outbreak of AHS diseases in endemic areas
- ✓ Further investigation should be undertaken to confirm molecular detection of AHSV in areas where there is no information on the epidemiology of the disease so that better control will be possible.
- ✓ Detail epidemiological investigations and characterization of virus from the vector and wild animals should be practiced.

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8. ANNEXS

Annex 1. Sample collection format sheet

Name of Clinic_____ Date_____

1. Owner name _____address_____Telephone_____
2. Species _____Breed_____sex_____ age _ID_____
3. History (duration, symptoms etc) _____
4. Physiological parameters:Hear Rate_____Respiration Rate _____Body T^{o} _____
5. Clinical signs observed:

- Acute case:
  - High fever
  - Severe respiratorystress
  - Profuse sweeting
  - Frothy discharge from nostrils
  - Head extended
  - Fore legs abducted
- Sub-acute case:
  - Fever
  - Oedema supraorbital fossa and head region
  - subcutaneousoedema
  - Congestion &hemorrhage conjunctiva
- Mixed Form: a combination of both pulmonary and cardiac form
- Mild form

  Transient fever Increased RR

  Inappetance

6. Disease suspected_____ Type of specimen (Blood, tissue) _____Transporting media used_____Tests requested _____

Annex 2. Extraction of total RNA from whole blood

- Takeout samples from +4^{o}C storage, left at room temperature and mix with a vortex for 15 seconds
- Mix well 100 µl sample and 400 µl of lysis buffer in 1.5 ml tube then mix thoroughly by vortexing
- Add 500 µl of 70% ethanol to the cleared lysate and mix well by vortexing for 15 seconds.
- Pipet 700 µl of the mixture in to the RNeasy spin column and centrifuge for 15 seconds at 4000 rpm and discard the flow through. pipet the remaining mixture and centrifuge again at4000 rpm for 15 seconds and discard the flow through.
- Add 700 µl of buffer RW1 and centrifuge for 15 seconds at 4000 rpm discard both the collection tube and flow through.
- Add 500 µl of buffer RPE and centrifuge with 4000 rpm for 15 seconds.
- Add 500 µl of buffer RPE again in the RNeasy spin column, then centrifuged at 12,000-rpm speed for 2 minutes.
- Transfer the RNeasy spin column in to anew 1.5 ml micro centrifuge tube and pipet 60 µl of RNase free water in to the RNeasy spini column membrane and centrifuge for 1 minute at 8000 rpm to elute.

Annex 3. Procedures for Gel preparation and electrophoresis process

- Prepare 1xTBE; mix 200 ml of 5xTBE with 800 ml distilled water
- Weigh 1 g of 2% agarose and add in to the flask with 200 1xTBE-buffers
- Melt agarose using microwave, run for 2 minute at highest setting
- Add 4 µl Gelred (4mg/ml) in to 200 ml agarose (2%) and swirl to mix
- Power gel slowly in to the tank and leave the gel settle for at least 15 minutes until it is solidified
- Transfer the agarose in to the gel tank and remove the comb gently
- Transfer 5 µl of each amplified DNA to afresh parafilm and mix with 2 µl loading buffer to each sample
- Load the first well with DNA marker and continue loading of samples and finish with a final lane of marker
- Close the gel tank, switch on the power supply and run the gel electrophoresis for 1 hour at 120 V.
- Switch off and unplug the gel tank and put the gel in the documentation system (gel box), adjust the gel and take picture and interpret the result.

Annex 4.Virus Isolation

Propagation of Vero cell for virus isolation,

- Wash the monolayer cell line by using PBS solution and add 2 ml of Trypsin incubated at 37ºC for 3 – 5 minutes.
- Add 10 ml of GMEM mediums to the flask to stop the trypsin action and mixed very well by pipe tting
- Add 10ml of GMEM up to the 30ml mark of the flask Then transfer 10 ml of the solution to five to each flask by using pipette and incubate the flasks at 37ºC for 24 hours 8.
- Takeout tissue samples from their storage and thaw at room temperature
- Measure about 1 gm tissue Sample and place on a mortar, washed for three times with PBS solution
- Cut the tissue into smaller pieces using scissors and forceps then grinded with pestles and mortar
- Add 9 ml of PBS in to the mortar and mix well then transfer in to test tubes
- Centrifuge the test tubes at 3500 rpm for 3 minutes then filter the supernatant using filter paper
- Wash monolayer cell lines with PBS and inoculate 1 ml of filtered supernatant 25 cm^2 flasks
- Finally incubated for one hour at 37ºC then added 2% GMEM medium to the flasks and incubated at 37ºC for daily follow up for any CPE on the cell lines

Annex 5: Sample origin information and molecular laboratory test result

S.no	Sample code	Lane No.	Districts	Spp	Sex	Age	Season	AHS
1	AHS/JrH01/PANVAC	15	Juru	Horse	M	<4yrs	November	-
2	AHS/JrH02/PANVAC	16	Juru	Horse	M	4-10yrs	November	-
3	AHS/JrD03/PANVAC	21	Juru	Donkey	F	<4yrs	November	-
4	AHS/JrD04/PANVAV	22	Juru	Donkey	F	4-10yrs	November	-
5	AHS/JrD05/PANVAC	24	Juru	Donkey	F	<4yrs	November	-
6	AHS/AdH01/PANVAC	31	Adullala	Horse	M	>10yrs	November	+
7	AHS/AdH02/PANVAC	32	Adullala	Horse	M	>10yrs	November	+
8	AHS/BGH01/PANVAC	26	Bale Gorro	Horse	M	>10yrs	December	-
9	AHS/BGH02/PANVAC	27	Bale Gorro	Horse	M	<4yrs	December	-
10	AHS/BGH03/PANVAC	28	Bale Gorro	Horse	M	4-10yrs	December	-
11	AHS/JmH01/PANVAC	2	Jimma	Horse	M	>10yrs	November	-
12	AHS/JmH02/PANVAC	3	Jimma	Horse	M	4-10yrs	December	-
13	AHS/JmH03/PANVAC	4	Jimma	Horse	F	>10yrs	December	+
14	AHS/JmH04/PANVAC	5	Jimma	Horse	M	4-10yrs	December	-
15	AHS/JmH05/PANVAC	6	Jimma	Horse	M	<4yrs	January	-
16	AHS/JmH06/PANVAC	7	Jimma	Horse	M	<4yrs	January	+
17	AHS/JmH07/PANVAC	8	Jimma	Horse	M	4-10yrs	January	-
18	AHS/JmH08/PANVAC	9	Jimma	Horse	M	4-10yrs	January	-
19	AHS/JmH09/PANVAC	29	Jimma	Horse	M	<4yrs	February	-
20	AHS/JmH10/PANVAC	30	Jimma	Horse	M	4-10yrs	February	-
21	AHS/NAH01/PANVAC	1	NegelleArsi	Horse	M	>10yrs	November	+
22	AHS/NAH02/PANVAC	10	NegelleArsi	Horse	M	4-10yrs	November	-
23	AHS/NAH03/PANVAC	17	NegelleArsi	Horse	F	4-10yrs	November	-
24	AHS/NAH04/PANVAC	18	NegelleArsi	Horse	M	4-10yrs	December	-
25	AHS/NAH05/PANVAC	19	NegelleArsi	Horse	M	>10yrs	December	-
26	AHS/NAD06/PANVAC	20	NegelleArsi	Donkey	F	4-10yrs	December	+
27	AHS/NAD07/PANVAC	23	NegelleArsi	Donkey	F	<4yrs	January	-
28	AHS/NAD08/PANVAC	25	NegelleArsi	Donkey	F	4-10yrs	March	-
29	AHS/shD01/PANVAC	13	Sheno	Donkey	F	>10yrs	January	+
30	AHS/shD02/PANVAC	12	Sheno	Donkey	F	4-10yrs	January	-
31	AHS/YbH01/PANVAC	11	Yabello	Horse	M	<4yrs	January	-
32	AHS/YbH02/PANVAC	14	Yabello	Horse	M	4-10yrs	January	-

From this table, the sample code denotes AHS: African horse sickness, the next for the study area where the sample was taken, H stands for horse and D for donkey, the number is to show sample no. and the last PANVAC for Pan African Veterinary Vaccine Center. the last column + is to denote positive result and – to the negative result

Annex 6. Reagent composition

Growth and Maintenance Media

Growth medium consist of GMEM supplemented with 10% tryptose phosphate broth (TPB), 0.63% of a 10% NaHCO3 solution, 1% of Antibiotic-Antimycotic Mixture 100X (Gibco, Grand Island, New York, USA), and 10 % foetal bovine serum (FBS). this medium will be replaced after 24 hours by the same medium but supplement with only 2-5% FBS.

Phosphate Buffer Saline (PBS)

1x Phosphate-buffered Saline (PBS: PBS; 137 mMNaCl, 2.7 mMKCl, 4.3 mM Na2HPO4·2H2O, 1.5 mM KH2PO4, 0.5% (v/v) Triton X-100

3x Protein Solvent Buffer (PSB): 188 mMTris-HCl (pH 6.8) 6% SDS, 30% Glycerol, 15% β-mercaptoethanol and 0.005%

1x TAE Buffer: 4 mMTris-HCl, 0.1% (v/v) Glacial acetic acid, 0.2 mM EDTA, pH 8.0

1xTBE Buffer: 0.1 M Tris-HCl, 0.1 M Boric acid, 2 mM EDTA, pH 8.0

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
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Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/33/06/13/2021

Name of Applicant: **Degu Fhetanegest (DVM, MVSc fellow)**

Address: Department of Microbiology, Immunology & Vet. Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Isolation and molecular detection of African Horse Sickness Virus from outbreaks in selected areas of Ethiopia*

Date of application:	**December, 2019**
Nature of the project:	**Mildly invasive**
Target animal species:	**Horses, donkeys, mules**
Number of animals involved:	**32**
Study area:	**Different out break areas in Ethiopia**

Minutes No. and date of review: **VM/ERC/03/12/020, 18/02/2020**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

Getachew Terefe (DVM, PhD, Professor)
Chairman

Signature

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