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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MOLECULAR SURVEILLANCE OF EQUINE HERPES VIRUS -1, 2, AND 5 CIRCULATING IN WORKING EQUIDS IN CENTRAL ETHIOPIA

BY

TUGE TEMESGEN HATAHU

JUNE, 2020

BISHOFTU, ETHIOPIA

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MOLECULAR SURVEILLANCE OF EQUINE HERPES VIRUS -1, 2, AND 5 CIRCULATING IN WORKING EQUIDS IN CENTRAL ETHIOPIA

A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Veterinary Science (MVSc) in Veterinary Epidemiology

BY

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DEPARTMENT OF CLINICAL STUDIES

MVSc IN VETERINARY EPIDEMIOLOGY

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by: Tuge Temesgen entitled "**Molecular Surveillance of Equine Herpes Virus -1, 2, and 5 Circulating in Working Equids in Central Ethiopia"** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of science in Veterinary Epidemiology

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TABLE OF CONTENTS

CONTENTS **PAGES**

STATEMENT OF THE AUTHOR

First, I affirm that this thesis is my solely work and that all sources of material used for this MVSc thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for Masters of Veterinary Science (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic award.

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LIST OF ABBREVIATIONS

A	Adenine
CD	Cluster of differentiation
CNS	Central nervous system
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-leukocyte
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Dpi	days post-infection
EHM	equine herpes myeloencephalopathy
EHV-1,-2,........9	Equidherpesvirus -1,-2,……………...9
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein Isothiocyanate
G	Guanine
GHV	Gazelle herpes virus
IFT	Immunofluorescence test
IgG	Immunoglobulin G
IgG	Immunoglobulin G
IPMA	Immuoperoxidase monolayer assay
LAMP	loop-mediated isothermal amplification
LATS	Latency-associated transcripts
MLV	Modified-live vaccines
NVI	National Veterinary Institute
OR	Odds ratio
ORF	Open reading frames
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
RT–PCR	Reverse transcriptase polymerase chain reaction

SiRNAs	small interfering Ribonucleic acid
SNP	Single nucleotide polymorphism
TCID50	Tissue culture infectious dose
TRITC	TetramethylrhodamineIsothiocyanate
UL	Unique long
URT	Upper respiratory tract
US	Unique short
USA	United state of America
VN	Virus neutralizing
VTM	Virus transport medium

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

ABSTRACT

Equine herpesvirus infections have a major economic, health, and welfare impact on working equids worldwide.This study was performed from October 2019 to April 2020 in the North Showa of Amhara regional state, East Shewa and West Arsiof Oromia regional state of Ethiopia,for molecular detection of the virus,and to identify the association between expected risk factors and occurrence of EHV -1,2 and 5 infectionsamong clinically suspected working equine population. A total of 58 samples were collected from 33 donkeys and 25 horses suspected with clinical signs of EHVs infection.Detection of EHV-1, 2, and 5 genes in the collected samples was done using polymerase chain reaction (PCR).Assessment of the associated risk factors was conducted using a multivariable logistic regression model.The results of this study showed that out of the 58samples, 36 (62%), 31(53%), and 15(25%) were found positive for EHV-1, 2, and 5, respectively. Concurrent infections with EHV-1 and EHV-2 (n = 18; 31%), EHV-1 and EHV-5 (n = 10; 17%), EHV-2 and EHV-5 (n= 9; 15.5%), and EHV-1, 2, and 5 (n = 8; 13%)were recorded.There wasno statistically significant difference ($P > 0.05$) of EHV-1, 2, and 5 infections with regard to age, sex, body condition score, and study sites, butstatistically significant difference ($P < 0.05$) between horse and donkeys were found.Our studyrevealed that EHVs was prevalent in the working equids in the study areas, which need serious attention for prevention and control actions. Equine herpes virus vaccines never have been practiced in Ethiopia, therefore other forms of prevention and control strategies must be considered.

Keywords: *Equids, Equine herpesvirus, Epidemiology, Polymerase Chain Reaction, Ethiopia*

1. INTRODUCTION

Working equids (horses, mules, and donkeys) have a great significance in the development, where they have an essential role in reducing poverty, providing food security, and enhancing rural development. These equids perform numerous activities on a daily basis, including the transportation of goods, people, and construction materials, as well as being used in agricultural and tourism activities (Gari *et al*., 2010).

Ethiopia has the largest equine population in the world, including 8.85million donkeys, 2.008 million horses, and 0.46 million mules (CSA, 2018). These equines play a significant role in the livelihood of small scale farmers, mainly used for transportation, draught power,and other purposes (Negussie *et al.*, 2017). Infectious diseases compromise the health and welfare of these working equids, which in turn threatens the livelihoods of the most vulnerable members of the society (Stringer *et al.*, 2015). Of these infectious diseases, EHVs are considered to be the most important due to their diverse clinical presentations and the potential cause of high economic losses (Lunn *et al*., 2009).

The impact of EHVs includes the economic losses associated with abortion and fatal neonatal disease, the loss of days in work as a result of respiratory infections, and the potentially dramatic effects on health and welfare of the increasingly prevalent neurological form of the disease (Schulman, 2016). The economic effects associated with veterinary treatment, vaccination,and preventative disease control strategies are considered. The EHVs also exert a significant, albeit indirect impact on the international horse industry as a consequence of the wastage associated with poor athletic performance or interference with the local and international movement of horses for breeding and competition purposes (Schulman, 2016).

Although there are currently nine EHVs described, two of the *alphaherpesviruses*, namely equine herpes virus1 (EHV-1) and equine herpes virus 4 (EHV-4), are clinically, economically, and epidemiologically the most important pathogens. These two viruses are associated with a range of disease manifestations of varying severity tha taffect equids of all ages and breeds (Paillot *et al*., 2014).

EHV-1 is a primary respiratory pathogen and its most serious consequences are late-term abortion, neonatal death, and equine herpes virus myeloencephalopathy (EHM), particularly when these manifest as epizootics. These significant sequelae occurrence is more commonly associated with cell-associated viremia and has a greater ability to infect a range ofcell types, including the tissues of the uterus and nervous system (Schulman, 2016).

Equine herpesvirus 2 (EHV-2) and equine herpes virus 5 (EHV-5) are distinct, but closely related, *equine gammaherpesviruses* that have been detected in equine populations worldwide (Allen *et al*., 2004). Outbreaks of severe respiratory disease in association with EHV-2 infection have been reported in groups of horses in many countries (Fortier *et al*., 2010; Sharp *et al*., 2007) with foals aged 6–10 weeks developing the most severe clinical signs (Léon *et al*., 2008). The molecular detection of these viruses, often confirmed by immunohistochemistry, has identified them in association with a range of previously unidentified pathologies, including abortion, endometritis, dermatitis, granulomatous dermatitis, and oral and esophageal ulcers (Marenzoni *et al*., 2013). EHV-5 has been associated with a number of different pathological conditions. Poor performance syndrome and respiratory signs in yearlings and adult horses have been reported (Fortier *et al*., 2009). The most relevant presentation of EHV-5 is development of a characteristic progressive interstitial pulmonary fibrosis, with a nodular appearance, now termed equine multinodular pulmonary fibrosis (EMPF) (Williams *et al*., 2007).

The key success in the epidemiology of EHVs in terms of its maintenance within a population is that, after the acute phase, they establish prolonged, possibly life-long, latent infections in asymptomatic equids which act, *via* periodic reactivation, as reservoirs of infection for new, susceptible equines. Latently infected equines are important biological reservoirs for EHVs. Because the periodic virus reactivation from latency leads to the production of infectious virus that serves as a source of infection (Tearle *et al*., 2003).

Equine herpes virus 1, 2, 4 and 5 are highly contagious and usually transmitted by direct contact mainly through the infected nasal discharge between infected animals or animals and an infected object. While rare, EHV-1 can be transmitted by aerosol or contaminated feed, water, and equipment (Patel and Heldens, 2005).

Even though EHVs, especially EHV-1 and EHV-4 were found to be prevalent in working equids in Ethiopia according to Getachew *et al*.,(2014) and epidemiology of EHVs in Ethiopian equids and invasion characteristics of EHV-1 and EHV-3 were studied by Negussie *et al*.(2017); those studies have limited epidemiological data and more investigation is essential to better understand the epidemiology. The information shortage concerning the epidemiological and molecular surveillance of EHVs in Ethiopian equids initiate to conduct this study. Except by Negussie *et al*.(2017), no previous results had been published on the epidemiology and molecular detection of EHVs infection in working equids in Ethiopia. Therefore, this study aims to investigate the epidemiology (risk factors associated with the occurrence of EHVs infection) and molecular surveillance of EHVs circulating in working equids in Ethiopia.

Specific objectives:

- To conduct molecular surveillance of EHV-1, 2, and 5 in working equids with suggestive clinical signs of EHVs
- To assess the association of expected potential risk factors and EHVs infection

2. LITERATURE REVIEW

2.1. Equine HerpesViruses

Equine herpes viruses (EHVs) are significant causes of serious illness and mortality in domestic equids population worldwide (Slater *et al.*, 2006). Taxonomically, the herpes viruses are currently classified within the order Herpesvirales that accommodates three families: the Alloherpesviridae, the Malacoherpesviridae and the Herpesviridae. The Herpesviridae has three subfamilies:Alpha, Beta and Gamma-herpesvirinae and contains the mammal, bird and reptile adapted viruses including the herpes viruses identified in association with the *equidae*. Herpes viruses infecting equines are named after the family of their primary natural host, the names ending in ‘id’, however,the historically applied synonym infrequent usage is ‘equine’ herpes viruses. Within the sub-family Alphaherpesvirinae, the genus *Varicellovirus* contains six species associated with the *equidae*: equine herpes virus 1 (EHV-1) or the ‘abortion virus, equine herpes virus 3 (EHV-3) or coital exanthema virus, equine herpes virus 4 (EHV-4) or rhinopneumonitis virus, equine herpes virus 6 (EHV-6) or asinine herpes virus 1, equine herpe svirus 8 (EHV-8) or asinine herpes virus 3 and equine herpes virus 9 (EHV-9) or gazelle herpes virus 1. In addition, equine herpes viruses 2, 5 and 7 (EHV-2, EHV-5, and EHV-7) and recently, zebra herpes virus 1 has been assigned the subfamily *Gammaherpesvirinae* (Griffin *et al*.,2010).

2.1.1. Structure of equine herpes viruses

Morphologically, herpes viruses are distinct from all other viruses. They contain a linear double-stranded DNA genome of 125-290 kbp in the form of a torus (Furlong *et al*., 1972). The DNA is contained within a icosadeltahedral capsid, which is surrounded by a proteinaceous matrix, designated the tegument. The latter is surrounded by a lipid envelope, containing membrane-associated proteins (Davison *et al.*, 2009).

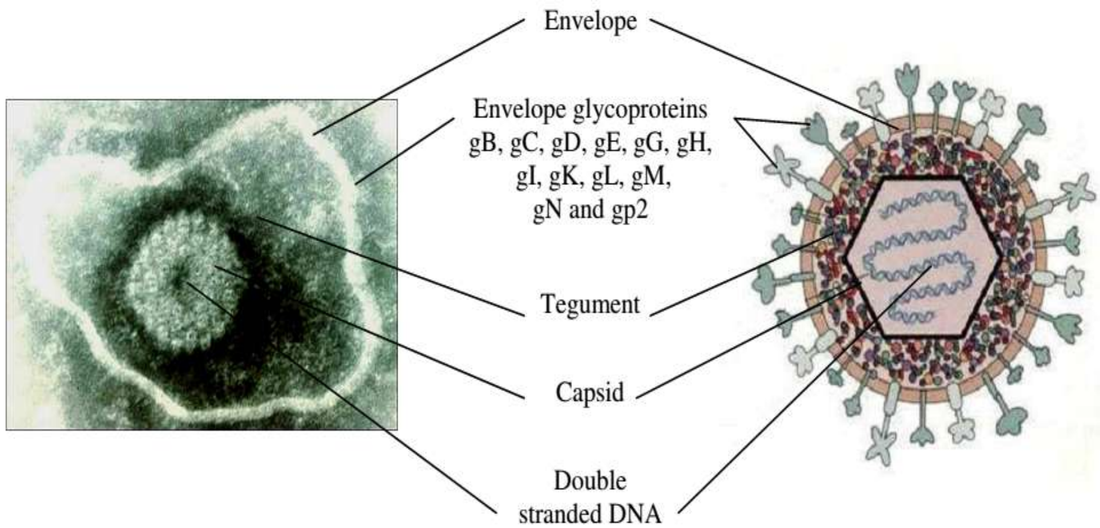

Figure 1:Electronmicroscopic photomicrograph (left) and schematic drawing (right) of an EHV-1 virion.Source:(Annick, 2011).

Electro-microscopic studies of thin sections of infected cells have shown that the envelope has a trilaminar appearance, which is to be expected as it is derived from membranes of the host cell (Mettenleiter, 2002). The envelope contains protrusions or spikes, which are more numerous and shorter than those appearing on the surface of many other enveloped viruses; the spikes consist of glycoprotein, the number and relative amounts of which vary in different herpes virus species. These glycoproteins play an important role in the infection process, mediating both entry of the virion into the host cell and cell-to-cell spread of virus (Roizman and Knipe, 2001).

The tegument is the structure between the capsid and envelope. It has no distinctive features in thin sections but may appear fibrous in negatively-stained preparations. It is frequently distributed asymmetrically and has varying thickness depending on the location of the virion within the infected cell, a factor affecting virion diameter (Furlong *et al*., 1972)

The icosahedra capsid, approximately 100 nm in diameter is composed of 162 morphological subunits (capsomers), 150 of which are hexameric and 12 of which, at the apices of the triangular facets on the icosahedrons, are pentameric (Trus *et al*., 2001).

The core located in the capsid of mature herpes virus particles contains the genome consisting of double-stranded DNA ranging in size from 124-235 kbp in association with viral proteins, and may assume a torus (doughnut) shape. In some herpes virus species, the torus appears to be suspended by a proteinaceous spindle extending from the inside surface of the capsid (Furlong *et al*., 1972).

2.2. Epidemiology

2.2.1. History of the EHVs disease

Equine Herpes virus 1(EHV-1) was firstly described at Kentucky agriculture experimental station in the beginning of the 1930s (Dimock and Edwards, 1933), after that, it was described as an outbreak caused by the same virus in Spain affecting nearly half of 125 mares (Ferrera, 1950). The first isolation of equine abortion virus from aborted fetus and 2 foals died shortly after birth had been recorded in Germany (Foote *et al*., 2003). The initial identification of the virus was done in the United States. The relation between the virus abortion and the respiratory infection was firstly assumed by a European observation. In Netherland, an outbreak of EHV-1 was recordedin 1991 at a Riding school. Since 1966, several equine myeloencephalopathy (EHM) and also, outbreaks have been described in veterinary literature from Europe, North America and recently from Australia *(Friday et al*., 2000). The virus was isolated from aborted fetus on the chorioallantoic membrane of embryonated chicken eggs as a first record (Hassanein *et al*., 2002). This trial was followed by other succeeding ones by some authors, who isolated and identified the local EHV-1 strain from aborted Arabian mares and internal organ of their foeti from a private stud with a history of recurrent abortion during 2005 and 2006 (Beshir Ata *et al*., 2018a).

The first *gammaherpesvirus (*γ-HV) in equids was isolated from a horse with upper respiratory tract disease in 1962. It was referred to as an equine cytomegalovirus. In 1987, other γ-EHVs were described. Although initially considered *betaherpesviruses*, they were subsequently reclassified as γ-EHVs and designated equine herpesvirus 2 (EHV-2) and equine herpesvirus 5 (EHV-5). The first asinine γ-HV was isolated from an asymptomatic donkey in 1988 (Plummer and Waterson, 1963).

2.2.2. Distribution and prevalence of EHVs

Estimates of the prevalence of EHVs infections strongly vary with viral detection technologies (Lunn *et al*., 2009). Increased numbers of EHVs cases have been reported from various parts of the world during the last decade with the majority of them from Europe and North America. European countries viz., France (Legrand *et al*., 2010), Germany (A. Fritsche and Borchers, 2011), Belgium (Gryspeerdt *et al*., 2011), Poland (Stasiak *et al*., 2015a), and Croatia (Starešina*etal*.,2012); North American countries viz., Canada (Burgess *et al*., 2012) and U.S.A (Nugent *et al*., 2006; Perkins et al., 2009a; Smith *et al*., 2010); Japan (Tsujimura *et al*., 2011) and Newzealand (McFadden *et al*., 2016); African countries viz., Ethiopia (Negussie *et al*., 2017) experienced outbreaks of EHV-1 infection by neuropathogenic strains of EHV-1. The incidence of neuropathogenic genotype from cases of neurological illness reported from different countries varies between 20% and 86% (Perkins *et al.*, 2009b, ; Legrand *et al*., 2010; Fritsche and Borchers, 2011). The prevalence of neuropathogenic strains in abortion outbreaks varies between 1.5% and 25.8%. The percentage prevalence was highest (25.8%) in France (Legrand *et al*., 2010) followed by 19.4% in USA (Smith *et al*., 2010), 10.6% in Germany (Fritsche and Borchers, 2011), 3.1% in Poland (Stasiak *et al*., 2015a) and 2.7% in Japan (Tsujimura *et al*., 2011).

2.2.3. Routes of transmission

Equine herpes virus-1, 2, 4, and 5 are transmitted by direct contact, mainly through the infected nasal discharge between infected animals or animal and an infected object. While rare, EHV-1 can be transmitted by aerosol or contaminated feed, water and equipment (Stephen M Reed and Toribio, 2004). Fetal and placental tissues from EHV-1 abortions typically contain large quantities of infectious EHV-1 that comprise an excellent source of infection to other equines. This can occur through either direct contact with the infectious material (e.g., for paddock mates) or fomites e.g., shoes or clothing of grooms, handlers and veterinarians (Gardiner *et al*., 2012). It is still unclear whether the virus can spread by the venereal route or not. Previous reports studied the possible risk of horizontal transmission of EHV-1 via semen and their effect on stallion fertility. By using conventional PCR, EHV-1 DNA in 51 out of 390 total semen samples (13%) were detected, however, the presence of EHV-1 did not appear to affect the fertility of infected stallions (Hebia-Fellah *et al*., 2009). In another study, using real-time PCR, EHV-1 shedding could be detected in semen on day 20 after the onset of fever in naturally infected stallions, which seems not to be directly associated with spermatozoa. EHV-3 is transmitted through direct skin-to-skin contact during coitus or through secretions containing live virus (Walter *et al*., 2012).

2.2.4. Risk factors for *EHVs infection*

The outcome of EHVs infection is influenced by several factors including the age, the physical condition, the immune status of the host, the type of infection (primary, re-infection and reactivation) and the pathogenic potential of the virus strain (Nugent *et al*., 2006), also the state of pregnancy and the different localities significantly affect the prevalence of EHVs infection. Stress, fall and spring seasons and animals older than 5 years of age had a period of pyrexia and a peak temperature occurring on day 3 of the febrile period. These risk factors were predictive of neuro-virulence and death. Mares and foals populations were found to be a reservoir of EHV-1, from which the virus can be transmitted before and after weaning and as young as 30 days of age (Lang *et al*., 2013).

Equine herpes virus-associated myeloencephalopathy could be developed during outbreaks of EHV-1 infection. The disease can affect up to 50% of the exposed equines. This percentage could fluctuate significantly among equine populations and it strongly depends on the demographics and risk factors. The risk of EHM development was shown to be high in older horses (Goehring *et al*., 2006), pregnant mares and mares with foals at foot (Barbić *et al*., 2012). Older animals (>20 years old) were also at high risk for development of EHM following experimental infection with EHV-1; while, EHM was experimentally induced in three out of four mature mares. While experimental infection of foals with a highly virulent strain of EHV-1 resulted in only mild respiratory disease (Goehring *et al*., 2006). Most reports of EHM outbreaks occurred more frequently among stabled equines than those at pasture, which may be related to conditions favoring transmission of any infectious agents due to presence of large numbers of clinically affected horses, higher traffic of both people and equines in and out of stable yards, using of common equipment that may act as fomites and stabled equines are also more likely to be used in training and competing and, thus, are presumably more stressed than those at pasture, which may facilitate reactivation of latent EHV-1 (Schrenzel *et al.*, 2008).

Recrudescence from latency will cause viral respiratory tract replication, which may then start further horizontal spread to other in-contact equines, this horizontal spread through respiratory droplet infection or fomites transmission but also contact with an aborted fetus or fetal fluids or membranes, is considered the second mechanism by which EHV-1 maintains presence in equine populations (Beshir Ata *et al*., 2018b).

Equine herpes virus1 **(**EHV-1) maintains its presence in equine populations via the latency, which is a non-replicative, non-immunogenic stage in a equine. The triggers for EHV-1 recrudescence, as well as the molecular mechanisms underlying this process, are poorly understood. The virus could be reactivated experimentally by the administration of high doses of glucocorticosteroids (Allen, 2002). Thus, in real life, stressful conditions such as transport, sales, competitions have the potential to induce EHV-1 recrudescence (Pusterla *et al*., 2009).

The virus may also recrudesce in immunocompromised animals. The latter may explain a silent circulation of EHV-1 among pregnant mares, as pregnancy has been shown to induce physiological immunosuppression in the equine. Recrudescence of latent EHV-1 may or may not be accompanied by clinical disease (Noronha and Antczak, 2012). In either case, latently infected equines become infectious following EHV-1 recrudescence in the respiratory tract and hence comprise a source of EHV-1 to susceptible animals. Another important consequence of latency is the fact that sporadic cases of abortion and possibly neurological disease, can occur in a closed group of horses, without an external source of EHV-1 infection. The fact that the majority of EHV-1 abortions occur as single events supports this view (Pusterla *et al*., 2009).

The source of the offending virus is thought to be EHV-1 that has reactivated locally within the blood vessels of the pregnant uterus and possibly, by extrapolation, the CNS. Such local reactivation can occur with or without the concurrent lytic respiratory infection and hence, with or without shedding of the virus in nasal secretions. The initial respiratory infection that led to the establishment of latency could have happened at any time in the past, possibly months to years before EHV-1 abortion or neurological disease (Allen, 2002). This provides an obvious challenge to the diagnosis and control of EHV-1-associated diseases. As with other herpes viruses, the ability of EHV-1 to infect horses and establish a long-term latent-carrier state in the face of the host immune responses assures in definite endemic EHV-1 infection in the equine population. Resistance to re-infection resulting from recovery from field infection with EHV-1 is short-lived, lasting only a few weeks or few months. After infecting the equines via the respiratory tract, EHV-1 rapidly becomes intracellular, including within circulating lymphocytes and passes directly from cell to cell without an extracellular phase so that the virus is not exposed to neutralizing antibodies and other protective components of the immune response. It is worth noting that the persistent (latent) infection site including peripheral blood mononuclear cells (PBMCs) and trigeminal ganglia, in order to be effective, EHV-1 vaccines must satisfy a challenging set of demands and activate a protective response that exceeds that provoked by natural infection (Pusterla *et al*., 2009).

Mechanism behind EHM is poorly understood. Studies on the evaluation of the risk factors associated with the development of EHM have been performed in Europe and in North America. Various factors viz., season, age, breed; sex, immunological status and latency have been found to be associated with EHM. A study in the Netherlands revealed a strong association between season and outbreaks of EHV neurological disease with all outbreaks occurring between mid-November and mid- May. However, this season specificity has not been observed in all countries (Paillot *et al*., 2008). Experimental infection proved that older horses are more predisposed to the development of neurological disease as compared to young to young/middle aged equines. Adult equines may develop viremia 100 times higher than young equines and they are 8 times more likely to develop the disease (Allen, 2008).

Equine herpes virus myeloencephalopathy (EHM) was present in equine population as early as 1950s, however, its importance came to limelight in the last decade after large outbreaks of EHM occurred in Europe and America (Stasiak *et al*., 2015b). Neurological disease can affect horses of all ages, including un-weaned foals, and often requires euthanasia of the affected animal. Horses exhibiting neurologic diseases can shed the virus in their nasal secretions and transmit the disease to in-contact animals (Henninger *et al.*, 2007a). The ORF30 spanning the nucleotide region 51,522 – 55,184 (3662nt) in EHV-1 genome encodes for a protein referred to as Pol, the putative DNA polymerase catalytic subunit which possesses DNA synthesis activity. This gene is highly conserved throughout its length. Recently, a single nucleotide polymorphism (SNP) of guanine (G) for adenine (A) at 2,254 nucleotide position of the ORF30 region resulting in an amino acid variation, from asparagines to aspartic acid (N/D752) have been proven to be associated with the neuro-pathogenic potential of the EHV-1 strain (Nugent *et al*.,2006). This DNA polymerase enzyme of EHV-1 has two sets of identical protein subunits each of which contains two catalytic pockets, serving as site for polymerase activity and the site for 3'- 5' exonuclease activity. In EHV-1, neuro-pathogenic strains, the point mutation results in a switch from no charge to a negative charge and induces a conformational change within the viral polymerase structure and thereby increases the replicative capacity of the virus and produce significantly higher viral loads (Liu *et al*., 2006).

The viral factors associated with the occurrence of abortion include strain variation; for example, there is a purported strong association of the N752 strain variant of the nonneuropathogenic ORF30 A2254 in natural abortions (Nugent *et al*., 2006). Nevertheless, while abortogenic potential reportedly varies between strains of EHV-1, most strains are presumed to be capable of inducing abortion, in contrast to the situation for neurological disease which is associated with only a few strains. Both the N752 and the D752 strains of the neuropathogenic ORF30 G2254 EHV-1 genotype have been associated with abortion, although neurotropic strains are apparently less commonly associated with abortion in the absence of neurological disease (Léon *et al*., 2008).

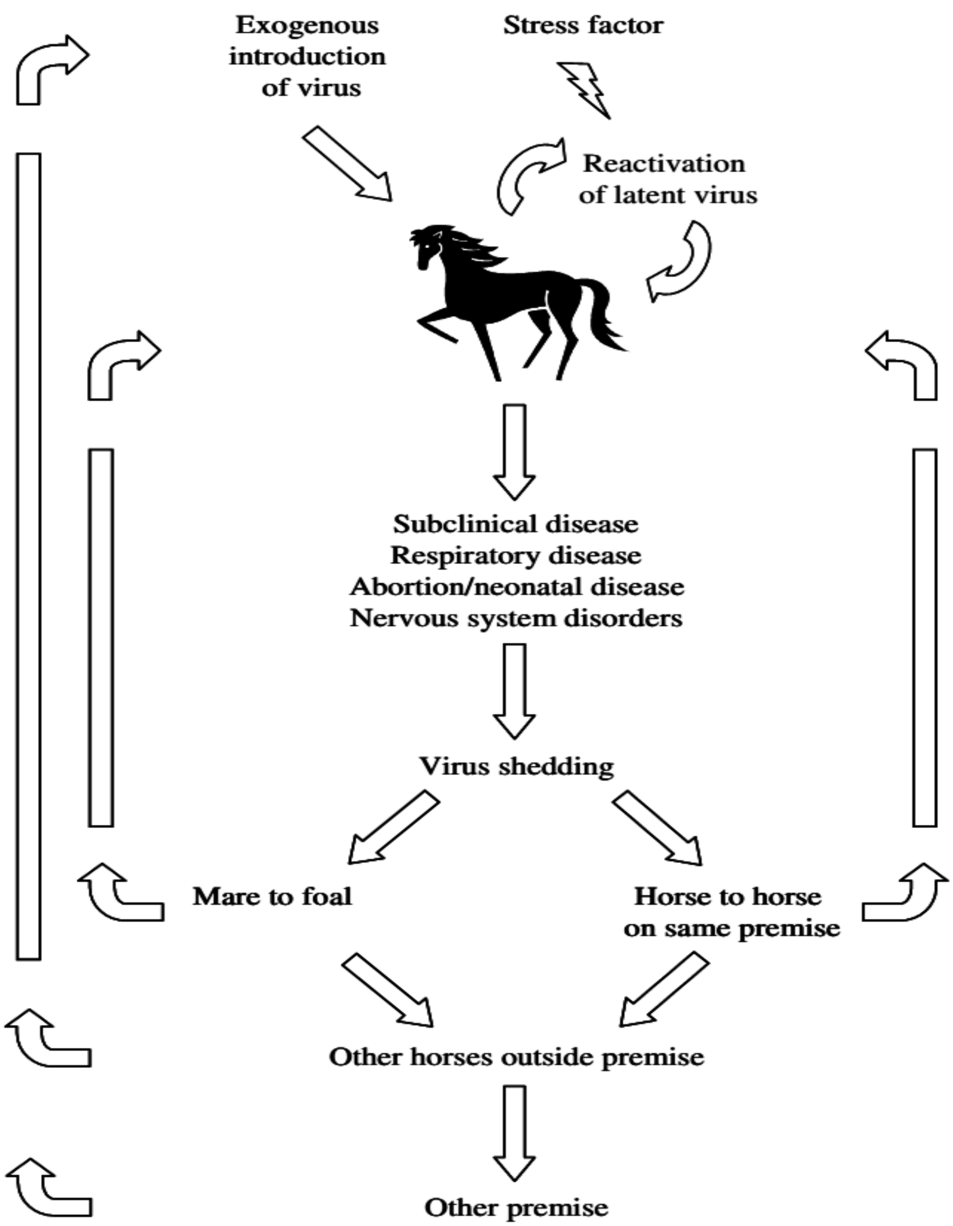

Figure 2: A schematic overview of the epidemiology of EHVs infection in the horse population. Source: (Annick, 2011).

2.3. Pathogenesis of Equine Herpes virus Infection

2.3.1. Equine alphaherpesviruses

Primary EHV-1 and EHV-4 infections occur at the respiratory epithelium, resulting in distinct herpetic lesions of the upper respiratory epithelial mucosa and viral shedding for 10 to 14 days after infection, or even longer in EHM-affected horses (Lunn *et al*., 2009).

2.3.2. Primary replication

Horses acquire an EHV-1 infection through direct contact or inhalation of infectious secretions (Allen, 2004). After inhalation, EHV-1 binds to and penetrates the epithelial cells of the upper and lower respiratory tract (Kydd *et al.*, 2006). Following a rapid replication, EHV-1 disseminates into the connective tissue, infecting local blood vessels and regional lymph nodes. How the virus penetrates the basement membrane and reaches the underlying connective tissue and lymphoid tissue remains unsolved to date (Van Maanen, 2002).

2.3.3. Viremia

Via a still unknown pathway, EHV-1 infects mononuclear cells which can enter the blood circulation resulting in a cell-associated viremia lasting for 8 to 18 days post-infection (Gibson *et al*., 1992). The exact identity of these carrier cells remains uncertain. A study from 1983 showed that T-lymphocytes, B-lymphocytes, and monocytes are all able to harbor EHV-1, but the predominant mononuclear cells harboring virus were found to be T-lymphocytes. Remarkably, after disruption of these cells, no infectious EHV-1 could be found during virus isolation, suggesting that EHV-1 exists in mononuclear cells in a non-infective form, or is blocked at a certain replication level until activation occurs (van der Meulen *et al*., 2006). A later study confirmed the latter hypothesis. The majority of infected PBMC does not show viral envelope proteins on their surface during viremia (van der Meulen *et al*., 2003). During a recent study using PBMC isolated from experimentally infected horses, viral genomic DNA was found using PCR techniques in allPBMC subpopulations, with CD8+lymphocytes and B-lymphocytes as most frequently infected cells (Wilsterman*et al.*, 2011).

2.3.4. Secondary replication

Transfer of EHV-1 from viremic cells to endothelial cells of target organs causes infection, followed by replication of EHV-1 and subsequent damage to these organs. Activation of adhesion molecules in endothelial cells of the nasal mucosa and the reproductive tract is a key step in transferring virus from infected leukocytes to endothelial cells. Indeed, an in vitro flow system using equine veins and arteries showed that EHV-1 was only transferred to endothelial cells if both leukocytes and endothelial cells expressed these surface molecules (Smith *et al*., 2002).

Secondary replication of EHV-1 in the nervous system of equines causes EHM. Different independent researchers agreed that lesions accompanying central nervous system disorders were characterized by a focal vasculitis with associated edema, hemorrhage and thrombosis. Axonal and myelin degeneration occurred focally and secondary to these vascular changes (Platt *et al.*, 2000). As actual isolation of EHV-1 from the nervous system is very rare, it was postulated that lesions could be induced by circulation immune complexes. However, Edington*et al.,*(1986) showed that although recovery of virus from the central nervous system (CNS) was low, immunofluorescence indicated that endothelial tropism of the virus was the initiating and persisting feature. The action of immune complexes in EHV-1 infections seems to be secondary and localized. In contrast to several other *alphaherpesviruses*, which can cause encephalitis through primary neurotropism, EHV-1 seems to be non-neurotropic and its propensity to induce myeloencephalopathy reflects a marked endotheliotropism (Wilson, 2007).

2.3.5. Myeloencephalopathy

Secondary replication occurs in endothelial cells of CNS-associated arterioles (in particular the vessels of the spinal cord), which may result in nervous system disorders 9-13 dpi. As a consequence, vasculitis, thrombosis, perivascular cuffing of lymphocytes at sites of endothelial infection occurs, probably caused by direct interaction of the host's immune system and infectious. This vascular damage leads to ischemia and reperfusion injury of the CNS. Neuropathogenic strains are capable of exhibiting longer and higher level viremia. This high-level viremia, interfere with the blood flow to CNS and resulting in the development of neurological diseases (Fritsche and Borchers, 2011).

2.3.6. Abortion

The pathogenesis of EHVs associated abortion is not fully defined, with two methods of viral transmission from the pregnant endometrium to the placenta having been described that may occur concurrently (Smith and Borchers, 2001). The key stage, namely endometrial endothelial cell infection, follows the viremia (an essential pathogenic prerequisite) associated with the initial respiratory lytic infection or reactivation. EHV-1-infected leukocytes reach the endometrium where they bind to endothelial cell surfaces, particularly in the smaller arterioles supplying the endometrial glands at the base of the micro-cotyledons. Subsequent sequence of vasculitis, thrombosis, micro-cotyledonary infarction with perivascular cuffing and trans-placental spread at the vascular lesion sites ensues (Gardiner *et al.,* 2012).

The transfer of infected leukocytes may occur in >50% of cases during EHV-1 abortion outbreaks. This may take the form of direct transmission via endometrial infarcts as endothelial cells lyses, freeing large amounts of free virus that enters the fetal trophoblasts, propagating widespread endometrial infarction and rapid detachment of fetal membranes with expulsion before the fetus is infected (Platt *et al*., 2010)**.** This may explain the phenomenon of viral-negative fetuses in ‘atypical’ cases where viral antigen and nucleic acids are found only in association with the fetal membranes. Alternatively or additionally, trans-placental cell-to-cell migration of the infected maternal leukocytes may occur, with infected monocytes transiting the chorion's epithelial layers, and small amounts of virus being slowly transferred from the endometrium via the fetal membranes. This latter scenario is probably seen in abortion cases where infarction results in milder lesions and with virus reaching the fetus prior to expulsion, generating the viral-positive fetuses reported most commonly. These cases with fetal infection are additionally associated with viral loads in fetal tissues far greater than in the fetal membranes (Gardiner *et al*., 2012). If this trans-placental infection occurs close to term, it is possible for an infected foal to be born alive; however, these neonates almost invariably succumb to interstitial pneumonia within a few days of birth. The uterine endothelial cells are reportedly more susceptible to EHV-1 infection in late than early gestation. Susceptibility during the early embryonic phase of gestation and any association with early embryonic death is currently unknown (Platt *et al*., 2010).

Endothelial and leukocyte cell surface adhesion molecules, the expression of which may be regulated by hormonal factors associated with late gestation, have been proposed to facilitate infection of the endothelium (Smith *et al*., 2002). The'less-virulent' EHV-1 strains are reported to have a reduced tropism for endothelial cells, nevertheless associated abortion is still a consequence of the vascular lesion and thrombo-ischemia due to viral replication in the endothelial cells as would be the case in the rare incidents of EHV-4 associated abortion which result when EHV-4 is able to replicate in the endometrial and trophoblastic endothelial cells (Bell *et al*., 2006). Vasculitis is a feature described at between five to nine months of gestation, suggesting that gestational age or additional host factors may be predictors of abortion as an outcome In short, a combination of both viral and host factors probably influence the outcome of EHV-1 infection of the gravid uterus, given that pregnant mares can become infected without abortion being an inevitable sequel (Slater *et al*., 2006).

2.3.7. The equine gammaherpesviruses

EHV-2 and EHV-5 are most frequently transmitted horizontally to a newborn foal from its damvia the nasopharyngeal route or subsequently, through contact with other foals (Bell *et al*., 2006). After inhalation, the virus infects the upper respiratory tract mucosal cells and B-lymphocytes probably at the site of draining lymphoid tissue (Gilkerson *et al*., 2015). Then, the lymphocyte-associated virus enters the circulation, disseminates systemically, and establishes latency (Allen *et al.*, 2004). Periodic reactivation of latent infections can occur throughout the life of the horse, resulting in the spreadof infection within a population. However, infections in later life appear mostly to be asymptomatic (Gilkerson *et al*., 2015).

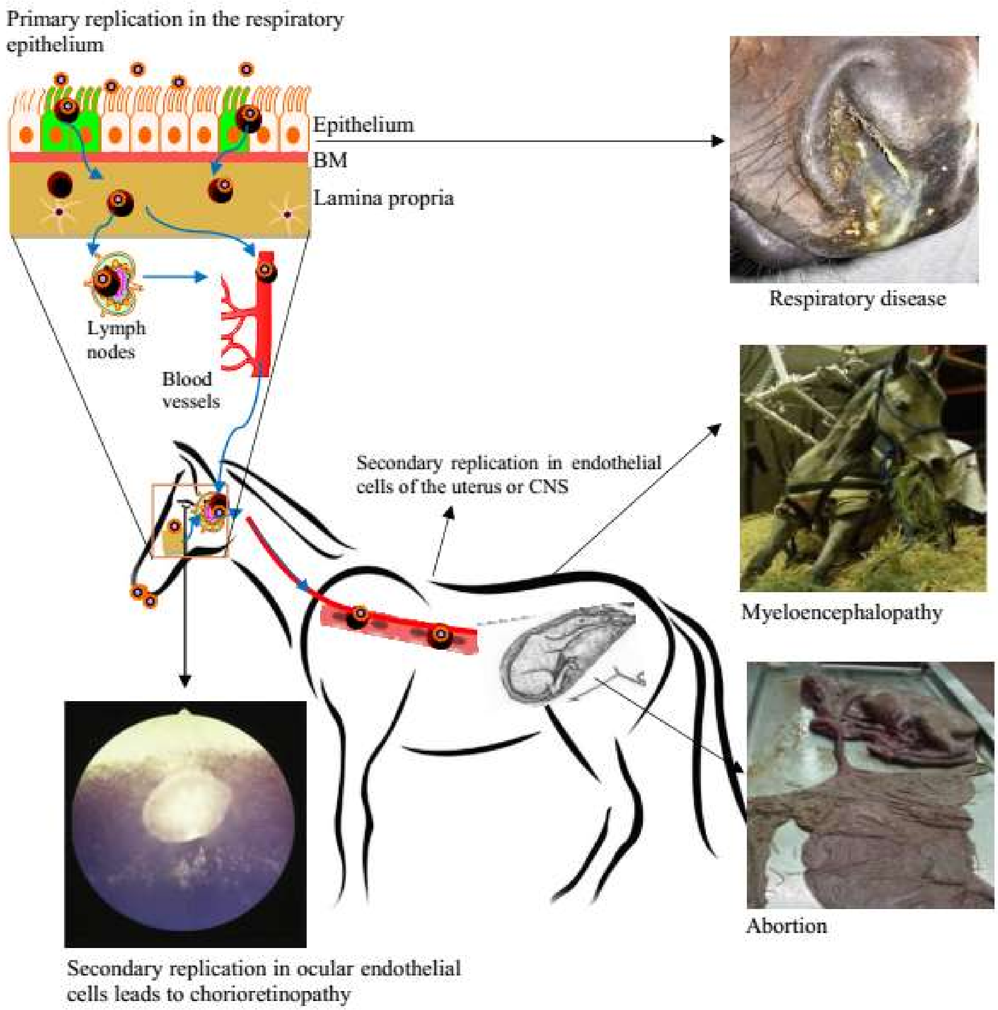

Figure 3: The pathogenesis of EHV-1. The virus enters into the respiratory mucosa by inhalation, causing erosions in the respiratory mucosa.Via a cell-associated viremia, EHV-1 spreads to the underlying tissues and further replicates in the local lymph nodes of the respiratory tract. Hereafter, the virus is transported to the endothelial cells of the pregnant uterus, the central nervous system, or the eye, hence leads to abortion, myeloencephalopathy, or chorioretinopathy. Source: Negussie *et al*. (2017).

2.4. Clinical Presentation of Equine Herpes virus Infection

2.4.1. Clinical signs associated with EHV-1 and EHV-4

Despite their close genetic and antigenic similarity, these two herpes viruses show markedly different pathogenicity (Osterrieder and Van de Walle, 2010). Equine herpes virus 4 is associated almost exclusively with upper respiratory tract (URT) infection, whereas EHV-1 typically has a systemic distribution and affects multiple organ systems with a consequently diverse range of diseases of varying severity (Ma *et al*., 2013).

Many infections with EHV-1 occur asymptomatically or are accompanied by respiratory disease of varying severity. However, EHV-1 infection can also result in comparatively more serious clinical outcomes such as abortion, neonatal death or neurological disease (Henninger *et al*., 2007a). The existence of EHV-1 viruses with different pathogenic potential has been described by several authors.; however, the virus, host and environmental factors that influence the clinical outcome of EHV-1 infection are poorly understood (Tearle *et al*., 2003).

2.4.2. Respiratory Form

Infection of presumed-naive horses with EHV-1 results in upper respiratory disease characterized by fever, depression, anorexia and progressive lymphadenopathy, as well as ocular and nasal discharges that often progress from serous to mucopurulent. Some animals may cough, although this is not a consistent clinical feature of EHV-1 infection. An initial leukopenia is followed by leukocytosis that develops approximately 1 week following EHV-1 infection, coinciding with the second peak of fever, and often with the change in the character of the nasal discharge. The clinical signs of upper respiratory disease usually subside within approximately 2 weeks of EHV-1 infection, although it may take longer for the lymph nodes to return to normal size (Pusterla *et al*., 2009). Severe rhinitis and bronchopneumonia described in association with natural EHV-1 infections could rarely be reproduced experimentally. Isolates sourced from animals showing severe respiratory disease typically induced only mild respiratory disease in experimentally infected animals (Allen, 204).

The incubation period observed for natural infections was alsolonger (Choi *et al*., 2014) than that observed under experimental settings (1–3 days) (Gibson *et al*., 2002). This suggests that the severe respiratory disease observed in some field outbreaks may reflect secondary bacterial infections, with mild or asymptomatic primary viral infections acting as a predisposing factor. Equines affected by respiratory disease usually recover uneventfully, although the time to recovery may be affected by the presence of concurrent or secondary infections. Some equines may show prolonged poor performance after apparent recovery from EHV-1 respiratory disease, which adds to the economical burden of disease (Pusterla*et al*., 2009).

2.4.3. Abortion form

Equine herpes virus 1 **(**EHV-1) is undisputedly the most important viral pathogen associated with equine abortion because of its prevalence, potential for epizootic spread particularly within naïve populations and economic impact (Lunn *et al*., 2009). The epizootic manifestation (abortion 'storm') can involve approximately 1/3 of exposed mares, and abortion rates of up to 87% have been reported for unvaccinated mares. Vaccination, heightened awareness and more effective disease reporting appear to have reduced the frequency of EHV-1 abortion, and in particular the incidence of abortion storms, in many countries (Damiani *et al*., 2014).

2.4.4. Neurological or myeloencephalopathy (EHM) form

The onset of clinical signs of EHM usually occur 6-10 dpi following the onset of viremia. Clinical signs depend on the number and size of affected sites, as well as relevance and location of affected nervous tissue (caudal spinal cord is most affected). Clinical signs usually include fever, ataxia, paresis/paralysis of hind limbs, bladder dysfunction, urinary incontinence and sensory deficit in the perineal area. In addition, ventral edema, scrotal or preputial edema in male horses, and limb edema are also noticed. In severe cases of EHM, paralysis may advance to tetraplegia and death of animal is observed (Pusterla and Hussey, 2014).

2.5. Diagnosis Techniques

2.5.1. Virus isolation

Virus culture and isolation remained as the gold standard for laboratory diagnosis of EHVs infections. The technique demonstrates the typical cytopathic effect (CPE) in susceptible cell cultures inoculated with sample supernatant and use concurrently with the rapid test of polymerase chain reaction (PCR) or immunoassays. The common cell used to isolate EHVs is Rabbit Kidney Cells (RK-13), Baby Hamster Kidney (BHK-21), Madin-Darby Bovine Kidney (MDBK), and Pig Kidney (PK-15) (Damiani *et al*., 2014). Nevertheless, it is important to understand that different cell lines have different sensitivity which could cause false-negative results (Balasuriya*et al*., 2012) and thus, the most susceptible cell must be favored especially if the infrequent cases of EHV-4 abortion are to be detected. The *alpha herpes viruses*, EHV-1 and EHV-4 produce CPE characterized by rounding and clumping of cells, the detachment of cells, and multinucleated syncytial cells within at least one to two days post-infection of high viral load sample. The presence of the virus in cell culture is confirmed with PCR (Lunn *et al*., 2009).

Isolation of EHVs from nasal swabs or peripheral blood mononuclear cells (PBMC) from clinically infected equines could provide robust evidence of the viral infection. For EHM suspected case, it is recommended to collect the nasal swab sample during the early stages as the neurological signs start to appear at the end of viremia phase. The virus also can be detected in PBMC during the viremia phase before the equines start to develop the neurologic signs. As for suspected case of EHV-1 abortion, tissues samples such as placenta, lung, liver, thymus and spleen from the aborted fetus the virus isolation can be attempted for virus isolation. Periodically, the virus is also presence in the cerebrospinal fluid (CSF) along with neurological signs (7-16 dpi) but it is rarely collected since isolation is difficult (Kohn *et al*., 2006).

The viability of the virus is required in the sample and it may be affected by factors such as sample collection and storage, timing of sample collection, postmortem interval and sample transport. For nasal swab, it is recommended to take the sample during the initial febrile phase and instantaneously transport them in sterile cold transport medium (Pusterla *et al.*, 2006). Blood sample intended for buffy coat separation should not be frozen while tissue samples should be kept at 4°C instead of -20°Cuntil inoculated into cell culture. Samples that will not be processed immediately should be stored at -70°C.The result of virus isolation may be negative due to procedural mistakes that could have affected the viral envelope integrity; a crucial component for host infection (Goehring*et al*., 2010a). It is also due to intermittent ofvirus shedding and interference of local antibodies (Harless and Pusterla, 2006).

2.5.2. Serology-based diagnosis technique

Serological testing for EHVs antibodies in serum or plasma has been the key tool to gain retrospective diagnosis and forms a valuable part of longitudinal surveillance. Serology helps to provide information on EHV-1 exposure in non-vaccinated equines which can be used to guide the management practices. Despite some limitations, most serologic assays are still reliable and useful for viral diagnosis, cost-effective and fairly easy to perform. Over the years, a variety of serology methods has been used in the detection of EHVs antibodies including the most common test formats for equine virus diseases, virus neutralization (VN) and enzyme-linked immunosorbent assay (ELISA) and other serologic tests (Irwin, 2007).

2.5.2.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA is one of the most common serologic tests used asa screen test in detection of many infectious diseases. InELISA, the antigen is bound to the wells of the plasticplate. The serum samples are added, and if EHV-1 or EHV-4 are present, they will bind to the antigens. When unbound antibody are washed, a secondary antibody against antibody such as anti-equine immunoglobulin G (IgG) conjugated to an enzyme like horseradish peroxidase is added afterward and becomes a complex with these antibodies. Unbound antibodies will be removed prior the addition of a substrate solution. The addition of colorimetric substrate produces a visible color change which indicates the positive reaction. The results can be interpreted visually or by microplate spectrophotometer (Abdullah *et al*., 2018).

Theoretically, detection of EHV-1 specific antibody would provide a more convenient way toidentify latently infected horses. Earlier ELISA assays, like other serologic assays, did not discriminate between EHV-1 and EHV-4 because of the close antigenic similarity between these two strains. Through recent advance, a type-specific ELISA using fusion proteins expressing variable regions of glycoprotein G (gG) homologues has been developed and able to distinguish antibody to EHV-4 and EHV-1 (Diallo *et al*., 2007). It has been shown to be a valuable diagnostic tool to identify horses infected with EHV-1 or EHV-4 when acute and convalescent sera are available. The IgG ELISA has been used in the epidemiological studies of EHV infection, as well as in the management of outbreaks of EHV-1 myeloencephalitis and abortion *(Studdert et al.*, 2003). A commercial ELISA test kit has been developed using EHV-1/4 recombinant glycoprotein G for the detection and distinguishing EHV-1 and EHV-4 infections such as Svanovir® and has been used broadly in to detect EHV-1 and EHV-4 infections (Ataseven*et al*., 2009).

2.5.2.2. Virus neutralization

Virus neutralization (VN) test detects antibodies capable of neutralizing the infectivity of the virus. This technique involves a serial dilution of heat-inactivated serum followed by incubation with 100-200 TCID50 virus which leads toneutralization of the virus with antibodies. The mixture is added into the cells to see any infection (Abdullah *et al*., 2018). The presence of the viral growth is examined by microscopic for the evidence of cytopathic effect (CPE). In the case of the low amount of virus present in the cell or no

CPE observed, immunofluorescence staining should be applied. By neutralizing the infectivity of the virus, antibody protects the cells against viral infection. The highest dilution that neutralized virus infectivity known as the titre (Marenzoni *et al*., 2008).

The detection of specific antibody in serum required more than a single blood collection as it is insufficient for a positive diagnosis of current and active infection. The diagnosis of acute EHV-1 cases by VN is reliable if the sampling is conducted during the onset of the disease. Therefore, it is recommended to collect a paired serum samples from in-contact equines since many of these animals seroconvert, showing indirect indication of EHV-1 infection (Pusterla*et al*., 2009). The acute phase sera sample should be taken after the beginning of clinical signs followed by convalescent phase sera taken two to four weeks after. Fourfold or greater increase in virus-specific antibody titre is needed to confirm the infection. According to American Association of Equine Practitioners, high titers in a single VN between 1:1024 to 1:2048 or greater are most probably demonstrate the recent infection rather than vaccination. Moreover, VN test is suitable to be used in prevalence surveys as it is capable of indicating the historical exposure of the infection (Smith and Borchers, 2001).

2.5.2.3. Complement fixation

The complement fixation (CF) is a method used to detect the presence of specific antibody such as IgM or antigen in the serum. CF test depends on formation of specific antibody-antigen complex in which during the reaction, complement will bind or fix to it. Sensitized sheep red blood cells are added to measure the complements that have bound in the reaction. If the complements have been fixed due to the antigen-antibody reaction, there will be no complements remain for the lysis of the sensitized sheep red blood cell and no haemolysis indicates the positive result (Abdullah *et al*., 2018).

Complement fixation (CF) along with other serological assays is suitable for ascertaining antibodies against EHV-1 or EHV-4. CF antibodies have low vitality which is normally untraceable for three months when infection happens. Hence, it is visibly effective during the outbreak by utilizing two pairs of collected sera within a fortnight apart as it is capable in providing the most powerful serological evidence for early EHV-1 infection (Hussey *et al*., 2006).

2.5.2.4. Immunofluorescence

Immunofluorescence (IF) is a technique to detect specific target antigens innasal or nasopharyngeal swab samples or in frozen (cryostat) sections from aborted fetal tissues (lung, liver, thymus and spleen) and placental tissue in the detection of EHV using the fluorescent-labeled antibodies. In IF techniques, the sections are mounted on microscope slides, fixed with acetone and incubated at 37°C treated with a proper dilution of the swine antibody specific for EHV-1 which formed chemically with fluorescent dyes such as Fluoresce inIsothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). These marked antibodies attach (directly or indirectly) to the antigen of interest which enables the detection of antigen via fluorescence microscope (Sarani*et al*., 2013).

2.5.2.5. Immunohistochemistry

Enzyme immunohistochemical staining such as immune-peroxidase has been established as a technique for detecting EHV-1 antigen paraffin embedded tissue of aborted equine fetuses or neurologically affected horses. Furthermore, it also can be attempted on infected cell monolayers for both EHV-1 and EHV-4 detection (Van Maanen *et al*., 2000).

2.5.3. Molecular approaches

Latency by *alphaherpesviruses* is seen as an important epidemiological plan to secure vitality while expanding inside the natural host population (Wilhelm and Pingoud, 2003). The latency site for EHV-1 have been found in the lymphoid tissue debilitating the respiratory tract and in the peripheral blood (Elia *et al*., 2006). Other studies claim latency is established mainly in the trigeminal ganglia (Barrandeguy and Carossino, 2018). During latency, the entire viral genome exists inside the host cells but only a few part is transcribing into detectable viral RNA, known as latency-associated transcripts (LATS). This features of EHV renders the conventional methods of detecting thevirus in the latent form. Molecular-based approaches are evidently more sensitive and specific, which have provided a rapid and accurate detection and characterization of EHV. By targeting these LATs, latency can be detected by using reverse transcriptase polymerase chain reaction (RT–PCR) or by real–time PCR. From the past decades, diagnosis by PCR including nested, multiplex PCR and quantitative PCR have form a vital part of the range of diagnostic tests currently available for EHV. The advent of the latest technology of loop-mediated isothermal amplification (LAMP) has great potential to emerge as a new approach for a rapid diagnostic tool for early detection and identification of EHVs (Abdullah *et al*., 2018).

2.5.3.1. Conventional polymerase chain reaction

Polymerase chain reaction (PCR) in detection of EHV-1 is considered superior to virus isolation in terms of rapidness, sensitivity and specificity (Marenzoni*et al*., 2008). The assay is able to detect 10 to 100 copies of target viral and produce a positive result although the testing with virus isolation is negative due to the low magnitude of viral load *(*Lunn*et al.*, 2009). The sensitivity of PCR is suitable for consensus study involving tissue samples in previously characterized EHVs as well as providing new sequence information for previously unreported EHVs (Leon *et al*., 2008).

2.5.3.2. Real time PCR

Quantitative PCR or real-time PCR (qPCR) was developed in 1996 using a probe-baseddetection system and enables the PCR amplification in a closed-tube system. Compared to conventional PCR, qPCR provide a lower risk of cross-contamination due to its close and automatized amplification system. The high technology offered by this assay, however, requires some initial high capital costs for instruments such thermal cycler which most of the low resource setting laboratory could not afford (Wilhelm and Pingoud, 2003).

The advanced in quantitive real-time PCR enables the discrimination between two viral states. The differentiation between latent and lytic infection rely on identification of the viral genome (DNA) and viral transcripts (mRNA) for one of the structural genes. Detection of viral DNA without the viral mRNA indicates the latent virus while detection of both viral DNA and mRNA demonstrate an active EHVs infection. In a previous study, the expression of the structural glycoprotein B in nasal swabs of the infected foal was detected only during the first week of the 28 days study period after the onset of the clinical signs. There is no persistence of LAT expression was recorded within the period, suggesting that the lytic infection of EHV-4 can be marked either by high DNA load or by detection of the transcriptional activity of the glycoprotein B (Pusterla *et al*., 2005).

2.6. Treatment

2.6.1. Antiviral therapy

Treatment of EHV-1 infections is limited to symptomatic care since no specific antiviral drugs are available for treatment of EHV-1 infections in horses to date. Experimental antiviral drugs have yet to prove clinical efficacy and several of them have been tested in field as well as under experimental conditions. During several natural outbreaks of EHV-1, treatment with the antiviral drug acyclovir has been reported with conflicting results (Henninger *et al*., 2007b). However, since therapeutic evaluations in case-control studies under field conditions are difficult due to a lack of good control animals, great care should be taken in interpreting these results. Acyclovir was also shown to have serious limitations due to poor oral bioavailability. Therefore, high therapeutic concentrations are needed in order to reach the desired effect, making thisdrug a costly medication with questionable efficacy. When using the pro-drug valacyclovir, sufficiently high acyclovir levels could be reached in plasma and nasal mucus after oral treatment, but no effect of this treatment was seen on clinical signs, viral shedding and viremia of EHV-1-infected ponies (Garré*et al*., 2009). The latter was in contrast to the results of another study, showing that oral treatment with valacyclovir significantly decreasedsigns of EHV-1 disease (Maxwell *et al*., 2008).

Recently, synthetic siRNAs against envelope glycoprotein B and the origin binding protein helicase were designed, and their efficacy to limit and prevent EHV-1 infection was tested in vitro and in a murine model. The results obtained during this study, indicated that siRNA treatment could be of great importance during an outbreak of EHV-1 in a horse population, as not only the severity of clinical disease but also the number of affected animals and the magnitude of nasal shedding could be reduced (Fulton *et al*., 2009). Although there was no significant difference in viral shedding and viremia between treated and control groups during an in vivo experiment with EHV-1-infected horses, euthanasia necessitated in case ofneurological disease was significantly reduced after application of EHV-1 specific siRNA (Brosnahan *et al.*, 2010).

2.6.2. Supportive therapy

Despite many experimental approaches, no systemic antiviral therapeutic agents are approved for the use in horses or have a proven efficacy. Therefore, supportive therapy is of paramount importance in the field. Treatment strategies are aimed at easing clinical signs and preventing secondary complications. The level of care necessary is dependent on the severity of clinical signs. Respiratory infections of immunocompetent animals are generally self-limitingand require only good nursing care. Broad-spectrum antibiotics can be administered toassist in combating secondary bacterial infections and high fever can be treated with antipyretics. Stress avoidance is paramount and horses in training should receive adequate rest before resuming work (van Maanen, 2002).

Abortion occurs suddenly with complete expulsion of fetus and placenta and complications are seldom. Therefore, after an uncomplicated partum, usually notherapy for the aborting mare is required. However, it is advisable to wash tail and hind quarters with disinfectants to avoidfurther viral contamination of the environment and/or other animals (Neubauer and Osterrieder, 2004).

Weak, but live born affected foals or, less frequently, foals that initially appear healthy but rapidly deteriorate due to intractable bacterial infections should be treated with intensive therapy like provision of warmth, oxygen, cardiovascular andnutritional support and appropriate drugs (van Maanen, 2002). However, these measures are almost always unsuccessful, as these foals are almostalways bound to die within a few days due to extensive damage in parenchymal and lymphoreticular organs and subsequent bacterial infection (Goodman *et al*., 2006).

Animals showing nervous system disorders should receive appropriate intensive care. Non-recumbent animals should be housed in a deeply bedded box, until coordination improves. Severely ataxic equines may be temporarily assisted by a slingto prevent secondary complications associated with recumbency. Regular urinary catheterization and rectum evacuation may be required in case of bladder or rectum paralysis (Friday *et al*., 2000). Equines which are able to stand, should be turned out or hand walked as soon as possible (Van Maanen *et al*., 2001).

Recumbent animals not able to be supported by a sling should be maintained in a sternal position in a well-bedded stable if possible. They should have easy access to food and water. Intravenous rehydratation may be necessary for horses unable to remain in sternal position. Horses in lateral recumbency should be manually rotated every few hours. Antibiotics are indicated because of the higher risk of developing aspiration pneumonia and/ordecubital ulcers. Horses can also be treated with corticosteroids in order to reduce thei nflammatory response in the nervous system. However, considerable care should be taken when treating horses with such drugs during an outbreak, as they induce immunosuppression and may stimulate a longer and higher spread of virus which canmake them even contra-indicated (Goehring and van Oldruitenborgh-Oosterbaan, 2001). Therefore, only short-acting corticosteroids, if at all, should be used, for a limited period (Reed and Toribio, 2004).

Other anti-inflammatory drugs such as dimethyl sulphoxide (DMSO) and non-steroidal anti-inflammatory drugs can be used, although their capacity to inhibit the development of the lesions of EHM is unknown. A recovery of non-recumbent animal is usually complete, although improvement is gradual and several months may elapse before maximal improvement. However, when a horse is recumbent for more than 24 hours, the prognosis is grave and euthanasia may be indicated (Van Maanen *et al*., 2001).

2.7. Control Strategies

2.7.1. Vaccination

Equine herpes virus 1(EHV-1) remains a leading cause of upper respiratory tract infection, viral abortion and nervous system disorders in horses worldwide, despite the customary use of commercial vaccines (Kydd *et al*., 2006). Following natural infection, EHV-1 efficiently induces a local mucosal humoral response, which offers the potential for immune exclusion of virus at the respiratorytract epithelium portal of entry. However, neither inactivated, nor attenuated vaccinesare able to induce detectable mucosal antibodies (Breathnach *et al*., 2001).

The currently available vaccines are not able to induce a significant level of protection against EHV-1-induced viremia. However, several vaccines are able to induce asignificant level of protection against either abortion or nervous system disorders (Breathnach *et al.,*2001). The purpose of vaccination is twofold. First, vaccination is meant to minimizevirus replication in the respiratory tract upon infection, thus limiting nasal shedding and the occurrence of respiratory disorders. Second, vaccination should prevent the occurrence of abortion and/or nervous system disorders. The first purpose ofvaccination seems to be fulfilled by the available vaccines, as demonstrated by reduced nasal virus titres and the reduced severity of respiratory disease up on challenge infection of vaccinated horses (Heldens *et al*., 2001).

Many studies have addressed the potential of inactivated vaccines in the battle against EHV-1. Inactivated virus vaccines can stimulate high titres of serum virus neutralizing (VN) antibody, which cannot only reduce the amount and duration ofvirus shedding but also prime the mucosal compartment (Breathnach *et al.*, 2001). However, they fail to induce cytotoxic T-leukocyte (CTL) responses which haveproven to be of paramount importance to limit EHV-1 infection (Minke *et al*., 2004). Several case-control studies have been performed with thecommercially available vaccine Duvaxyn EHV1,4® (Foote *et al*., 2002). This inactivated vaccine clearly reduced clinical symptoms, the duration ofvirus shedding and the quantity of virus shed. Additionally, a significant reduction inthe occurrence of abortion was noticed. However, vaccination under field conditionscould not prevent continuous circulation of both EHV-1 and EHV-4 in vaccinated populations (Foote *et al*., 2006). Two other studies were performed with another inactivated and oil-adjuvanted vaccine Pneumabort K®. Neither of these studies could demonstrate a difference in febrile responses, clinical signs and subsequent abortion rates between vaccinated and control mares (Bryant *et al*., 2003).

However, a recent study with the latter vaccine did not only show a significant reduction of clinical signs and nasal shedding after three vaccinations, but alsodemonstrated a significant reduction in the number of days of viremia (Goehring *et al*., 2010b). A possible influence on abortion or nervous system disorders was not evaluated during this study. Modified-live vaccines (MLV) probably stimulate CTL responses and prime theanimals for mucosal antibodies, making them valuable candidates to limit EHV1infections. They induce a rapid onset of immunity which is both broad and longlasting. However, care should be taken when using these vaccines as reversion tovirulence remains a possible risk (Minke*et al*., 2004).A study of (Bürki *et al*., 2000) showed that all horses became infected and developed viremia upon challenge aftervaccination with the MLV Prevaccinol®. Two out of 4 maresaborted, but the study lacked a control group of non-vaccinated mares. Another study compared a commercially available inactivated vaccine (Flu-vac Innovator 6®) and a commercially available modified-live vaccine (Rhinomune®) (Goodman*et al.*, 2006).

The modified-live vaccine induced significantly lower VN antibodies, suggesting a bias towards a cytotoxic immune response and virus shedding was significantly lower. Although the lymphocyte viral gene copies were similar, viremia lasted for a shorter period after vaccination with Rhinomune®. Upon challenge, none of the horses showed nervous system disorders, in contrast to the horses vaccinated with Flu-vacInnovator 6® (3/5) and the control group (3/5). Also a recent study showed a great decrease in clinical symptoms and nasal shedding after vaccination with Rhinomune®. However a decrease in duration of viremia could not be noticed (Goehring *et al*., 2010b). Other alternative approaches for potential vaccination against EHV-1-induced disease such as DNA vaccines, live-vectored vaccines and gene-deleted mutants arenot yet commercially available, but have undergone pilot studies. A study of (Soboll *et al*., 2006), demonstrate a limited immune response and protection following DNA vaccination with plasmids encoding gB, gC, gD, IE and early proteins of EHV-1. A recent study showed that a vaccine with the IE gene of EHV-1 in a recombinant modified-live vaccine vector provides not only a reduction inclinical disease, but also a reduction of cell-associated viremia (Soboll *et al*., 2006).

Unfortunately, until now, the currently available vaccines do not provide completeprotection as they especially stimulate high titers of circulating antibodies and areunlikely to stimulate cytotoxic effector lymphocytes. As EHV-1-induced protective immunity is only short-lived, vaccination has to be repeated every 6 months. Future vaccination strategies should be aimed at stimulating both CD8+ andCD4+ elements of cell-mediated immunity, in particular CTLs, but at the same time maximize stimulation of mucosal and plasma VN antibody (Kydd *et al*., 2006).

2.7.2. Management

The overall goals in management of EHV-1 infections are to minimize exposure of equines to exogenous virus, to maximize immunepreparedness to forestall disease in the event of exposure, and to decrease the likelihood of latent virus recrudescence under influence of stress(van der Meulen *et al*., 2007). Management has to be concentrated mainly on prevention ofthe more serious sequel of an EHV-1 infection like abortion storms and outbreaks of neurological disease (Van Maanen, 2002).

Retrospective analysis of equine herpes viral disease outbreaks often can be linkedto a source of exogenous virus. Age segregation of horses on a farm is one of the best tactics to limit spread of herpes viruses. In particular, pregnant mares should beseparated from young stock and transient horses. Ideally, the pregnant mare population should be routinely subdivided into small groups according to expected delivery dates. Personnel should handle pregnant mares beforecontacting other horses on the premises. New horses should be isolated for a period of 3 weeks, with daily monitoring of rectal temperature and general health, before mixing with the resident population (Allen*et al*., 2008).

Vaccination programs should be instituted on a farm-wide basis and should include adult horses and non-breeding animals as well as young stock and pregnant mares. Although some stress is unavoidable, efforts can be made to avoid unnecessary stress and to be diligent in routine disease surveillance among animals known to berecently subjected to stress. Stress factors such as poor feeding conditions, overcrowding, travelling in late pregnancy and separation from mates should be minimized (Van Maanen, 2002).

The three priorities for management in caseof an outbreak of EHV-1 are an early diagnosis, prevention of further spread andthorough management of clinical cases (Pusterla*et al*., 2009). The importance of an early diagnosis of EHV-1 is crucial, as several specificinterventions may be implemented in case of an EHV-1 induced outbreak. Measures designed to contain potential EHV-1 spread are necessary until EHV-1 is either confirmed or excluded (Lunn *et al.*, 2009).

To prevent further spread of virus to other horses or facilities, quarantine measurements of the whole facility are necessary for at least 4 weeks after the lastclinical case. A recent paper even describes the necessity of isolating actively EHV-1 shedding horses in a separate air space under strict biosecurity and isolation procedures (Goehring *et al*., 2010a). All affected animals should be kept isolated andno horses should be allowed to enter or leave the property until quarantine is lifted (Pusterla *et al*., 2009). Areas contaminated by virus should be carefully cleaned with detergents. In the face of an EHV-1 outbreak, vaccination can be used for horses at increased risk of exposure and to reduce the spread of infectious virus. There is some controversy associated withthis practice, because of the concern that EHM might be associated with a history of frequent vaccination (Henninger*et al*., 2007a). However, the latter statement should be interpreted with care, as the majority of horses that was vaccinated 3-4 times a yearwere older than 5 years. The latter has been described as a risk factor for the development of neurologic disease (Goehring*et al*., 2006).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Angolela Tera, Kembibit and DebreBrahanZuria districts of the North Shewa zone of Amhara regional state and Ade'a district of East Shewa zone and Arsi Negele district of West Arsi zone of Oromia regional state. North Showa zone of Amhara regional state is located between 8°44' and 10°44' North latitude and 38°40' and 40° 6' East longitude, located 130 km northeast of Addis Ababa, Ethiopia. Ade'a district is found in the East Shewa of Oromia regional state, which is located at 9 N and 40^0 E in the central highland of Ethiopia at 47 km southeast of Addis Ababa. Arsi Negele district is one of the districts located in the West Arsi zone of Oromia regional state. The district is located at south 231km from Addis Ababa, Ethiopia. The district is located between $07^0$09'N- $07^0$42'N latitude and $38^0$25' E - $38^0$54'E longitude (NMSA, 2003). Those study areas were selected based on previous EHVs outbreak published by Negussie*et al*.(2017) and seroepidemiology report of EHVs by Mekonnen *et al.* (2017) and Laing *et al.*(2018).

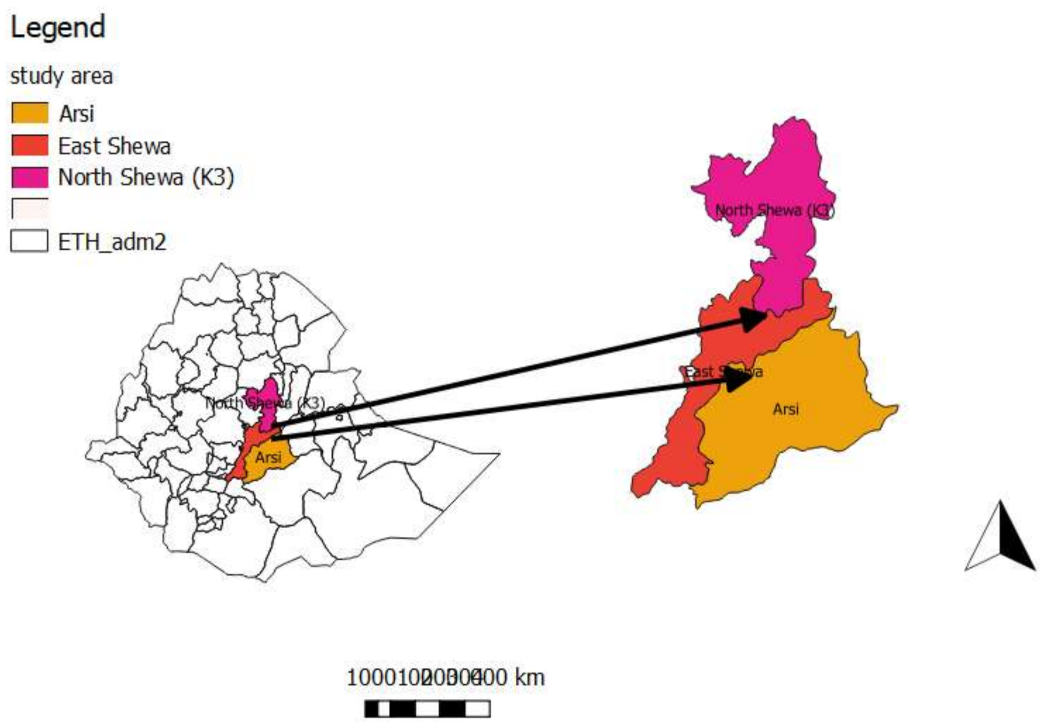

Figure 4: Map of the study area where samples were collected from equids (North Showa of Amhara regional state, East Shewa and West Arsi of Oromia regional state, Ethiopia)

3.2. Study Population and Study Design

Equine herpes viruses (EHVs) disease outbreak investigation was carried out in the purposively selected study sites. The study population comprised all ages of working equids (horses, donkeys, and mules) with the history of fever (rectal temperature ≥ 38^{o}C for donkeys and ≥ 38.5^{o}C for horses and mules), respiratory problems, edema at the ventral region of the abdomen/limbs, ataxia, incoordination, lethargy, abortion, neonatal foal death and neurological signs suspected as clinically diseased were included in the study.

3.3. Sampling and Processing Protocol

Within the target zones, North Showa (AngolelaTera, Kembibit and DebreBerhanZuruia districts), East Shewa (Ade'adistrict) and West Arsi (Arsi Negele district) were purposively selected based on previous information of EHVs disease published by different scholars and suspected EHVs case reported by veterinary clinicians from the area.

A total 58 biological samples from clinically suspected equids;15 nasopharyngeal swabs (donkeys =5; horses = 10), 42 whole blood (donkeys =19; horses =22) from live equids, and 1 tissue sample from dead donkey were collected for the detection of EHV-1, 2, and 5. Nasopharyngeal swabs were collected using sterile cotton swabs and kept in tubes with 2ml viral transport medium (VTM) containing phosphate-buffered saline (PBS) (pH of 7.2 - 7.6) with antibiotics. Whole blood samples (10 ml) were collected using EDTA vacutainer tube. One tissue pool (spleen, lung, kidney, and liver) sample was collected in a bottle with VTM from the deceased donkey. All collected samples were immediately placed in a cool-box and transported under cold-chain to National Veterinary Institute (NVI), Ethiopia. Whole blood samples and both Nasopharyngeal swabs and tissue samples were stored at -20 ^{O}C and -70^{O}C, respectively, until processed. Around 10% (w/v) pooled homogenates of the tissue sample was used for virus detection. This tissue sample was prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petridish with dissecting scissors, followed by macerating the tissue further in serum-free PBS with antibiotics using mechanical tissue grinder (mortal and pistil). Epidemiological information such as species, sex, age, and body condition score of the sampled equids, zones, and districts from where the sample collected was noted and appropriately recorded

3.4. DN AExtraction, PCR Amplification, and Gel Electrophoresis

Equine herpes virus DNA was extracted from 200µl of nasopharyngeal swaps, whole blood and tissue suspension samples using aDNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions with a final DNA elution volume of 50µl.

PCR amplification was performed using virus specific primers for the detection of EHV -1, 2, and 5. DNA amplification was carried out in a total volume of 25µl PCR reaction mixtures. Each of the 25µl PCR mixtures contained 12.5µl of nuclease-free water, 5µl of 5 x Herculase II reaction buffer, 0.5µl Herculase II fusion DNA polymerase, 0.5µl of 25 mM each deoxynucleoside triphosphate (dNTP) mix, 1µl of each forward and reverse primers, 2.5 µl of dimethyl sulphoxide (DMSO), and 2µl template DNA. In each reaction, a negative control (nuclease-free water) was included. The region of interest targeting EHV-1, ORF30 was amplified with an initial denaturation step of 94^{o}C for 5 min, followed by 40 cycles of denaturation at 94^{o}C for 30 s, annealing at 64^{o}C for 30 s, extension at 72^{o}C for 1 min and 30 s, and a final extension at 72^{o}C for 10 min. The region of interest targeting EHV-2 and EHV-5, gB genes were amplified using the following thermocycling conditions: an initial denaturation step of 95°C for 5 min, followed by 40 cycles of amplification, using denaturation at 95°C for 30 s, annealing at 60^{o}C, and extension at 72^{o}C for 45 s and followed by a final extension at 72 °C for 10 min.

Table 1**:**Primers used for amplification of specific regions of the genome of EHV-1,2 and 5

Virus	Region	PCR primers	Size	References
EHV-1	ORF30	FW:5'GCTACTTCTGAAAACGGAGGC-3' RV:5'-TATCCTCAGACACG GCAACA-3'	466bp	Goodman*etal*. ,2007
EHV-2	gB	FW:5'-GCCAGTGTCTGCCAAGTTGATA-3' RV:5'ATACGATCACATCCAATCCC-3'	444 bp	Diallo*et al*. ,2008
EHV-5	gB	FW: 5' ATGAACCTGACAGATGTGCC 3' RV: 5' CACGTTCACTATCACGTCGC 3'	293 bp	Holloway*etal*., 1999

Amplified DNA (PCR products) were analyzed by gel electrophoresis on a 1.5% agarose gel at 100 V for 120 min in 1 × TAE buffer (40 mMTris-acetate, 1 mM EDTA pH 8.0). The gel was stained with 0.5µg of ethidium bromide and DNA bands visualized using a UV transilluminator. The PCR products were identified by 100-base pair (bp) DNA size marker.

3.5. Statistical Data Analysis

The collected data were entered into Microsoft Excel 2003 spread sheets and analyzed using the Stata-13 version. A multivariable logistic regression model was used to measure the association between EHVs infection and potential risk factors. The effects of risk factors were reported as statistically significant if P-value is less than 5 %.

3.6. Ethical Clearance

Ethical clearance was obtained from the animal research ethical review committee of Addis Ababa University College of Veterinary Medicine and agriculture for collecting samples from equines during this study.

4.RESULTS

4.1. PCR Amplification of EHVs Conserved Genes Using Specific Primers

In this study, the detection of EHV-1, EHV-2, and EHV-5 genes was done by PCR using specific primers. A total 58 (42 whole blood, 15 nasopharyngeal swabs, and 1 tissue) biological samples were collected from 33 donkeys and 25 horses that showed suggestive clinical signs of EHVs (Table 2). The result showed that EHV-1 was detected at the highest percent (62%; n = 36) from which 19 (75%) were from donkeys and 17 (51.52%) were from horses (Figure 5), followed by EHV-2 (53%; n = 31) from which 6 (24%) were from donkeys and 25 (75.76%) were from horses (Figure 6). EHV-5 was detected with lowest percent (25%; n = 15) from which 7 (28%) were from donkeys and 8 (24.2%) were from horses (Figure 7) (Table:2). Mixed infections with EHV-1 and EHV-2, EHV-1 and EHV-5, EHV-2 and EHV-5, and EHV-1, 2, and 5 were recorded in 18 (31%), 10 (17%), 9 (15.5%) and 8 (13. 8%) working equids, respectively.

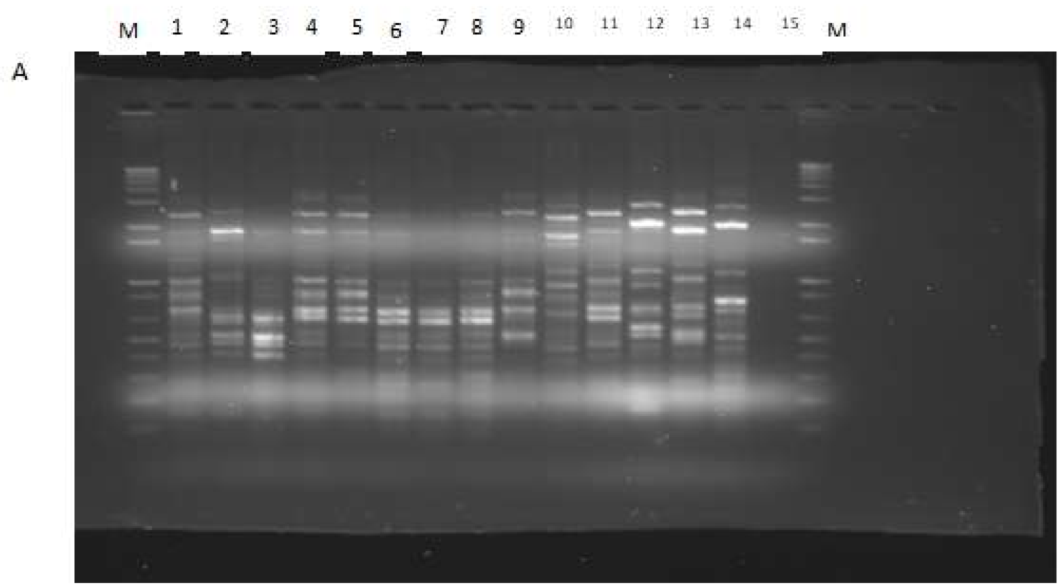

Figure 5: Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products for EHV-1. Lane M: 100-base pair DNA marker; lane: 5 and 9 were negative samples; lane 1,

2, 3, 4, 6, 7, 8, 10, 12, 13, and 14 were positive samples; lane:15 negative control (nuclease-free water).

B

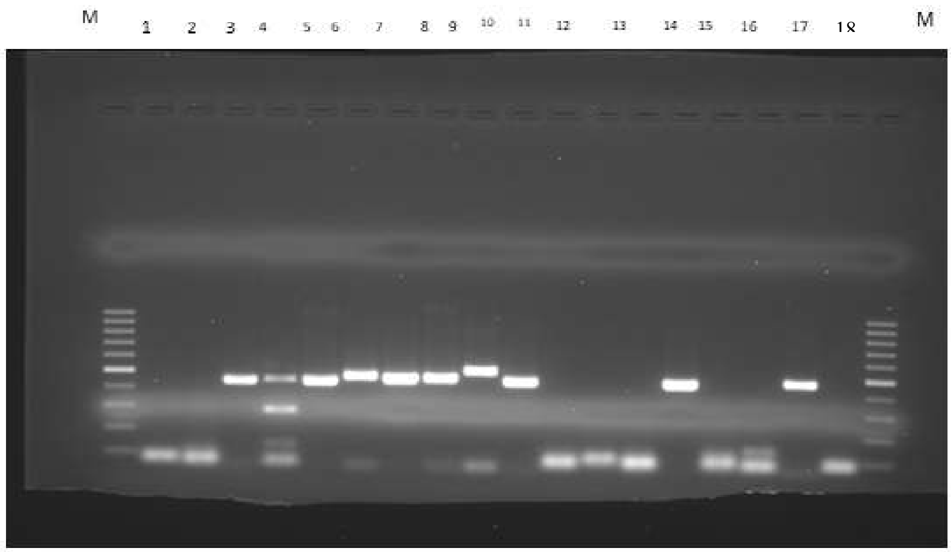

Figure 6: Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products for EHV-2. Lane M: 100-base pair DNA marker; lane: 1, 2, 11, 12, 13, 15, and 16 were negative samples; lane 3, 4, 5 ,6, 7, 8, 9, 10, 14, and 17 were positive samples; lane 18: negative control (nuclease-free water).

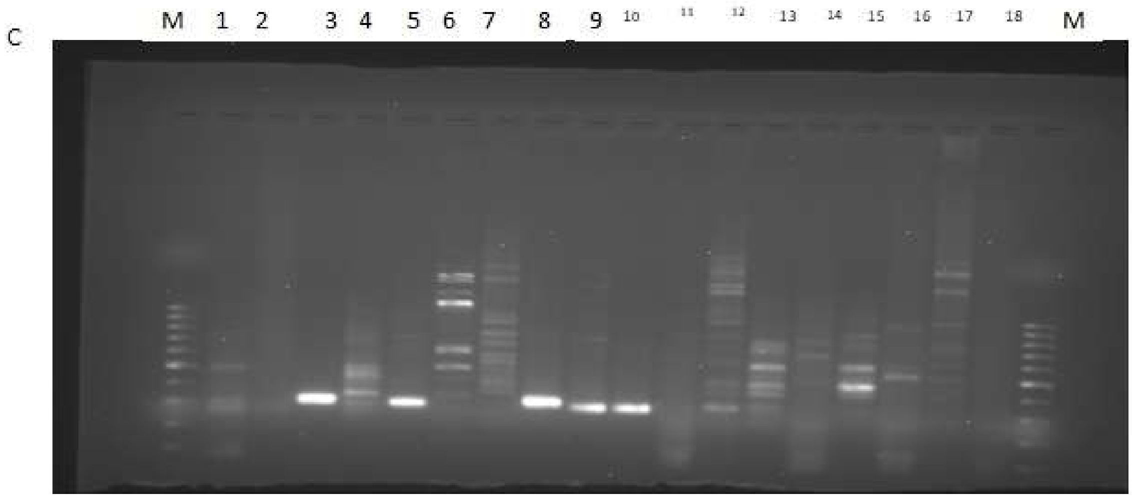

Figure 7: Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products for EHV-5. Lane M: 100-base pair DNA marker; lane: 2, 4, 6, 7, 11, 13, 14, 15, 16,and 17were negative samples; lane 1, 3,5,8,9,10,and 12 were positive samples; lane: 18 negative control (nuclease-free water).

Table 2: EHV-1, 2, and 5 detected from donkeys and horses suspected with EHVs

Risk factors	No. equids	EHV-1	EHV-2	EHV-5
Species				
Donkeys	25	19 (76%)	6 (24%)	7(28%)
Horses	33	17 (51.52%)	25(75.76%)	8(24. 24%)
Sex				
Male	32	17 (53.13%)	21 (65.63%)	9(28.13%
Female	26	19 (73.08%)	10 (38.46%)	6 (23.08%)
Age				
≤ 4yrs	24	15 (62.5%)	14(58.33%)	7(29.17%)
5-9 yrs	25	15 (60%)	13(52%)	6(24%)
≥ 10yrs	9	6 (66.67%)	4(44.44%)	2(22.22%)
BC				
Poor	29	15 (51.72%)	15(51.72%)	8 (27.59%)
Medium	26	18 (69.23%)	15(57.69%)	7 (26.92%)
Good	3	3 (100%)	1(33.33%)	0 (0%)
Zones				
West Arsi	5	1 (20%)	3(60%)	3(60%)
East Shewa	7	3 (42.86%	5(71.43%)	1(14.29%)
North Shewa	46	32(69.57%)	23(50%)	11(23.91%)
Districts				
ArsiNegele	5	1 (20%)	3 (60%)	3(60%)
Ade'a	7	3 (42.86%)	5 (71.43%)	1(14.29)
AngolelaTera	36	24(66.67%)	18(50%)	10(27.78)
Kembibit	5	5 (100%)	2 (40%)	0(0%)
DebreBrahanzuria	5	3 (60%)	3 (60%)	1(20%)
Total	58	36(62%)	31(53%)	15(25%)

*BC: body condition*yrs: years *No. equids: numbers of equids

4.2. Association of the Risk Factors with Occurrence of EHV-1, 2, and 5

The current study revealed higher number of EHV-1 was detected in donkeys (n = 19; 76%) than horses (n = 17; 51.52%). In contrast, the occurrence of EHV-2 was statistically higher (P =0.001) in horses (n =25; 75.76%) than in donkeys (n = 6; 24%); this difference was statistically significant (P = 0.001). Similarly, although not statistical significant (P > 0.05), EHV-5 (n = 8; 24%) was detected in higher number in horses than in donkeys(n = 7; 28%) as shown in table 3 and 4.

Table 3**:** Multivariable logistic regression model for the association between selected potential risk factors and individual equidswithEHV-1 positive status.

Risk factors	**No. equids**	**EHV-1**	**OR**	**P**	**95% Conf. Interval**	
Species						
Horses	33	17 (51.52%)	0.2878501	0.097	0.0662086	1.251464
Donkeys	25	19 (76%)				
Sex						
Female	26	19 (73.08%)	1.765462	0.456	0.396051	77.869824
Male	32	17 (53.13%)				
Age						
≥10yrs	9	6 (66.67%)	0.6463703	0.677	0.0831257	5.026057
5-9 yrs	25	15 (60%)	0.816876	0.786	0.1895949	3.519537
≤4yrs	24	15 (62.5%)				
BC						
Good	3	3 (100%)	1			
Medium	26	18 (69.23%)	2.68843	0.157	0.6844984	10.55906
Poor	29	15 (51.72%)				
Zones						
West Arsi	5	1 (20%				
East Shewa	7	3 (42.86%	4.026483	0.356	0.0557592	44.01276
North Shewa	46	32(69.57%)	6.647169	0.228	0.015097	10.27178
Districts						
Arsi Negele	5	1 (20%)				
Ade'a	7	3 (42.86%)	1			
AngolelaTera	36	24(66.67%)	1.128319	0.915	0.1239704	10.26943
Kembibit	5	5 (100%)	1			
DebreBrahanzuria	5	3 (60%)	1			

*BC: body condition * yrs : years * No. equids: numbers of equids

Table 4: Multivariable logistic regression model for the association between selected potential risk factors and individual equidswith EHV-2 positive status

Risk factors	No.ofequids	EHV-2	OR	P	95% Conf.Interval	
Species						
Donkeys	25	6 (24%)				
Horses	33	25(75.76%)	13.65879	0.001	3.118921	59.81639
Sex						
Male	32	21 (65.63%)				
Female	26	10 (38.46%)	0.4818989	0.317	0.1153555	2.013138
Age						
≤ 4yrs	24	14(58.33%)				
5-9 yrs	25	13(52%)	1.068244	0.929	0.2474998	4.610695
≥10yrs	9	4(44.44%)	1.167931	0.877	0.1624897	8.39476
BC						
Poor	29	15(51.72%)				
Medium	26	15(57.69%)	1.355964	0.667	0.3393961	5.417378
Good	3	1(33.33%)	.0779918	0.120	0.0031182	1.950728
Zones						
West Arsi	5	3(60%)				
East Shewa	7	5(71.43%)	1.566561	0.792	0.055759244.01276	
North Shewa	46	23(50%)	0.3937925	0.575	0.015097	10.27178
Districts						
Arsi Negele	5	1 (20%)				
Ade'a	7	3 (42.86%)	1			
AngolelaTera	36	24(66.67%)	3.038669	0.342	0.3073354	30.04375
Kembibit	5	5 (100%)	3.19366	0.468	0.1383687	73.7122
DebreBrahanzuria	5	3 (60%)	1			

*BC: body condition * yrs : years * No. equids: numbers of equids

The detection of EHV-2 was higher in males (n = 21; 65.63%) than females (n = 10; 38.46%). Similarly, a higher number of EHV-5 was detected in males (n = 9; 28.13) as compared to females (n = 6; 23.08). Higher EHV-2 (n = 14; 58.33%) and EHV-5 (n = 7; 29.17%) were detected in young (≤4 years) than adults (5-9 years) (EHV-2: (n = 13; 52%), EHV-5: (n = 6; 24%)) and old equids (> 10 years) (EHV-2: (n = 4; 44.44%), EHV-5: (n = 2; 22.22%)). Higher EHV-1 (n = 15; 62.5%) was recorded both in young (≤4 years) and adults (5-9 years) than old equids (≥10years) EHV-1: (n = 6; 66.67%). The highest occurrence of EHV-1 (n = 8; 69.23%) was found in medium body condition score followed by poor (n = 15; 51.72%), and good body condition scores (n = 3; 100%). Based on the origin of the animals, higher detection of EHV-1:32 (69.57%), EHV- 2: 23(50%), and EHV- 5: 11(23.91%) were recorded at North Shewa. Followed by EHV-2: 5(71.43%), EHV-1: 3 (42.86%), and EHV-5: 1(14.29%) were detected at East Shewa and both EHV-2 and EHV-5:3(60%) and EHV-1:1(20%) were detected at West Arsi. Similarly, although not statistical significant (P > 0.05), EHV-1: (n=19: 73.08%) was detected in higher number in females than in males (n=17; 53.13%). The statistical analysis showed that there was no statistically significant difference (P> 0.05) of EHV-1, 2, and 5 infections based on all considered risk factors exception of species of the equids.

Table 5: Multivariable logistic regression model for the association between selected potential risk factors and individual equidswith EHV-5 positive status.

Risk factors	No. equids	EHV-5	OR	P	95% Conf. Interval	
Species						
Donkeys	25	7(28%)				
Horses	33	8(24.24%)	0.8755353	0.856	0.2089047	3.669434
Sex						
Male	32	9(28.13%				
Female	26	6 (23.08%)	0.7852378	0.749	0.178955	3.44555
Age						
≤ 4yrs	24	7(29.17%)				
5-9 yrs	25	6(24%)	0.9121862	0.899	0.2199762	3.782607
≥10yrs	9	2(22.22%)	0.6643336	0.689	.0893514	4.939363
BC						
Poor	29	8 (27.59%)				
Medium	26	7 (26.92%)	0.9241899	0.906	0.24937	3.42514
Good	3	0 (0%)				
Zones						
West Arsi	5	3(60%				
East Shewa	7	1(14.29%)	0.1238987	0.160	0.0067501	2.274154
North Shewa	46	11(23.91%)	0.1831084	0.270	0.0089554	3.743968
Districts						
ArsiNegele	5	3(60%)				
Ade'a	7	1(14.29)	1			
AngolelaTera	36	10(27.78)	1.436302	0.778	0.115686 1	7.83244
Kembibit	5	0(0%)	1			
DebreBrahanzuria	5	1(20%)	1			

*BC: body condition * yrs : years * No. equids: numbers of equids

5. DISCUSSION

Equine herpes viruses have a major economic, health, and welfare impact on working equids worldwide. They are incriminated in several disease forms; including respiratory, abortion, and neurological forms of disease that greatly affect the performance, breeding, and competition ability of equids (Slater *et al*., 2006). Although the Ethiopian working equids were considered as the most proficient and valuable in reducing poverty, there is little information about the previous and current epidemiological situation of EHVs in Ethiopia.

In this study, molecular surveillance of EHVs in Ethiopian working equids was conducted to unravel the epidemiology and associated risk factors of EHVs. In the present study, EHV-1, 2, and 5 were detected from clinically diseased equids in North Shewa, East Shewa, and West Arsi using PCR. Analysis of the results obtained from EHVs cases outlines that EHV-1 is the most prevalent type detected, with an overall percentage of 36 (62%). This is roughly in line with average apparent prevalence (AP) in Egypt 64% (Beshir *et al*., 2018) and Detection and molecular characterization of equine herpes viruses in horses in the Republic of Serbia, EHV-1;59.1% by **(** Radalj*et al*.,2018), but relatively greater than the prevalence of EHV-1: 12 (7.5%) in Ethiopia studied by (Negussie *et al*.,2017). Similarly, 31 (53%) and 15 (25%) equids with clinical signs of EHVs were infected with EHV-2 and EHV-5, respectively. The current detection rates of EHV-2 in Ethiopian equids in line with previously studied by (Negussie *et al*., 2017) and roughly similar with elsewhere studies conducted in Egypt by (Amer *et al*., 2011). Unlikely the current detection of EHV-5 was lower than the prevalence reported by (Negussie *et al*., 2017). In the present study, concurrent infections with EHV-1, EHV-2, and EHV-5 were recorded in 8 (13. 8%) from which 3 (21.77%) were from donkeys and 5 (36.25%) were from horses. This mixed infection is in contrast to other reports (Ataseven*et al*., 2010; Negussie*et al*., 2017) co-infection was not detected in donkeys. However, concurrent infections detected in both donkeys and horses with suggestive clinical signs of EHVs, their synergistic effect remains unknown.

A multivariable logistic regression model was built to test the relationship between potential risk factors: species, sex, age, body condition scores, and location of equids with EHV-1, -2, and -5 infection. The overall current detected percentages of EHV-1 and EHV-5 infections in Ethiopian working equids were 62% and 25%, respectively.I n the present study, EHV-1 was higher prevalent in donkeys compared to horses. The number of EHV-2 and EHV-5 detected were higher in horses than donkeys. This is in line with the report of neurological EHV-1 outbreaks in Ethiopia (Negussie*et al*., 2015). According to Negussie *et al*., (2015) the higher prevalence of EHV infections in Ethiopian donkeys might be associated with (1) host factors (Ethiopian donkeys may be more susceptible to EHV infections than horses) and/or (2) stress (donkeys are more subjected to a heavy workload, travel longer distances, are generally in a poor nutritional state, and have a heavy parasite burden).

In the current study, a significant variation was not observed among the gender of equids with EHVs infections. However, the highest EHV- 2, and 5 was detected in male equids. This is in line with what has been reported in Egypt (Besher*et al*., 2018), but a higher number of EHV-1 was detected in females. Regarding age groups, the highest EHV-1 was detected at an age ranging from 5 to 9 years. This agrees witha study done by Negussie *et al*. (2015) where the highest proportion of equines were affected at the age ranging from 7 to 10 years. Higher EHV-2 and EHV-5 were detected at an age range ≤4 years. This is generally indicate young and adult Ethiopian working equids are at a greater risk of getting EHVs infections.This might be due to Ethiopian equids at this age range are more subjected to a heavy workload and prone to stress. Based on animal location higher EHV-1, EHV-2, and EHV-5 infections were detected at North Shewa than East Shewa and West Arsi. Eventhough epidemiology and molecular characterization of EHVs infection was published from North Shewa, Ethiopia by (Negussie*et al*., 2017), the present study is the first molecular surveillance of EHVs infections incorporating East Shewa and West Arsi.

There was no statistically significant difference in the occurrence of EHV-1 and EHV-5 based on all considered potential risk factors. This is in agreement with seroprevalence reported in Egypt by (Beshir Ata *et al*., 2018), but in contrast with higher seroprevalence recorded in working equids of age group between 3 to 8 years by Mekonnen *et al*., (2017) and the highest proportion of equines were affected at the age ranging from 7 to 10 years (Negussie*et al*., 2015). In this study, horses were fourteen times at higher risk of getting EHV-2 infection than donkeys. Significant variation was not noticed with age, body condition scores, and location of equids in EHV-2 infection.

6. CONCLUSION AND RECOMMENDATION

In conclusion in working equids with suggestive clinical signs of the disease, EHV-1 (n= 36; 62%), EHV-2 (n = 31; 53%), and EHV-5 (n = 15; 25%) was detected indicating the widely distribution of EHV-1, 2, and 5 through out the equine population in the study areas. Besides, mixed infections with EHV-1, 2, and 5 were recorded. EHVs disease is an important disease of working equids which need serious attention for prevention and control actions in Ethiopia.

Based on the above conclusion the following recommendations are forwarded:

- Further research are required to investigate the epidemiology of EHVs in both healthy and diseased equids.
- Equine herpes virus vaccines never have been practiced in Ethiopia therefore;other options of prevention must be considered.

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8. ANNEXES

Annex 1**:** Data collection sheet

Investigators Name ________________Veterinary Contact Information**:**Clinic name____________ phone. No______

A. ID	D. collected	Addres	Animal Spp	Sex	BC	Age	Sample Type	Ataxia	Nasal Discharge	T(o^c)	Hind. Weakness	Inco-ordination	Lethargy	Limb Edema	Abortion

Annex 2:DNA extraction Procedures

1. 200 µl of anticoagulant-treated whole blood, nasopharyngeal swabs and tissue suspension sampleswere added to 2ml collection tube.
2. 200 µl Buffer AL was added and mixed thoroughly byvortexing. Then the samples wereincubated at 56°C for 10 min.
3. 20 µl proteinase kinase K was added to each sample
4. 200 µl of ethanol (96–100%) was added and mixed thoroughly by vortexing.
5. The mixture was Pipeted into a DNeasy Mini spin column and placed in a 2 ml collection tube. Then Centrifuged at ≥6000 x *g* (8000 rpm) for 1 min. The flow-through and collection tube was discarded.
6. The spin column was placed in a new 2 ml collection tube, then 500 µl of Buffer AW1 was added and centrifuged for 1 min at ≥6000 x *g*. The flow-through and collection tube was discarded.
7. The spin column was placed in a new 2 ml collection tube, then 500 µl Buffer AW2 was added to and centrifuged for 3 min at 20,000 x *g* (14,000 rpm). The flow-through and collection tube was discarded.
8. The spin column was transferred to a new 2 ml collection tube.
9. The DNA was eluted by adding 50 µl Buffer AE to the center of the spin column membrane.
10. Then incubated for 1 min at room temperature (15–25°C) and Centrifuged for 1 min at ≥6000 x *g*

Annex 3: DNA extraction at National Veterinary Institute of Ethiopia