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Molecular Detection and Epidemiology of Equine Herpesviruses-1 (EHV-1) in Working Equids in Central Ethiopia

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Abstract: Equine herpesvirus 1 (EHV1) is an economically important viral pathogen of equines and causes respiratory disease, neonatal foal mortality, late-term abortion and sporadic encephalomyelitis aka equine herpes myeloencephalopathy (EHM) in affected horses. It has a major economic and welfare impact on all sectors of the horse industry worldwide bringing direct clinical effects on equine. This study aimed to report the molecular detection of EHV-1 and to assess the risk factors associated with infection in working equids in central Ethiopia. A total of 58 equids composed of 25 donkeys and 33 horses with suggestive clinical signs of EHV were purposively selected. Accordingly Nasopharyngeal swab, whole blood and pooled tissue sample were collected, viral DNA extracted using the QIAamp DNA Mini Kit and Virus specific PCRs targeting the DNA polymerase gene (ORF30) was performed using specific primers to amplify a region of 466bp of EHV-1. Out of 58 samples subjected to virus-specific PCR assay 13.8% (n = 8) of them were found positive for EHV-1. The detection rate of EHV-1 was relatively lower in donkeys (12%) than in horses (15.1%), higher in males (15.6%) than females (11.5%) with (OR=1.42; 95% CI=0.31-6.59), higher in age group 4-10 years old as compared to the age group ≤3 years old and the old. >10 years. On the other hand, yearling and young equids with age group ≤3 were relatively protected than other age groups. The ability of the virus to establish clinical disease was found 15.2% from overall of laboratory confirmed EHV-1 cases with no statistically significant (P >0.05) variation. Agro ecological factor for the occurrence of EHV-1 reveal nearly 2 times higher in midland areas. All tissue samples taken from equines died after showing symptom of EHV-1 found severely infested by adult liver fluke in their liver during postmortem examination and confirmed positive for EHV-1 in PCR assay. Results of the present study demonstrate that EHV-1 infections are prevalent in most parts of central Ethiopia with no variation among Equid species, age group and agro–ecology. All cases of EHV-1 infections cannot establish clinical disease unless the equine are immune compromised. The interaction of EHV-1 and liver fluke in infection and fatality in Equine needs further investigation.

Key words: Equine Herpesvirus 1 · Central Ethiopia · Equid · Molecular Detection · Orf30

INTRODUCTION

Equine herpes viruses (EHVs) are ubiquitous viral pathogens of all members of the Equidae family with a worldwide spread of infections [1-3]. It belongs to an enveloped double-stranded DNA virus comprising nine verity types [4] each with distinct envelope glycoproteins, of which EHV-1 is a member of the Varicellovirus genus within the subfamily Alpha herpesvirinae [5]. Genetically the EHV-1 is approximately 150 kbp in size [6, 7] and the genome contains 76 open reading frames (ORFs) predicted to encode functional proteins [5].

Equid herpesvirus 1 (EHV-1) can cause a wide spectrum of diseases ranging from inapparent respiratory infection to the induction of abortion, chorioretinopathy, in extreme cases, neurological disease (myeloencephalopathy) resulting in paralysis and ultimately death [8-10]. The severity of disease resulting

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from EHV-1 infection is likely to be influenced by a number of factors, including the age and physical condition of the host; whether the infection is primary, reinfection, or a reactivation of latent virus; the immune status of the host; and the pathogenic potential of the strain involved [1, 8]. There are several EHV-1 strains with different clinical outcomes. Restriction endonuclease analysis of purified EHV-1 DNA over 35 years sequence reveals 78 EHV-1 strains isolated in the field [2].

Infection with EHV-1 occurs through the respiratory tract by inhalation of the aerosolized infectious virus, nose-to-nose contact, or contact with fomites [11, 12]. Differences in clinical outcomes between EHV-1 strains are related to the variance of replication in leukocytes and endothelial cells in which the neuropathogenic strain typically caused a prolonged high level of viremia and widespread endothelial cell infection, whereas the abortogenic strains typically cause a more short-lived, low-level viremia and restricted or absent endothelial cell infection [2, 13]. The neuropathogenicity of EHV-1 strains is associated with single nucleotide polymorphism (A→ G 2254) in the open reading frame 30 (ORF30), of the viral DNA polymerase enzyme catalytic unit of gene, resulting in an amino acid variation from aspargine to aspartic acid (N/D752) [2, 14, 15].

The EHV-1 has a major economic and welfare impact on all sectors of the horse industry worldwide through their direct clinical effects on equine [16, 17]. The establishment of lifelong latency and period of reactivation in a large proportion of infected animals ensures the survival of herpes viruses in equine populations and enables the virus to be shed sporadically throughout the life time of the host [18, 19]. Even though horses are considered as natural hosts to EHV-1[20], other members of the equidae family, including mules and donkey are found to be affected and establish typically silent infections serving as reservoir hosts [21, 22].

Equine have an essential role in the livelihoods of millions of people in Ethiopia mainly for transportation, draught power, other social values and contributions ranging from festivals and entertainment to ceremonial decoration during funeral services [23] However, recently Ministry of Agriculture in Ethiopia frequently received reports of overwhelming death of working equids after showing clinical feature of EHVs in some specific regions. Previous studies on the occurrence and the epidemiology of EHV-1 in Ethiopian working equids reveal the highest prevalence [24-26]. But the current situations of EHV-1 especially evidence concerning to the prevalence and associated risk factors were not available in most parts of Ethiopia.

Therefore, the aim of this study was

- Molecular detection of EHV-1 infections in working equids in central Ethiopia
- Determination of risk factors associated with EHV-1 infections.

MATERIALS AND METHODS

Description of the Study Areas: The study was conducted from November 2019 to February 2020 in selected districts of the central part of Ethiopia. These are AngolelanaTera (Chacha), BasonaWerana (DebreBerhanZuriya), Kembebit (Sheno), Bishoftu (including Adea kebeles) and Arsi Negele districts (Fig. 1). These districts were selected based on previous respiratory disease reports and the presence of high equine population.

Samples were collected from six districts of different altitudes and locations of the sampling sites recorded by GPS. The study areas consist of two agroecological zones: highlands (above 2500 m above sea level (masl) and midland (between 1500–2500 masl). The study was conducted in the highlands of Angolelana Tera (Chacha) of Amhara region with an altitude of 2812 masl and in Kembibit of Oromia region with an altitude ranges from 2630–3020 masl. The study was also conducted in the midlands of Basona Werena (Debre Berhan zuria) of Amhara region with an altitude of 2360 masl and Ada'a (Bishoftu) and Arsi Negele districts of Oromia region which are located at an altitude of 1900 masl and an altitude ranges from 1500 to 2300 masl, respectively.

Study Design and Sample Collection: Based on reports of the disease outbreaks, this study used a cross-sectional study design to collect samples. Field level investigation was conducted purposively at problem reported areas and related districts on Equids with suggestive clinical signs of EHV for short period of time. In each study site, equids were clinically examined for evidence of respiratory distress, coughing and nasal discharge. Equids without noticeable respiratory clinical signs were also included in this study. Epidemiological data such as age, sex, species of the host, presence of clinical signs and geographical locations were also collected to determine whether viral detection was associated with potential risk factors. A total of 58 equids composed of 25 donkeys and 33 horses were enrolled in this study. The study population was also categorized into three age groups: ≤3 years (yearlings and young equids), 4– 10 years (adults) and above 10 years (old). Nasopharyngeal swabs were

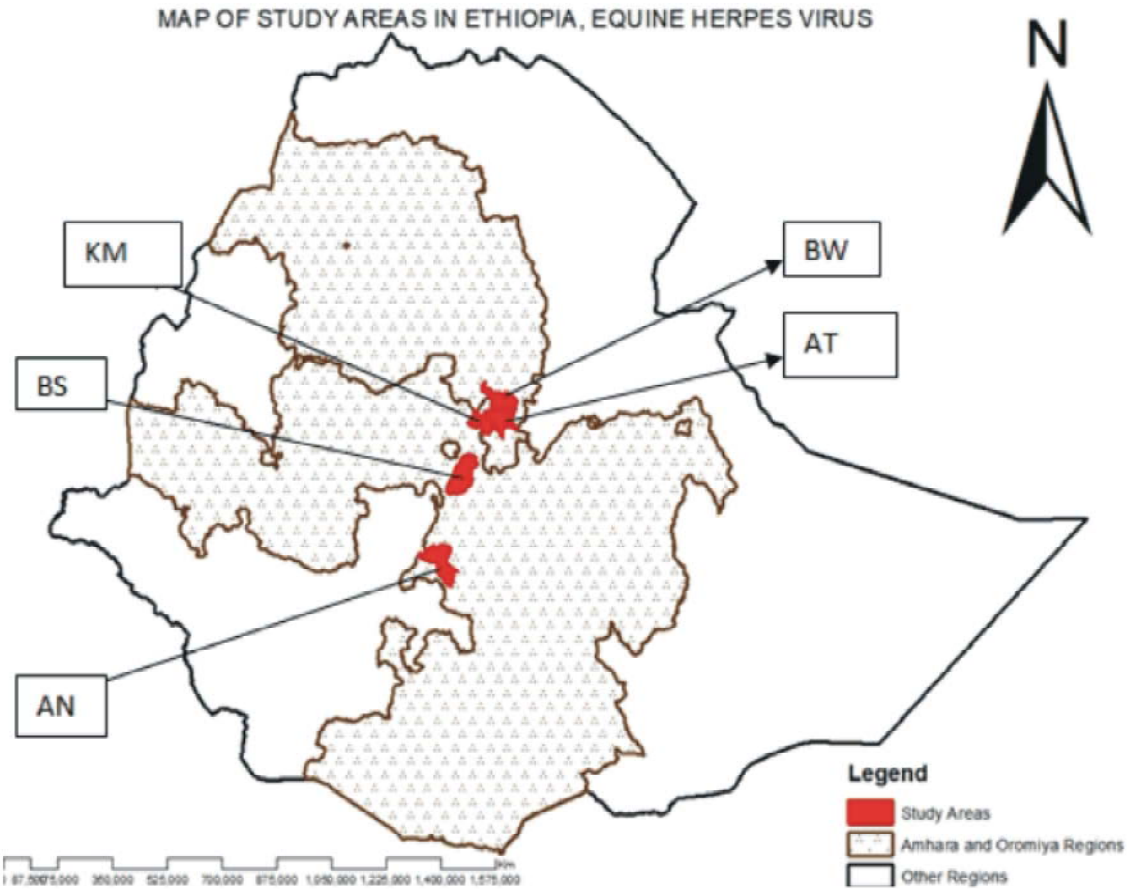

Fig. 1: Study areas in selected parts of Central Ethiopia. BW: Basona Werana (DebreBerhan zuria), AT: AngolelanaTera (Chacha), Kimbibit (Sheno), BS(Bishoftu, Adea kebeles) and AN (Aresi Negele)

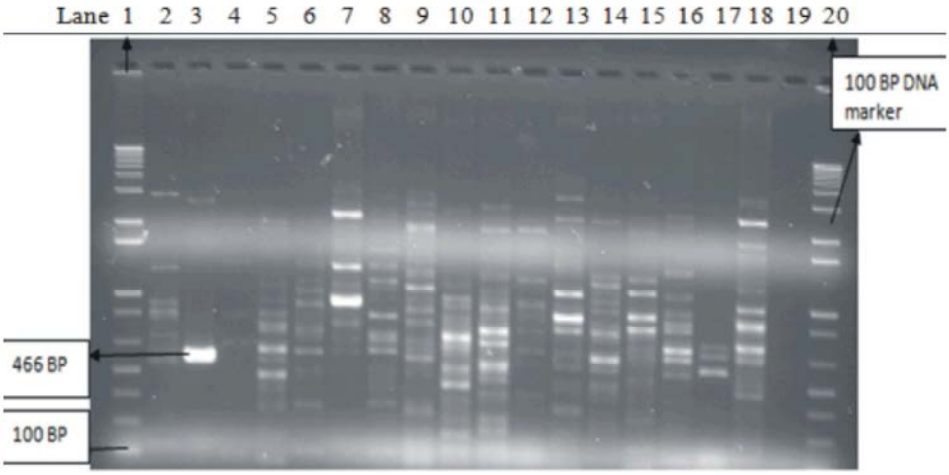

Fig. 2: EHV-1 ORF30 Viral DNA polymerase gene with 466 BP. It illustrates one Panel of PCR products run on 1.5% agarose gel electrophoresis for EHV-1. Lane1 and Lane 20: 100-basepair DNA molecular weight marker; molecular bands lane 3 and 17 positive samples, Lane 2, 4-16 and lane 18 are negative samples with non specific bands, lane19: negative control (distilled water)

Fig. 3: Post mortem examination of Equine died off showing symptom of EHV-1 infection in Angolelana Tera district of central Ethiopia (image taken on January 2021)

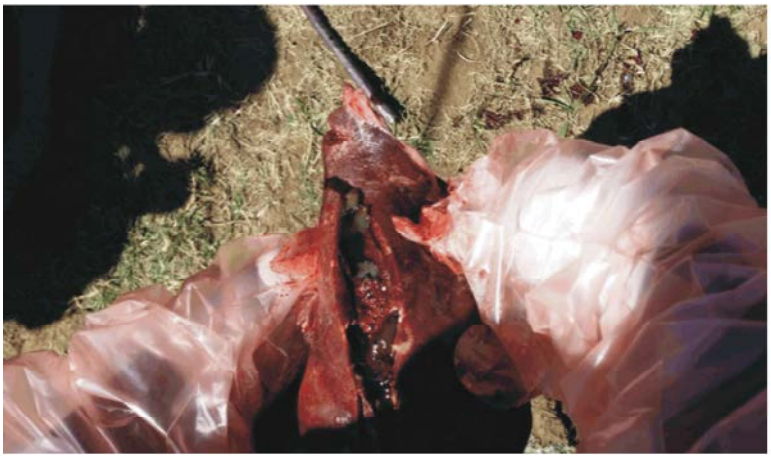

Fig. 4: Severe infestation of liver fluke in the liver of donkey died after showing clinical sign of EHM in Angolelana Tera district of central Ethiopia (image taken on January 2021)

collected from equids with clinical signs of respiratory disorder and from those equids without noticeable clinical signs. Nasopharyngeal swabs were collected using standard Sigma Virocult® swab - 15 cm long with a cellular foam bud. Each collected swabs was placed into three ml viral transport medium containing PBS with a pH of 7.2–7.6 and antibiotics.

According to OIE [27] 20 ml whole blood sample was collected from the jugular veins of each animal using anticoagulant EDTA coated vacutainer tube. Pooled tissue sample of spleen, liver, lung, sub-maxillary lymphnode was also aseptically taken when the equids found dead after showing the clinical feature of EHV (Fig. 3, 4). These pooled tissue sample were placed in a bottle containing a viral transport medium composed of an equal amount of glycerol and 0.04M PBS at pH 7.2–7.6 with some antibiotics and antifungal. Epidemiological information and disease conditions were also recorded during sampling. All collected samples were labeled and immediately placed in a cooler containing ice and transported to the National Veterinary Institute, Bishoftu. Samples were kept at - 20°C until further processing.

DNA Extraction and Polymerase Chain Reaction (PCR) Assay: Viral DNA extraction was performed purposively from 200µl of either of nasopharyngeal swabs, whole blood or tissue suspension using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until processed. Virus specific PCRs targeting the DNA polymerase gene (ORF30) was performed using specific primers to amplify a region of 466bp of EHV-1, according to Goodman *et al.* [15].

PCR amplification was performed using Agilent's Herculase II fusion DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA). Each of the 25 µl PCR mixtures contains 12.5 µl of nuclease-free water, 5 µl of Herculase reaction buffer, 0.5 µl Herculase II fusion DNA polymerase, 0.5 µl of 25 mM each deoxynucleoside triphosphate (dNTP) mix, 1 µl of each forward and reverse primers, 2.5 µl of DMSO and 2 µl template DNA. In each reaction a negative control (nuclease-free water) was included.

The region of interest ORF30 targeting EHV-1 was amplified with an Initial denaturation step of 95°C for 15 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55.5°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The primers used for amplification of a specific region of the EHV-1 is FW: 5'-GCTACTTCTGAAAACGGAGGC-3' & RV: 5'-TATCCTCAGACACG GCAACA-3'.

Analysis of the PCR products was performed in 1.5% horizontal agarose gel electrophoresis using electrolyte buffer Tris Acetate EDTA (TAE) and stained with gel red under UV light. The PCR products were identified by 100bp DNA size marker (Fermentas, Germany). The gels then examined for speci?c size bands using a UV trans-illuminator.

Statistical Methods: Data generated from laboratory investigations were recorded using Microsoft Excel spreadsheets and analyzed using STATA version 14 for Windows (Stata Corp. College Station, TX, USA). Pearson chi-square test was used to compare the significant difference between the risk factors and EHV infection. Odd ratio (OR) was used to assess the degree of association of risk factors with disease occurrence as indicated by 95% confidence intervals. The logistic regression analysis was employed to analyze and regress those factors having a significant effect on the occurrence of disease based upon P value < 0.05 as a significance threshold for entries and removals.

RESULTS

A cross sectional study was conducted from November 2019 to February 2020 for molecular detection of EHV-1 in working equids in central Ethiopia. From a total of 58 collected samples subjected to virus specific PCR assay, 13.8% (n = 8) was found positive for EHV-1 (Table 1, Fig. 2). Relatively higher percentage of EHV-1 was detected in horses (15.1%) than donkeys (12%) without statistically significant difference. During sample collection none of the mules was found to fulfill the selection criteria hence couldn't be sampled.

Table 1: Multivariable logistic regression analysis of risk factors associated with EHV-1 detection

Variables	No. of Equids	EHV-1 positive (%)	OR	95% Conf. interval	P-value
Species					
Donkey	25	3 (12%)	0.76	0.16-3.55	0.73
Horse	33	5 (15.1%)	1.0		
Sex					
Female	26	3 (11.5%)	1.0		0.655
Male	32	5 (15.6%)	1.42	0.31-6.59	
Age group					
≤3	14	1 (7.1%)	0.37	0.39-4.64	0.38
4-10	35	6 (16.7%)	1.0		
>10	9	1 (11.1%)	0.60	0.06-5.77	0.66
Agro-ecology					
Mid land	17	3 (17.6%)	1.54	0.32-7.33	0.59
High land	41	5 (12.2%)	1.0		
Respiratory disease					
Yes	33	5 (15.2%)	1.30		0.731
No	25	3 (12%)	1.0	0.28-6.10	

In the comparison of EHV-1 infection by sex difference, it was detected relatively at a higher percentage in males (15.6%) than females (11.5%). The age of equids was categorized into three groups for convenience purposes: ≤3 years (yearlings and young equine), 4-10 years (adult) and above 10 years (old). In this study, EHV-1 was found relatively higher at age group of 4-9 years (16.7%) followed by above the age of 10 years (11.1%). But, the difference is not statistically significant (P > 0.05).

The test result reveals a relatively highest prevalence of EHV-1 in mid lands (17.6%) than high land areas (12.2%). From a total of 58 equine samples subjected for molecular detection, 15.2% of equine tested for EHV-1 show signs respiratory problems while they are positive without significant difference in determining the ability of the virus to establish clinical disease. This result reveals equines with the signs of EHV-1 infection are 1.31 times more than those without respiratory problem while they are infected by EHV-1. All 4 tissue samples subjected to PCR assay was found positive for EHV-1. Equids from which tissue samples taken were found severely infested by adult liver fluke in their liver during postmortem jjeld as compared to the age group =3 years old and the old age group >10 years. On the other hand, yearling and young equids with age group =3 were relatively protected than other age groups. But this result is not statistically significant (P >0.05). Agro ecological factor for the occurrence of EHV-1 reveal nearly 2 times higher in midland areas (Angolelanatera, Ade'a and and Arhesi negele) than high land areas (Angolelana Tera and kembibit).

DISCUSSION

Despite the largest equine population in Ethiopia [28] probably with the highest density per square kilometer in the world [29] and their essential role in the livelihoods of millions of people [23]. North Shewa Zone Livestock and Fishery Production and Development Agency reported that substantial number of equines especially donkeys have died after showing suggestive clinical signs such as depression, anorexia, ataxia and lameness especially during the cold season in the last five years in some districts of the region [30]. The epidemiological and clinical signs observed in equids in these areas point out EHV-1 infections and it reminds McCartan & Russell [31] and Van Maanen [32] suggestions that once equine show sever clinical sign and recumbent, the prognosis is poor unless intensive care is provided.

Results of the present study reveals out of the total number of 58 nasopharyngeal swabs samples subjected to virus specific PCR assay 13.8% was found positive for EHV-1 and it is nearly consistent with the report of 13.20% Hafshejani *et al*. [33] in Iran, 7.5 % Negussie *et al*. [26] in Ethiopia, 19.4% Smith *et al*.[34] in U.S.A, 10.6% [35] in Germany, 7% [36] in Argentina, but inconsistent to the report of 71.9% [24] in the same region of Ethiopia, 81.7% [37] in Turkey and 52.25% [38] in the same region Turkey. The discrepancy may be due to smaller samples size enclosed in this study or likely due to the use of different detection methods in which the later study utilized ELISA serological tests that provide only retrospective evidence of infection, but virus-specific PCR can identify also active infection [39, 40]. Estimates of the prevalence of EHV infections strongly vary with viral detection technologies [20].

Low level of EHV-1was recorded in the present study as compared to the previous report of the outbreak in the same area. This may be associated with the short time period covered in the study and it was conducted at end of chilling weather condition. However, EHVs has the characteristic nature of latency [18, 19] that reactivate when animal gets stressed (transported, raced, starved, chilled, etc.) and immune-compromised [41, 42]. A higher percent detection rate of EHV-1 on tissue samples collected from equids died after showing typical symptoms of EHM. These equids were found severely infected adult liver fluke in their liver during postmortem examination. These may be associated with immune suppressing effect of concurrent infection two agents. The liver fluke promotes its own survival through several strategies to down-regulate the immune response of the host during the early phase of infection and advanced chronic infection characterized by a persistent immune suppression [43]. The present result open door for studying the interaction of parasites like Fasciola and EHV-1 infection in equines.

In the current study, there is species difference in the level of EHV-1 infection in which horse are more affected (15.1%) than donkeys (12%) but the difference was not statistically significant (p=0.731). This finding is failed to be supported by other reports of similar studies such as Negussie [26] reported (19.5%) donkeys were affected than horse (3.4%), Mekonnen [24] reported 74.7% donkeys affected than horse (66.7%) and Yildirim [38] reported 85% donkeys than 52.48% horse infected by EHV-1. This disparity show over time adaptation ability of the virus in different host range and it needs further investigation to give generalization.

At least one or all of clinical signs such as fever with rectal temperature up to 41 ^{0}C and depression, ataxia and respiratory problems were recorded in 15.2 % of the equine recognized positive for EHV-1on the present study. This is in agreement with the result of Negussie [26] even though many scholars such as Stasiak [44], Akhalesh [45], Van Maanen [22] and Gilkerson [15] linked EHV-1 with several clinical outcomes. Henninger [46] reported that fever occurs days before the onset of neurological signs, but is often absent during the neurological disorders. On the other hand, 37.5% equids in the study did not show any of the clinical signs but detected positive for EHV-1. This might be associated with latent infection, host or virulence factors. It was stated that the outcome of EHV-1 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 [47, 48].

Based on the present study equine in the age group between 4 and 10 years old has got 63% higher probability to be affected by EHV-1 than the yearling/young ones with no statistically significant difference by age group (Table 1). This result is inagreement with report of Allen [49] where adult horses develop viremia 100 times higher than young horses and they are 80 times more likely to develop the disease. More over Negussie [25] reported that mainly equids in the age group of 3-14 years (median 9 years) were clinically affected than the other. This is due to the fact that equine in the adult age group are more likely subjected to work overload in Ethiopian context. Gilkerson's [18] finding also suggest that as the age of equine increase, the probability of getting EHV-1 positive will increase due to the establishment of lifelong latency and being silent carrier of the virus. But the present finding is incompatible with the reports of other studies where the highest prevalence was recorded at age less than three years [11, 18].

CONCLUSION

Results of the present study demonstrate that EHV-1 infections are prevalent in most parts of central Ethiopia with no variation among Equid species, age group and agro–ecology. All cases of EHV-1 infections cannot establish clinical disease unless the equine are immune compromised. The interaction of EHV-1 and liver fluke in infection and fatality in Equine needs further investigation.

Declarations: Availability of data and materials the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions: KW performed sample collection, laboratory analysis and wrote the manuscript. ML participated in laboratory analysis. MB participated in sample collection. KA analyzed the data and participated in reviewing the manuscript. HN set up the study designs, participated in sample collection and laboratory analysis, coordinated the work and wrote the manuscript. All authors read and approved the manuscript.

Ethical Declarations

Ethical Approval and Consent to Participate: Ethical approval for this study was granted from the animal research ethical review committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University (Reference number: VM /ERC/08/01/12/2020). All methods were performed in accordance with relevant guidelines and regulations. All protocols were approved by animal research ethical review committee. Before conducting the research, equine owners were informed with the objectives and the benefits of the study and they gave consent for their animal's inclusion in the study. The informed consent was obtained from all participating equine owners prior to sample collection and this was approved by the ethics committee. The consent from equine owners was verbal because they are unable to write and read. These consents were taken in the presence of a third independent party.

Consent for Publication: Consent to publish the finding of the data was obtained verbally from all equine owners during sampling and data collection.

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