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ORIGINAL RESEARCH

Molecular Identification of Equine Herpesvirus 1, 2, and 5 in Equids with Signs of Respiratory Disease in Central Ethiopia

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Background: Equine herpesvirus (EHV) infections have major economic, health, and welfare impacts on equids. This study was performed in three selected zones of central Ethiopia with the objectives of detecting EHV-1, -2, and -5 in horses and donkeys with suggestive signs of respiratory tract disease and to assess epidemiological risk factors associated with infections.

Methods: A total of 58 nasopharyngeal swab samples were collected from donkeys and horses showing clinical signs of respiratory disease. Polymerase chain reaction (PCR) was used to detect EHV-1, -2, and -5. Evaluation of the associated risk factors was conducted using a multivariable logistic regression model.

Results: Among the 58 equids tested, 36 (62%), 31 (53%), and 15 (25%) equids were positive for EHV-1, -2, and -5, respectively. Concurrent infections with EHV-1 and EHV-2 (31%), EHV-1 and EHV-5 (17%), EHV-2 and EHV-5 (15.5%), and EHV-1, -2, and -5 (13%) were recorded. EHV-1 was detected significantly in higher proportion in donkeys (76%; 95% CI: 1.066–2.251; P = 0.047) compared with horses (51.5%). In contrast, horses had fourteen times more likely to be positive for EHV-2 (OR: 13.66; 95% CI: 3.119–59.816; P = 0.001) compared to donkeys. Detection of EHV-1, -2, and -5 was no significant association with age, sex, and body condition score.

Conclusion: The present study revealed the molecular evidence of EHV-1, -2, and -5 infection in donkeys and horses with signs of respiratory disease. It also documented that donkeys and horses have varying levels of susceptibility to EHVs. This species-specific in susceptibility difference to EHVs infections should be further elucidated.

Keywords: equids, equine herpesviruses, epidemiology, PCR, Ethiopia

Introduction

Working equids have a great significance in the development of Ethiopia, where they have an essential role in reducing poverty, providing food security, and enhancing rural development.[1] These animals are especially important to vulnerable groups, landless communities, and to women, where they can provide an effective entry point to income-generating activities. Numerous infectious diseases negatively impact the health and productivity of working equids. Respiratory viral diseases have been identified as one of the major health threats to equids, which cause significant economic losses.[2,3] Respiratory diseases are consistently ranked amongst the top three health problems of the equids in Ethiopia.[4] Many viral agents have been associated with respiratory disease of equids, although the level of

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evidence of causation for some of these agents is tenuous. Equine herpesviruses (EHVs) have major economic and welfare impacts on the equine industry worldwide. Despite their importance, studies on the epidemiology of these respiratory viruses in Ethiopian working equids are limited. Thus, understanding the distribution of these viral diseases is helpful to implement appropriate control strategies.

EHV-1 belonging to the subfamily *Alphaherpesvirinae* is one of the most important and prevalent viral pathogens in equids and a major threat to the equine industry throughout the world.[5] EHV-1 infection may induce several clinical forms of disease including respiratory infection associated with pyrexia, cough, and respiratory distress.[3] The virus may spread to distant organs enables the development of more severe sequelae such as abortion in pregnant mares, stillbirth and neonatal death, and neurological disorders including Equine Herpesvirus Encephalomyelitis.[3,6] However, the severity of the disease resulting from EHV-1 infection is likely to be influenced by several factors, including the age and physical condition of the host, whether the infection is primary, secondary, or a reactivation of a latent virus, the immune status of the host and the virulence of the strain involved.[7]

Equine herpesvirus 2 (EHV-2) and equine herpesvirus 5 (EHV-5) belong to the *Gammaherpesvirinae* subfamily, which has been detected in equids worldwide.[2] These viruses typically cause upper respiratory tract disease (eg pharyngitis),[8,9] or keratoconjunctivitis[10,11] accompanied by clinical signs such as nasal and ocular discharge, tachypnea, coughing, fever, enlarged lymph nodes, anorexia, poor body condition, and depression. EHV-5 is commonly associated with fatal equine multinodular pulmonary fibrosis.[12,13]

The epidemiological features associated with EHVs in equids are a high incidence of respiratory infection early in life, the establishment of lifelong latency, and reactivation of latent virus with subsequent shedding.[5,14,15] Latently infected equids experience reactivation episodes, during which infectious virus shed into respiratory tract secretions, resulting in transmission to naive hosts.[16] The physiological factors responsible for the activation of the latent virus are associated with stressors including weaning, commingling, transportation, and concurrent infections.[3]

Infectious diseases compromise the health and welfare of working equids, which in turn threatens the livelihoods of the most vulnerable members of society.[1] EHVs are among the infectious viral diseases that have a significant socio-economic impact on the equine industry. Previous studies have documented that respiratory disease particularly coughing and nasal discharge, is one of the major health concerns for working equids in Ethiopia.[4] Equine herpesviruses are important pathogens that are involved in the respiratory disease of varying severity and an important cause of serious morbidity and mortality in Ethiopian equids.[17,18] Despite their importance, information regarding the epidemiology of EHVs associated with outbreaks of respiratory disease in working equids in Ethiopia is limited. Thus, investigation of EHVs and associated determinants in clinically respiratory diseased equids in different regions of the country would be a valuable input to the current understanding of EHVs epidemiology in the different geographical settings and unvaccinated equine populations. Understanding the risk factors that contribute to the development and/or distribution of these viral diseases is also helpful to develop appropriate control strategies. Therefore, the present study was conducted with the objectives of identifying EHV-1, -2, and -5 from horses and donkeys with suggestive signs of respiratory tract disease and assessing the risk factors associated with infections.

Materials and Methods

Study Area

This study was conducted in three selected zones such as the North Shewa, East Shewa, and West Arsi zones as shown in Figure 1. In the North Shewa zones of Amhara Regional State, three districts were selected for sampling such as Angolela Tera, Kembibit, and Debre Berhan Zuria, whereas from East Shewa and West Arsi zones of Oromia Regional State Ada'a and Arsi Negele districts, respectively, were included for this study.

Study Population and Study Design

Investigation of equine respiratory disease outbreaks in equids was performed in selected districts of the study sites. The study population comprised all ages of horses and donkeys with respiratory clinical signs such as unexplained fever (rectal temperature ≥ 38 °C for donkeys and ≥ 38.5 °C for horses), depression, nasal discharge, and coughing. Nasopharyngeal swabs were collected with viral transport medium (VTM) containing

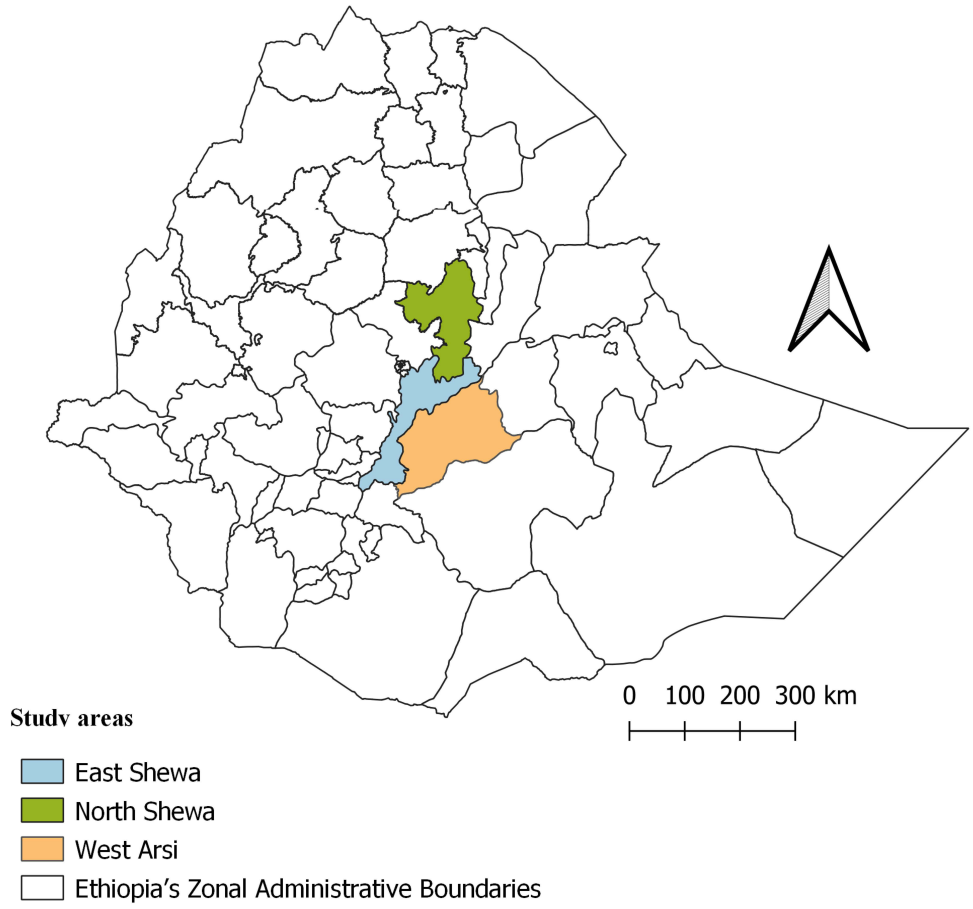

Figure 1 Map of Ethiopia illustrating the study areas. This map was developed from Ethiopian's Zonal Administrative boundaries shape file 2021 using QGIS version 3.1.1.2.

an equal amount of glycerol and 0.04M of phosphate-buffered saline (PBS) supplemented with 1μg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 μg/mL amphotericin B (Bristol-Myers Squibb, New York, USA). From equids with signs of respiratory disease, a total of 58 nasopharyngeal swabs were collected using sterile cotton swabs and kept in tubes containing 2 mL of VTM. All samples were immediately placed in a cool box and transported under a cold chain to the National Veterinary Institute (NVI) Ethiopia for testing. During sampling, information such as species, sex, age, and body condition score of the sampled animals was recorded. The age of the animals was variable ranged from 6 months to 14 years. The age was categorized as ≤4 years (young), 5–9 years (adult), and ≥10 years (old) as previously described by Cozzi et al[19] with some modification. The body condition of the animals was also scored as poor, medium, and good body condition according to Henneke et al.[20]

DNA Extraction and Thermal Amplification

The total DNA was extracted from 200 μL of nasopharyngeal swabs using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions with a final DNA elution volume of 50 μL.

Primers that are specific to EHV-1,[21] EHV-2,[22] and EHV-5[23] were employed for PCR thermal amplification as shown in Table 1. 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 25 μL PCR reaction mixtures. Each of the 25 μL PCR mixtures contained 12.5 μL of nuclease-free water, 5 μL of 5 x Herculase II 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 dimethyl sulphoxide (DMSO), and 2 μL

Table 1 Primers Used for Amplification of Specific Regions of the Genome of EHV-1, -2, and -5

Virus	Target	Oligonucleotides	Size
EHV-1	ORF30	FW: 5′GCTACTTCTGAAAACGGAGGC-3’ RV: 5′-TATCCTCAGACACG GCAACA-3’	466 bp
EHV-2	gB	FW: 5′-GCCAGTGTCTGCCAAGTTGATA-3’ RV: 5′ATACGATCACATCCAATCCC-3’	444 bp
EHV-5	gB	FW: 5′ ATGAACCTGACAGATGTGCC 3’ RV: 5′ CACGTTCACTATCACGTCGC 3’	293 bp

Abbreviations: FW, forward; RV, reverse.

template DNA. In each reaction, a negative control (nuclease-free water) was included. The region of interest of ORF30 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 PCR assay targeting the gB genes of EHV-2 and EHV-5 was 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°C, and extension at 72°C for 45 s and followed by a final extension at 72 °C for 10 min.

The positive PCR products were identified based on amplified band size on a 1.5% agarose gel. The band sizes of 466 bp, 444 bp, and 293 bp are specific to EHV-1, EHV-2, and EHV-5, respectively (Figure 2).

Statistical Analysis

Data were organized in Microsoft Excel 2010 spreadsheets and analyzed using the STATA version 13 for Windows (Stata Corp. College Station, TX, USA). The strength of the association between outcome and explanatory variables was assessed by using the odds ratio (OR). Multivariable logistic regression analyses were used to model the effects of potential risk factors on the outcome variables. The effects of risk factors were considered statistically significant when P-value was less than 0.05.

Results

In this study, among the 58 equids tested, 36 (62%), 31 (53%), and 15 (25%) equids were positive for EHV-1, -2, and -5, respectively (Table 2). EHV-1 was detected at the highest proportion (62%), of which 75% were from donkeys and 51.52% were from horses, followed by EHV-2 (53%), of which 24% were from donkeys and 75.76% were from horses. EHV-5 was detected with the lowest proportion (25%), of which 28% were from donkeys and 24.2% were from horses. Concurrent infections with EHV-1 and EHV-2 (31%), EHV-1 and EHV-5 (17%), EHV-2 and EHV-5 (15.5%), and EHV-1, -2, and -5 (13.8%) were recorded.

The current study revealed that significantly (P = 0.047) higher proportion of EHV-1 was detected in donkeys (76%; 95% CI: 1.066–2.251) compared with horses (51.5%) (Table 3). In contrast, the occurrence of EHV-2 was statistically (P = 0.001) higher in horses (75.8%; 95% CI: 3.119–59.816) compared with donkeys (24%) (Table 4). Although

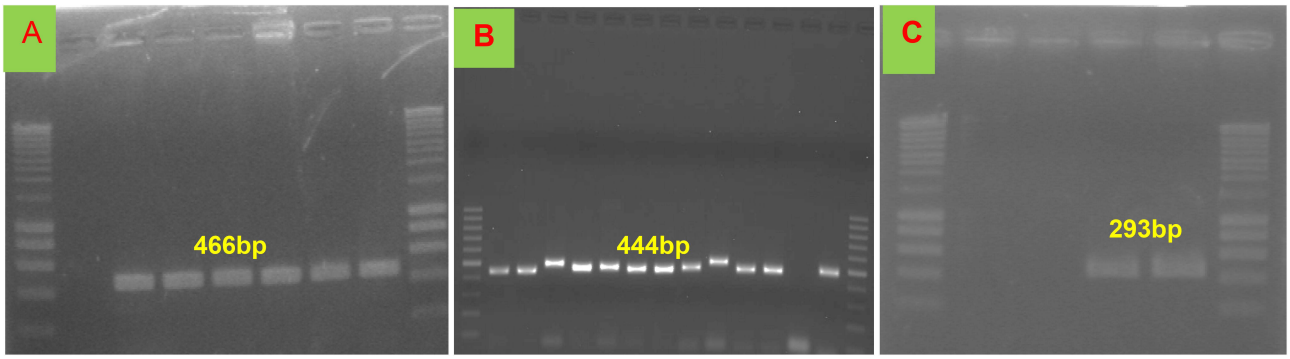

Figure 2 Representative agarose gel electrophoresis of DNA-amplified product generated by targeting specific regions of ORF30 EHV-1 (466 bp) (**A**) on 1.5% agarose gel with a DNA Molecular Weight Marker (MWM) of 200 bp, and EHV-2 gpB gene (444 bp) (**B**) and EHV-5 gpB gene (293 bp) (**C**) on 1.5% agarose gel with a DNA Molecular Weight Marker of 100 bp.

Table 2 The Proportion of Equids That are Positive for EHV-1, -2, and -5 from Donkeys and Horses with Signs of Respiratory Diseases

Risk Factors	No. of Equids	EHV-1 Positive (%)	EHV-2 Positive (%)	EHV-5 Positive (%)
Species				
Donkeys	25	19 (76)	6 (24)	7 (28)
Horses	33	17 (51.5)	25 (75.8)	8 (24. 2)
Sex				
Male	32	17 (53.1)	21 (65.6)	9 (28.1)
Female	26	19 (73.1)	10 (38.5)	6 (23.1)
Age				
≤ 4 years	24	15 (62.5)	14 (58.3)	7 (29.2)
5–9 years	25	15 (60)	13 (52)	6 (24)
≥ 10 years	9	6 (66.7)	4 (44.4)	2 (22.2)
Body condition score				
Poor	29	15 (51.7)	15 (51.7)	8 (27.6)
Medium	26	18 (69.3)	15 (57.7)	7 (26.9)
Good	3	3 (100)	1 (33.3)	0 (0)
Total	**58**	**36 (62)**	**31 (53)**	**15 (25)**

not statistically significant (P = 0.856), EHV-5 was detected in a lower proportion in horses (24%; 95% CI: 0.209–3.669) compared to donkeys (28%) as shown in Table 5.

EHV-1 was detected at a higher proportion in females (73.1%; 95% CI: 0.39–77.86) compared to males (53.1%), but the difference was not statistically significant (P = 0.456) as shown in Table 3. Although not statistically significant (P = 0.317), EHV-2 was detected in higher proportion in males (65.6%; 95% CI: 3.119–59.816) compared to females (38.5%). Similarly, EHV-5 was detected a higher proportion in males (28.1%; 95% CI: 0.179–3.445) compared to females (23.1%), however, a statistically significant association (P = 0.749) was not observed.

In this study, the difference in EHV infection among the different age groups was not statistically significant (P > 0.05). A higher proportion of EHV-1 was recorded in older equids (66.7%) compared to other age groups (Table 3). In contrast, a higher proportion of EHV-2 (58.3%) was detected in young equids compared to adults (52%) and older age groups (44.4%) as illustrated in Table 4. Similarly, EHV-5 was detected in a higher proportion in young equids (29.2%) compared to adults (24%) and older age groups (22.2%) as shown in Table 5.

EHV-1 was recorded in a higher proportion in equids with medium body condition scores (69.2%) followed by poor (51.7%) and good body condition scores (100%). Similarly, EHV-2 was recorded at a higher proportion in equids with medium body condition scores (57.7%) as compared to good (33.3%) and poor (51.7%) body condition scores. EHV-5 was detected in a relatively higher proportion in equids with poor body condition as compared to other body condition scores. However, there was no statistically significant (P > 0.05) difference among the different body condition scores with EHVs positivity.

Table 3 Multivariable Logistic Regression Model for the Association Between Potential Risk Factors with EHV-1 Positive Status

Risk Factors	No. of Equids	EHV-1 Positive (%)	OR (95% Conf. Interval)	P-value
Species				
Horses	33	17 (51.5)	0.29 (1.066–2.251)	0.047*
Donkeys	25	19 (76)	Ref	
Sex				
Female	26	19 (73.1)	1.77 (0.396–77.869)	0.456
Male	32	17 (53.1)	Ref	
Age				
≥10 years	9	6 (66.7)	0.65 (0.083–5.026)	0.677
5–9 years	25	15 (60)	0.82 (0.189–3.519)	0.786
≤4 years	24	15 (62.5%)	Ref	
Body condition score				
Good	3	3 (100)	1	
Medium	26	18 (69.2)	2.69 (0.684–10.559)	0.157
Poor	29	15 (51.7)	Ref	

Note: *Represent statistically significant.
Abbreviations: Ref, reference; OR, odds ratio.

Table 4 Multivariable Logistic Regression Model for the Association Between Potential Risk Factors and EHV-2 Positive Status

Risk Factors	No. of Equids	EHV-2 Positive (%)	OR (95% Conf. Interval)	P-value
Species				
Donkeys	25	6 (24)	Ref	
Horses	33	25 (75.8)	13.66 (3.119–59.816)	0.001*
Sex				
Male	32	21 (65.6)	Ref	
Female	26	10 (38.5)	0.48 (7.192–41.163)	0.317
Age				
≤ 4 years	24	14 (58.3)	Ref	
5–9 years	25	13 (52)	1.07 (0.247–4.611)	0.929
≥10 years	9	4 (44.4)	1.18 (0.162–8.395)	0.877
Body condition score				
Poor	29	15 (51.7)	Ref	
Medium	26	15 (57.7)	1.36 (0.339–5.417)	0.667
Good	3	1 (33.3)	0.078 (0.003–1.951)	0.120

Note: *Represent statistically significant.
Abbreviations: Ref, reference; OR, odds ratio.

Table 5 Multivariable Logistic Regression Model for the Association Between Potential Risk Factors and EHV-5 Positive Status

Risk Factors	No. of Equids	EHV-5 Positive (%)	OR (95% Conf. Interval)	P-value
Species				
Donkeys	25	7 (28)	Ref	
Horses	33	8 (24.3)	0.88 (0.209–3.669)	0.856
Sex				
Male	32	9 (28.2)	Ref	
Female	26	6 (23.1)	0.79 (0.179–3.445)	0.749
Age				
≤ 4years	24	7 (29.2)	Ref	
5–9 years	25	6 (24)	0.91 (0.219–3.783)	0.899
≥10 years	9	2 (22.2)	0.66 (0.089–4.939)	0.689
Body condition score				
Poor	29	8 (27.6)	Ref	
Medium	26	7 (26.9)	0.92 (0.249–3.425)	0.906
Good	3	0 (0)		

Abbreviations: Ref, reference; OR, odds ratio.

Discussion

In the present study, EHV-1, -2, and -5 were identified from clinically respiratory diseased equids. A high proportion of equids (77.6%) in the present study was positive for more than one type of EHVs. This might be associated with either reactivation of the latent equine herpesvirus or primary infection. In the present study, mixed infections with EHV-1 and EHV-2 (31%), EHV-1 and EHV-5 (17%), EHV-2 and EHV-5 (15.5%), and EHV-1, -2, and -5 (13.8%) were recorded. Although co-infection of EHVs was detected from equids with suggestive clinical signs of respiratory disease, their synergistic effect on the clinical outcomes remains unknown.

Analysis of the results obtained from EHVs cases outlines that EHV-1 is the most prevalent type detected, with an overall percentage of 62%. This correlates with other studies done in Egypt (64%)[24] and the Republic of Serbia (59.1%),[25] but relatively higher than the findings in Ethiopia (7.5%)[18] and Algeria (2%).[26] In the current study, identification of EHV-2 in 53% equids with signs

of respiratory disease is relatively higher than other studies reported around the world. In previous studies, EHV-2 was identified in 20% of equids with signs of acute upper respiratory disease in Ethiopia[18] and 19.2% of equids in Turkey.[27] Similarly, EHV-5 was detected in 25% of respiratory diseased equids in the present study. This is consistent with a report from Turkey (21.9%)[27] and Ethiopia (23.1%),[18] but considerably higher than reported in New Zealand (71%)[28] and France (63.4%).[29] EHVs might directly cause respiratory illness or predispose to other opportunistic pathogens. There has been unequivocal evidence that EHV-1 is the major cause of respiratory disease in equids globally. However, in the previous studies, EHV-2 and -5 were detected from immunocompetent equids without signs of respiratory disease.[9,30,31] Thus, our results support the notion that EHV-2 and/or EHV-5 in equids may either compromise the host immunity and increase susceptibility to opportunistic infections[8,32] or play a direct role in the development of respiratory disease in equids.

In the present study, the proportion of equids positive for EHVs varied with the species of equids. Donkeys had 0.26 times (OR: 0.26; 95% CI: 1.066–2.251; P = 0.047) more likely to be positive for EHV-1 compared to horses. This is comparable to the results of a recent study done by Negussie et al[18] who reported that a larger proportion of donkeys were positive for EHV-1 than horses. The higher EHV-1 infections in Ethiopian donkeys might be linked with the genetic susceptibility of the host and/or immunosuppression associated with stress-related to heavy workload, long-distance travel, and poor nutritional status in the donkeys. Long-distance transport can increase the risk of respiratory disease as a consequence of immunosuppression and stress-associated viral reactivation.[33] In contrast, horses had fourteen times more likely to be positive for EHV-2 (OR: 13.66; 95% CI: 3.119–59.816; P = 0.001) compared to donkeys. This is in agreement with previous studies where EHV-2 and EHV-5 were predominantly found in horses.[34–36] At present, a possible explanation of why donkeys and horses have varying susceptibility to EHV infection could not be given. More work is needed to elucidate the exact relationships between host susceptibility to EHVs positivity.

In the present study, the proportion of EHVs positive equids varied for different age groups. In the current study, a high proportion of EHV-1 was detected among adults. This is in agreement with a recent report from Ethiopia[18] and Algeria[26] where EHV-1 was most commonly detected among adults. The possible explanation is that working equids are exposed to a heavy workload during the adult age, which subsequently results in reactivation of the latent virus, but this requires further investigation. In contrast, EHV-2 and EHV-5 were detected in a higher proportion among young equids. In agreement with our results, EHV-2 and -5 were the most common infections detected from the respiratory tract of young horses.[26,28,37] Young foals might be exposed to EHV-2 and -5 infection from the mares during the first months, after which the virus is also spread horizontally to their contact foals, as has been suggested by others.[29,38,39]

Conclusions

The present study revealed the molecular evidence of EHV-1, -2, and -5 infection in donkeys and horses with signs of respiratory disease. Concurrent infection with EHV-1 and EHV-2, EHV-1 and EHV-5, EHV-2 and EHV-5, and EHV-1, -2, and -5 were recorded. It also documented that donkeys and horses have varying levels of susceptibility to EHVs. This species-specific difference in susceptibility to EHVs infections should be further elucidated.

Ethical Consideration

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 the 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. Equine owners gave verbal consent for their animal's inclusion in the study because they are unable to write and read. These consents were taken in the presence of a third independent party.

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Disclosure

The authors declare that they have no competing interests in this work.

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