ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

SEROEPIDEMIOLOGICAL STUDY OF BOVINE HERPES VIRUS-1 IN DAIRY CATTLE HERDS OF ADDIS ABABA, ETHIOPIA

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By:

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BISHOFTU, ETHIOPIA

SEROEPIDEMIOLOGICAL STUDY OF BOVINE HERPES VIRUS-1 IN DAIRY CATTLE HERDS OF ADDIS ABABA, ETHIOPIA

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Epidemiology

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DEDICATION

This thesis is dedicated to my late father, Demissie Shewie, and my mother, Tsehay Muaraga, and also to all my brothers, Samuel Demissie, Biniyam Demissie, Tsega Demissie, and Yohannes Demissie and all sisters, Tsion Demissie, Meseret Demissie, and Selam Demissie.

STATEMENT OF AUTHOR

First, I declare that this thesis is my actual work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AA	Addis Ababa
AHI	Animal Health Institute
AI	Artificial Insemination
AIC	Akaike's Information Criteria
AP	Apparent Prevalence
AUC	Area Under Curve
BIC	Bayesian Information Criteria
BoHV	Bovine Herpes Virus
BRDC	Bovine Respiratory Disease Complex
BRSV	Bovine Respiratory Syncytial Virus
BVDV	Bovine Viral Diarrhea Virus
CI	Confidence Interval
CNS	Central Nervous System
CPE	Cytopathic Effect
CRIB	Cell line resistant to infection with bovine viral diarrhea virus
DIVA	Differentiating Infected from Vaccinating Animals
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
HF	Holstein Friesians
IBR	Infectious Bovine Rhinotracheitis
IPB	Infectious Pustular Balanoposthitis
IPV	Infectious Pustular Vulvovaginitis
MDA	Maternally Derived Antibody
MDBK	Madin–Darby Bovine Kidney
OD	Optical Density
OIE	Office International des Epizooties
OR	Odd Ratio
PCR	Polymerase Chain Reaction

LIST OF ABBREVIATIONS (*Continued*)

PI-3	Parainfluenza-3 virus
QGIS	Quantum Geographic Information System
R_0	Basic reproduction ratio
R^2	Coefficient of Determination
ROC	Receiver Operating Characteristic
TP	True prevalence
UK	United Kingdom
UL	Unique Long
US	Unique Short
VNT	Virus Neutralization Test
WOAH	World Organization for Animal Health

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ABSTRACT

Bovine herpes virus-1 (BoHV-1) is the cause of respiratory disease, abortions, and genital disorders in cattle. Although BoHV-1 has been known to cause severe economic damage to the dairy industries, little is known about its epidemiology in dairy cattle of Addis Ababa (AA), Ethiopia. A cross-sectional study was undertaken in the dairy cattle herds of AA from November 2021 to May 2022 to estimate the seroprevalence and associated risk factors for BoHV-1. A total of 369 dairy cattle from 115 dairy herds were included in this study, and a proportional stratified random sampling method was employed. Blood samples were collected and subjected to ELISA test to detect antibodies against BoHV-1. Semi-structured questionnaires were also administered to determine the risk factors and association of reproductive disorders with BoHV-1 seropositivity. Univariate and multivariate mixed effect logistic regression analyses were used. The animal and herd level prevalence were 21% (95% CI: 17-25%) and 32% (95% CI: 24-42%), respectively. In a multivariable mixed effect logistic regression model, adult cattle had fourteen times (OR = 14.32; 95% CI: 2.53–81.5; P=0.003) more likely to increase the risk of being BoHV-1 seropositive than young cattle. Purchased cattle had four times (OR = 4.15; 95% CI: 1.36–12.66, P=0.012) more likely to increase the risk of being BoHV-1 seropositive than homebred cattle. Less than 100m distance between cattle herds were eleven times (OR=10.75; 95% CI: 1.47–78.3; P=0.019) more likely to increase the risk of being BoHV-1 seropositive than beyond 100m. The risk of being BoHV-1 seropositive was twenty-four times higher (OR = 23.92; 95% CI: 1.93–296.33; P=0.013) on farms that did not control visitors than on farms that did. The risk of being BoHV-1 seropositive was significantly higher in herds using bulls (84%; 95% CI: 36-99; P=0.01) than herds using AI. BoHV-1 seropositivity was significantly associated with cows which had a history of abortion (OR = 6.89; 95% CI: 1.97–22.76; P=0.002), a history of retained placenta (OR = 3.26; 95% CI: 1.32–8.07; P = 0.010), and a history of repeat breeding (OR = 3.64; 95% CI: 1.08–12.18; P=0.036). A practice of BoHV-1 free certified bull selection and routine biosecurity measures on the farm should be adopted, and the risk factors should also be included in designing the prevention and control strategy of BoHV-1.

Key words: AA, BoHV-1, Dairy cattle, ELISA, Risk factors, Seroprevalence

1. INTRODUCTION

Ethiopia has the largest livestock population in Africa, with an estimated 70 million cattle. The local breeds account for 97.4% of all cattle in the country while hybrids and exotic breeds accounted for remaining 2.3% and 0.31%, respectively. There are 7.5 million dairy cows in Ethiopia producing approximately 4.96 billion liters and on average of 1.48 liters per cow per day over a lactation period of 210 days (CSA, 2021). Despite huge numbers of livestock population and significant contribution towards dairy producers as well as the country's economy, the attention given to the dairy cattle industry in Ethiopia is quite minimal. Efficient reproduction is critical for the economic success of dairy industry. However, the dairy sector is still underdeveloped for several reasons including the preponderance of infectious diseases that impede the productive and reproductive performance of dairy cattle.

Numerous infectious pathogens are known to have a direct impact on the reproductive health of cattle. Among reproductive pathogens, BoHV-1 is the most important infectious pathogen that has a significant impact on the dairy industry and the national economy (Yoo, 2010), and it is one of the most important infectious agents that has a direct impact on the respiratory health (Andrews *et al*., 2004) of cattle. It is a member of the bovine respiratory disease complex (BRDC) pathogens, which is the leading cause of cattle death around the world (Hou *et al*., 2017). The virus causes three different types of diseases, namely Infectious Bovine Rhinotracheitis (IBR), Infectious Pustular Balanoposthitis (IPB), and Infectious Pustular Vulvovaginitis (IPV) (Biswas *et al*., 2013). Respiratory infection is a highly contagious disease in cattle (Kathiriya *et al*., 2018). The diseases are List B diseases of cattle in the World Organization for Animal Health (WOAH) (OIE, 2021).

BoHV-1 can cause financial and economic losses due to reduction of milk yield, failure of conception, abortion, loss of newborn calves, loss of body condition, insufficient feed conversion, secondary bacterial pneumonia, and high cost of treatment (Majumder *et al*., 2019; Wedajo *et al*., 2021). BoHV-1 affects the milk compositions and plays the role of bovine mastitis due to its immunosuppressive nature (Rola *et al*., 2015) and it causes a trading restriction in the

cattle industry (Muylkens *et al*., 2007). BoHV-1 is host-specific (Kaur and Chandra, 2016) and primarily cattle breeds of all ages are affected, but different domestic and wild animals are susceptible naturally and experimentally (More *et al*., 2017).

BoHV-1 is transmitted mainly through secretions of the respiratory, ocular, or genital tract during direct contact between animals and is also spread through fresh or frozen semen from infected bulls (Nuotio *et al*., 2007), and it may also be transmitted vertically across the placenta (Mahmoud and Salem, 2012). Bovine species display different clinical signs such as rhinotracheitis, balanoposthitis, pustular vulvovaginitis, infertility, conjunctivitis, and encephalitis when infected with BoHV-1 (Yoo, 2010), and abortion (Graham, 2013; Brock *et al*., 2020).

When cattle are infected with BoHV-1, the virus can form latent in the trigeminal or sacral ganglia of the peripheral nerves, which is a unique property of the virus. Therefore, animals with a latent BoHV-1 infection serve as a potential source of infection for susceptible animals in the herd when the virus is reactivated by immunosuppressive conditions or treatments (Nuotio *et al*., 2007), and once the cattle are infected, they remain carriers of BoHV-1 for the rest of their lives and become intermittent shedders of the virus (Brock *et al*., 2020).

In Ethiopian cattle herds, a few seroprevalence investigations of BoHV-1 have been conducted so far by the different researchers; Lefevre. (1975) reported a seroprevalence of 41.8% in Harar and Sidamo provinces; Bekele *et al*. (1989) reported a 67% seroprevalence in Gobe and Ghibe in central Ethiopia and Recent seroprevalence studies in the central, southern, and southwest regions (Sibhat *et al*., 2018), Kombolcha and Dessie (Wedajo *et al*., 2021), and northwestern region (Zewde *et al*., 2021), revealed that their overall seroprevalence was 41%, 25.6%, and 77.6%, respectively. Although these much studies were conducted in different regions of the country, many data gaps still exist and more investigation needs to be done to better understand the epidemiology of the virus in the different geographical settings and dairy cattle population. Due to the large number of improved dairy cattle breeds that exist in AA, an increasing demand for dairy producers from other regions of the country to buy improved breeds, which is a major driver of disease transmission over large distances via infectious animal movement.

Furthermore, AA dairy herds are one of the sources of male calves for government organizations that produce semen to improve local breeds of cattle through AI. For effective control and prevention measures, identification of epidemiological factors, estimation of the seroprevalence of the virus, understanding of farmers' awareness of the disease, and understanding of the geographic distribution of the disease through effective and regular surveillance are necessary. Thus, this epidemiological information is crucial in order to design more effective intervention strategies. At present, there is no vaccine available against BoHV-1 in Ethiopia, and outbreaks of this viral disease are controlled by proper management adaptations. Therefore, this study was carried out with the following objectives:

- To estimate the current status of seroprevalence of BoHV-1 in dairy farms of Addis Ababa.
- To identify risk factors affecting the occurrence of the BoHV-1.
- To assess the role of the virus on the reproduction problem.

2. REVIEW ON BOVINE HERPES VIRUS-1

2.1. Etiology

From eight known herpes viruses which infect cattle (Table 1), BoHV-1 is one of the most important virus (Muylkens *et al*., 2007; Nettleton and Russell, 2017). BoHV-1 belongs to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae*. Four subtypes of virus are known based on viral peptide pattern and restriction endonuclease analysis: BoHV-1.1 and BoHV-1.2a (associated with IBR), BoHV-1.2b (associated with IPV and IPB), and BoHV-1.3 (associated with encephalitis) (OIE, 2008; Kathiriya *et al*., 2018), but BoHV-1.3 was reclassified as the distinct species bovine herpesvirus type 5 (BoHV-5) due to differences in its antigenic and genomic properties. The risk of new antigenic variants arising is low because BoHV-1 isolates are antigenically and genetically similar and stable (Nettleton and Russell, 2017). The BoHV-1.2 subtypes are less virulent than subtype 1.1 (OIE, 2008).

Table 1: Eight known herpesviruses that naturally infect cattle

Herpes virus	subfamily	Natural host	Disease in cattle
BoHV-1	alpha	Cattle	IBR, IPV, and IBP
BoHV-2	alpha	Cattle	Bovine mammillitis/pseudo LSD
BoHV-4	gamma	Cattle/African buffalo	Uncertain
BoHV-5	alpha	Cattle	Bovine herpes virus encephalitis
BoHV-6 (BLH)	gamma	Cattle	Uncertain
AlHV-1	gamma	Wildebeest	Malignant catarrhal fever
OvHV-2	gamma	Sheep	Malignant catarrhal fever
SuHV-1	alpha	Pigs	Pseudorabies encephalitis

BoHV (Bovine herpes virus), AlHV (Alcelaphine herpes virus), OvHV (Ovine herpes virus), SuHV (Suid herpes virus), BLH (bovine lymphotropic herpes virus), LSD (lumpy skin disease)

Source : (Nettleton and Russell, 2017)

The BoHV-1 has a typical alpha herpes virus structure, being 150 nm in diameter with an enveloped icosahedral capsid which contains 162 capsomeres, which codes for approximately 73 proteins and it is made of a long double-stranded linear Deoxyribo Nucleic Acid (DNA) molecule arranged as a class D genome which comprise a unique long (UL) and a unique short (US) sequences. gB is dominant envelope glycoprotein (Muylkens *et al*., 2007). BoHV-1 genome codes for 33 structural proteins, 13 of which are probably associated with the envelope and 10 of these have the potential to encode glycoproteins which are labelled g (for glycoprotein) followed by a letter that designates their identity among herpes virus proteins. Among the 10 glycoproteins (Table 2), gB, gC, gH, gK, gL, and gM are in the UL segment and the remaining four, namely gG, gD, gI, and gE are in the US segment. Vhs (virion host shut down), VP8, VP16, BICP0, and BICP4 are other major proteins of BoHV-1 (Biswas *et al*., 2013).

Table 2:Some of the major proteins of the BoHV-1 and their functions

#	**Protein name**	**Functions**
1	gB (glycoprotein gB)	Attachment, entry, direct cell-to-cell spread and fusion
2	gC (glycoprotein gC)	Attachment
3	gD ((glycoprotein gD)	Entry, cell-to-cell spread
4	gI (glycoprotein gB)	Cell-to-cell spread
5	gH (glycoprotein gH)	Cell-to-cell spread
6	Vhs (virion host shut off)	Rapid shut off of host cell protein synthesis
7	VP16 (a TIF - transinducing factor of alpha genes)	Transactivator ofBHV-1 immediate early (IE) genes
8	BICP0	Activator of viral promoters
9	VP8	Protein kinase; enhances a TIF function
10	BICP4	Transactivator ofsome gene promoters

Source :(Biswas *et al*., 2013)

The viral glycoproteins located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity (OIE, 2008). The gene has been coined as essential and/or

non-essential for virus growth in culture after deleting individual gene from BoHV1. Glycogen E (gE) is one of the non-essential gene which are used to make marker vaccine. No antibody response occur for gE after the animal vaccinated with this virus unlike cattle infected with a field strain of BoHV-1 (Robinson *et al*., 2008).

Experimentally, BoHV-1 isolates do not show specific tropism for either the respiratory tract or genital tract because IPV strains can replicate in the nasal epithelium and IBR strains can replicate in the genital epithelium efficiently. Therefore, to determine the disease presentation route of infection is important than strain of the virus (Nettleton and Russell, 2017). BoHV-1 is resistant to environmental influence. The virus is an enveloped virus and prone to disinfectants which destroy its lipid envelope and is readily inactivated by 0.5% NaOH, 0.01% $HgCl_2$, 1% chlorinated lime, 1% phenolic derivatives, 1% quaternary ammonium bases, and 10% Lugol's iodine. Formalin (5%) inactivates BoHV-1 within 1 minute. In feed, the virus can survive for more than 30 days. (Biswas *et al*., 2013).

2.2. Epidemiology

2.2.1. Host range

BoHV-1 is host specific (Kaur and Chandra, 2016) and cattle, all ages, are primarily affected (Biswas *et al*., 2013). Cattle, sheep, goat, camelids, water buffalo, and pig are naturally susceptible domestic species with BoHV1 and Red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), reindeer (*Rangifer tarandus*), and feral pig are naturally susceptible wildlife species. There are experimentally susceptible wildlife species these are Deer, Reindeer (*Rangifer tarandus*) and Muledeer (*Odocoileus hemionus*) and Rabbits are experimentally infected in domestic species. There is no recognized wild animal reservoir species for BoHV-1. In domestic animal species such as goats and sheep, BoHV-1 is able to establish a latent infection in the trigeminal ganglia from which it can be reactivated but they are not considered to play a role as an alternative reservoir for the virus. Latency, but not reactivation, has also been demonstrated in water buffaloes (*Bubalus bubalis*) but it is not proven as the reservoir (More *et al*., 2017).

2.2.2. Distribution

BoHV-1 is a worldwide distributed and showing differences in incidence and prevalence (Muylkens *et al*., 2007). The virus is distributed in the North America like United States of America (Newcomer and Givens, 2016),Canada (Baillargeon *et al*., 2017) and Mexico (Solis-Calderon *et al*., 2003) and from Latin America Brazil (Campos *et al*., 2009) and Costa Rica (Raizman *et al*., 2011). The virus is distributed in Europe (Waldeck *et al*., 2021) but a number of European countries like Austria, Denmark, Finland, Sweden, and Switzerland are officially free of IBR using Test and slaughter strategy (More *et al*., 2017). BoHV-1 is distributed in Asia such as China (Nikbakht *et al*., 2015, Zhou *et al.,* 2020), India (Majumder *et al*., 2019) and Iran (Yang *et al*., 2012). In North America, South America, and Europe Subtype 1 virus isolates are prevalent. These subtypes are frequently detected in cattle suffering from IBR and the respiratory tract of aborted fetuses. Subtype 2a strains are prevalent in Brazil and are associated with IBR, IPV, IPB, and abortions. Subtype 2b strains are prevalent in Australia and Europe and associated with IBR.IPV, IPB, but not abortion (Jones, 2019). The BoHV-1 are prevalent in different parts of Africa (Table 3).

Table 3: Seroprevalence of BoHV-1 in different parts of Africa

Study area	Prevalence (%)	Diagnostic test	Study design	Study period	References
Algeria	14.16	gB ELISA	Cross-sectional	May,2014- Sep, 2015	(Kaddour *et al*., 2019)
Cameroon	16.7 ± 1.7%	indirect ELISA	Cross-sectional	-	(Daniel *et al*., 2018)
Egypt	27.89%	indirect ELISA and PCR	Cross-sectional	winter 2017	(Zeedan *et al*., 2018)
Ghana	69%	ELISA	Cross-sectional	-	(Adu-Addai et al., 2012)
Kenya	17.4 %	gB ELISA	Cross-sectional	2016-2017	(Kipyego *et al*., 2020)
Morocco	50%	indirect ELISA	Cross-sectional	-	(Lucchese *et al*., 2016)
Namibia	80%	ELISA	Cross-sectional	1984-1987	(Geiger *et al*., 1990)
S. Africa	74.47%	ELISA	Cross-sectional	-	(Njiro *et al*., 2011)
Sudan	8.75%	ELISA and PCR	Cross-sectional	2015	(Elhassan *et al*., 2015)
Tanzania	42.0%	ELISA	Cross-sectional	August,1985-May, 1986	(Lyaku *et al.*, 1991)
Tunisia	25.9%	ELISA/VNT	Cross-sectional	-	(Ghram and Minocha, 1990)
Zambia	23.28%	ELISA/direct FAT	Cross-sectional	-	(Mweene *et al*., 2003)

Bovine herpes virus-1 status in Ethiopia

A few Sero-prevalence investigations of BoHV-1 have been conducted so far in the dairy herds of Ethiopia (Table-4) and recent studies displayed in Figure-1. A variety of ELISAs, namely Micro-ELISA (Bekele *et al*., 1989), Blocking-ELISA (Asmare *et al*., 2018; Sibhat *et al*., 2018), and competitive ELISA (Wedajo *et al*., 2021) have been used for studies of BoHV seroprevalence in cattle in Ethiopia . This Serological investigation ascertained that, this virus is widespread among local and exotic cattle in different parts of the country and the first report was in 1975 (Lefevre, 1975). In those studies the antibody induced from the cattle against the BoHV-1 is due to natural infection but not due to vaccination because no vaccination had been used in Ethiopia. Age, Herd size, type of farm, geographic region, agro-ecology, parity, and purchased animals are mentioned risk factors of BoHV-1 in those studies.

Even though the research conducted on BoHV-1 in the country low, the disease is prevalent and economically important and with other bovine respiratory disease complexes, it causes impediments to livestock development in Ethiopia. BoHV-1 is not considered as one of the most important respiratory and reproductive diseases of cattle. Studies are undertaken on BoHV-1 so far revealed the existence of the virus in different parts of the country with a prevalence that ranges from 5.9% in Sebeta (Sibhat *et al*., 2018) to 81% in Ghibe (river valley between Shoa and Jimma) (Bekele *et al*., 1989). These seroprevalence variation reported could be due to differences in agro ecology, animal management, production system, population density, the types of tests used to evaluate the seroprevalence of BoHV-1 and also there may be bias and variation.

According to different investigation conducted in Ethiopia so far, the Seroprevalence of BoHV-1 is high in both individual animal and herd level and it is significantly associated with reproductive disorders which affects the dairy industry in Ethiopia but still attention is not given for prevention and control (Lefevre, 1975; Bekele *et al*., 1989; Asmare *et al*., 2018; Sibhat *et al*., 2018; Wedajo *et al*., 2021).

Table 4:Seroprevalence of BoHV-1 in cattle from different locations of Ethiopia since 1975

Study area	Prevalence	Breed of cattle	Diagnostic test	Study design	Study period	References
North Western Ethiopia	77.6% (Overall)	Indigenous zebus	I-ELISA	Cross-sectional	November 2020 to June 2021	(Zewde *et al.*, 2021)
Kombolcha	21.1	Local and cross breed	C-ELISA	Cross-sectional	-	(Wedajo *et al.*, 2021)
Dessie	30.9					
Addis Ababa	32.1	HF, Jersey, and HF-Zebu crossbred	B-ELISA	Cross sectional	-	(Sibhat *et al.*, 2018)
Holeta	45					
Sebeta	6					
Debrezeit	26					
Ambo	45					
Adaberga	61.9					
Adama	32					
Jimma	56					
Alagae	53					
Hosanna	35					
Wolyta sodo	62					
Hawassa	32					
Arsinegelle	45					
Shashemene	61.5					
Wondogenet	26.3					
Central, South and South-Western Ethiopia	43.8 (Overall)	HF, Jersey, and HF-Zebu crossbred	B-ELISA	Case- control	-	(Asmare *et al.*, 2018)
Gobe(arsi)	58	Indigenous zebus	Micro-ELISA	Cross sectional	-	(Bekele *et al.*, 1989)
Ghibe	81					
Harar and Sidamo province	41.8 (Overall)	-	-	Cross sectional	-	(Lefevre, 1975)

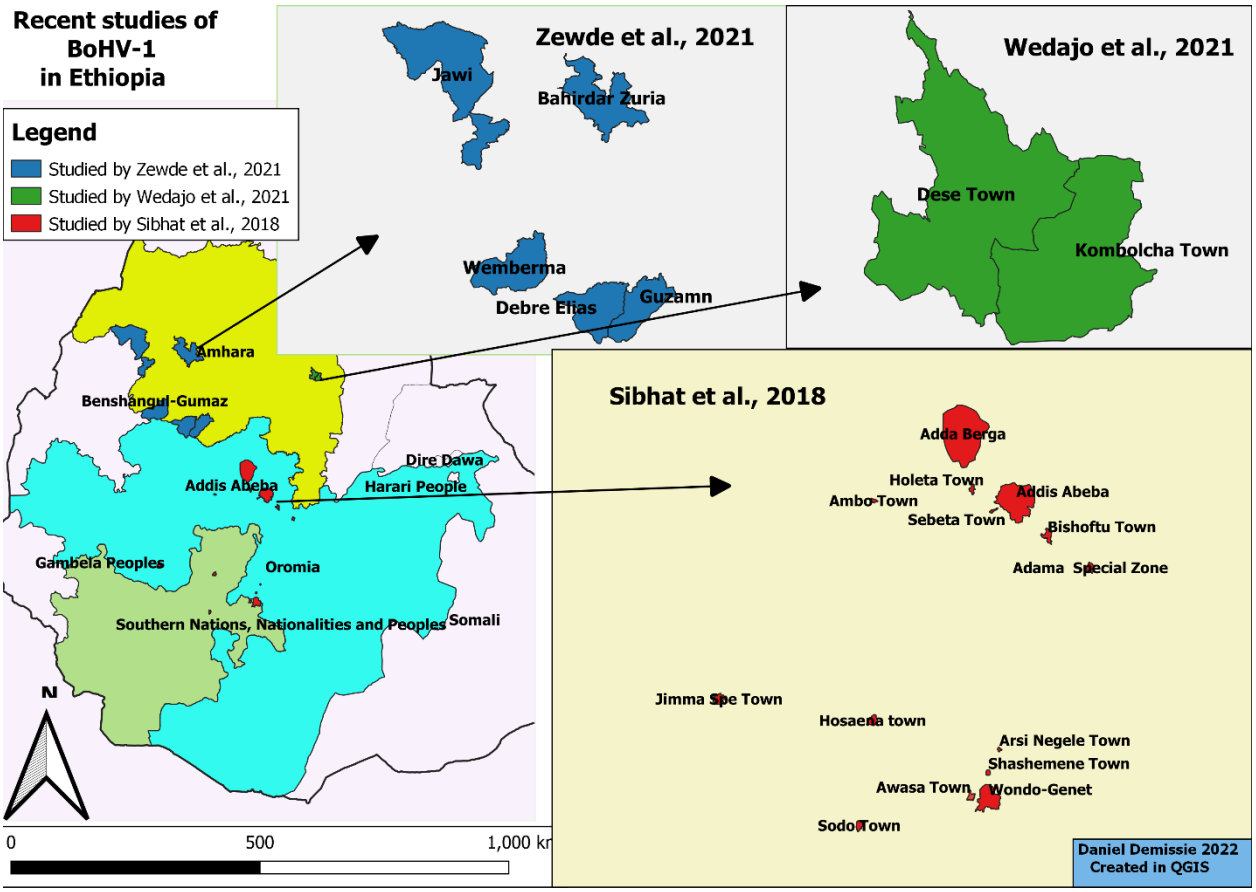

Figure 1: Map of BoHV-1 studied area in Ethiopia using QGIS Version 3.16.8-Hannover

The map was sketched using quantum geographic information system (QGIS) software to show the sero-prevalence of the BoHV-1 in Ethiopian districts, which are studied by (Sibhat *et al*., 2018; Wedajo *et al*., 2021; Zewde *et al*., 2021).

2.2.3. Risk factors

A condition, characteristic, behaviour that increases the probability of getting a disease is called a risk factor**.** Age, herd size, sex, direct animal contact, farm density or cattle density are important risk factors for the introduction of BoHV-1. Older and male cattle are at high risk of being seropositive. Purchase of the cattle, larger herd size (Boelaert *et al.*, 2005) and parity also important risk factor and multiparous cows are other important risk factors (Wedajo *et al.*,

2021). Risk factors which found in one country not be the risk factor for another country. Waldeck *et al.*, (2021) classified risk factors of BoHV-1 in to seven groups based on herd factors (Herd size, herd type), management factors (Seasonal calving, Presence of a bull, Borrowing machinery), animal factors (Breed, Sex, Age), purchase related factors (Purchase of cattle, Rejected export cattle), direct animal contact factor (Cattle shows,(Communal) grazing, Housing, Other species), neighborhood factors (Herd density, Distance between herds, Escaping and mingling) and indirect transmission factors (Visitors, Vaccination). But vaccination history were not have any association with BoHV-1 seropositivity (Raaperi *et al*., 2010; Sayers *et al*., 2015).

Presence of a bull for breeding purpose, concentrated herd in a given region, cattle parade, professionals visitors that are not wearing protective clothing are also another risk factors. Larger herds are prone to the infection because mostly they have contact within the farm, and contacts with other farms through purchasing of cattle and also they have different types of visitors. In the first few months of life, calves are protected against the BoHV-1 due to maternally derived antibody (MDA) and it is common to remain uninfected until they join the adult herd but thereafter infection can occur at any age. High level of herd immunity induced by periodic reactivation limits the spread of virus among adults in an infected herd, but naive replacements will soon encounter infection and may experience clinical disease at this time. Virus is likely to circulate periodically in high-yielding dairy herds with annual replacement rates of 25 per cent (Nettleton and Russell, 2017).

2.2.4. Transmission

During acute respiratory infection, BoHV-1 is shed in nasal discharge of cattle for 10–14 days (Biswas *et al.*, 2013). BoHV-1 transmitted directly through respiratory, ocular or genital secretions during contact with acutely infected animals and latently infected animals in which reactivation of the virus takes place and indirectly through contaminated fresh or frozen semen from infected bulls semen, humans, contaminated materials, Airborne transmission (Nuotio *et al*., 2007). Direct nose to nose contact is the best way of transmission of BoHV-1 (Muylkens *et al*., 2007). It also transmitted during embryo transfer and also through placenta of aborted

animals (Kaur and Chandra, 2016). Airborne transmission of BoHV-1 is possible at a distance of 3.85 meters under experimental conditions (Sibhat *et al*., 2018). From milk and faeces, BoHV-1 has been isolated but they are not significant source of transmission route in the field. If the cattle infected with the BoHV-1, the value of basic reproduction ratio (R_0) are ≥ 9 and ≥ 7 for calves and adult cattle, respectively (More *et al*., 2017). R_0 is a threshold value indicating infection dynamics in a population (Average number secondary infections generated by a single infectious individual (primary infection) introduced into a totally susceptible population.

Cattle breeders primary concern is that a lot of pathogens are transmitted through bovine semen, of which BoHV-1 is one of the most common viral pathogen and causes significant treat for the cattle industry as it can cause reproductive disorders such as abortion, infertility, repeat breeding and the like. Thus, semen should ideally be collected from bulls that are serologically negative for BoHV-1 infection (Kaur and Chandra, 2016). Sheep may play a crucial role in the epidemiology and maintenance of BoHV-1 in the environment, this is due to the detection of antibodies against BoHV-1 but they are unlikely to play any role in BoHV-1 transmission (Biswas *et al*., 2013).

2.2.5. BoHV-1 role in bovine respiratory disease complex

Bovine respiratory disease complex (BRDC) is a multifactorial disease caused by multiple deleterious management practices, and interactions among viral and bacterial pathogens. The primary pathogens are bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBR), parainfluenza-3 virus (PI-3), and *Mycoplasma bovis*. BoHV-1 is one of the most important causes of respiratory disease complex called shipping fever. Following these primary pathogens, damaged lungs are invaded by secondary bacterial pathogens of which the principal organism is *Mannheimia haemolytica* which causes severe lung damage, pneumonia and even death. Further bacterial involvement follows, usually *Pasteurella multocida* and *Arcanobacterium* (*Actinomyces*) *pyogenes*, resulting in chronic lung pathology. As a result of virus infection of the upper respiratory tract, bronchi, lower trachea and lung two important events occur that increase the colonization of the lower lung with bacterial pathogens, leading to the development of severe pneumonia (Andrews *et al*., 2004; Majumder *et al*., 2019).

Field and Experimental evidence supported that BoHV-1 involves in promoting bacterial superinfections leading to severe bronchopneumonia. The modifications induced by BoHV-1 may act at three levels of the physiological responses to pathogens. Mucosal clearance by mucous secretion and ciliary activities are affected because the epithelium damages occur by the BoHV-1. Polymorphonuclear neutrophils and of alveolar macrophages activities are affected by BoHV-1 and also changes in leukocyte trafficking and selective depletion of CD4+ lymphocyte populations encountered. Another possible mechanism that might explain BoHV-1/bacterial synergism is the exposure of leukocytes to inflammatory cytokines released in response to BoHV-1 infection (Muylkens *et al*., 2007).

2.2.6. Latency and reactivation

Latency establishment is a unique feature of the virus (Biswas *et al*., 2013). As with all Alphaherpesvirinae, BoHV-1 is able to establish a latent infection in the trigeminal or sacral ganglia when infected with a the virus or vaccinated with a live viral vaccine. It may also occur in tonsils and peripheral blood lymphocytes. Therefore, animals with a latent BoHV-1 infection serve as a potential source of infection for susceptible animals when the virus is reactivated *(Nuotio et al*., 2007). There is no viral DNA replication and also shut down of viral gene expression occurs during Latency which is maintained by virally encoded genes, notably the expression of the BoHV-1 latency-related transcript, which prevents initiation of viral replication (Nettleton and Russell, 2017). Cattle recovering from BoHV-1 infection become a silent carrier. These animals remain carriers of BoHV-1 for the rest of their life and immunosuppressive treatments or conditions might reactivate virus replication, leading to spread of the infection to the rest of the herd (Biswas *et al*., 2013). Up to 10 per cent of clinically normal animals become latently infected and most of these cattle will show antibody levels to the BoHV 1 virus, some do not (Andrews *et al*., 2004).

Reactivation may be absent for long periods or occur frequently. The reactivation from latent infection may occurs with or without showing clinical signs (Gu and Kirkland, 2008). It depends up on the antibody titer. If the animals with high antibdy titers are less likely to re-excrete the

virus but reactivation and re-excretion in animals with low antibody titres will boost their antibody levels so, the frequency of reactivation and virus spread cannot be predicted. Once the virus is reactivated it travels through nerves to the original site of the entry where virus amplification occurs, which may or may not lead to virus re-excretion and clinical signs. Reactivation from latency occurs when animals are stressed (Ackermann and Engels, 2006) due to transport, debilitating weather, poor nutrition, mixing with new animals, change in the herd structure, calving, mating, onset of lactation, intercurrent disease and also it can also be induced consistently by high doses corticosteroids given (Muylkens *et al*., 2007; Nettleton and Russell, 2017).

The preimmune status and the progeny of the virus phenotype are the two factors that influences the virus re-excretion. The cattle exposed to the BoHV-1 either naturally or following the vaccination program, primary immune response, can successfully control the re-excretion of any latent carrier. So that, the secondary immune response is effective at inhibiting virus re-excretion. The other factor that influences re-excretion of the virus is that the progeny virus phenotype has an influence on the ability for a virus to be re-excreted or not. Experiments demonstrated that a BoHV-1 gC-gE- null mutant established latency but was not re-excreted following a reactivation treatment (Muylkens *et al*., 2007).

2.3. Pathogenesis

Virus are introduced in the animal through direct contact with the nasal secretion or aerosol route and through semen then causes respiratory and genital tract infections, respectively. Mucus membrane of the upper respiratory and genital tracts are the natural portal of entry of BoHV-1 and inoculation of the virus through the conjunctival epithelium also the possible way of entry (Majumder *et al.*, 2019).

After penetrated into the target epithelial cells, BoHV-1 sets up the lytic replication cycle which corresponds to the sequential expression of viral genes and leads both to the production of new progeny viruses and to cell death. The rise of intranuclear inclusions and cell ballooning are the characteristics of BoHV-1 cytopathic effect (CPE). Necrosis and apoptosis processes during the

BoHV-1 replication cycle cause the cell death. Massive virus production of BoHV-1 infection results at the natural portal of entry then the new progeny viruses are shed in the nasal mucus at high excretion titers and are responsible for the rapid dissemination of the infection within a cattle herd. R_0 was estimated to be at least 7 in a dairy cattle herd in experimental conditions. The new progeny also spreads using the local dissemination, the systemic spread by viremia and eventually the neuroinvasion into the infected animal (Muylkens *et al*., 2007).

The spreading of new progeny viruses in the infected mucosa permissible by two different ways. First, the viruses which are released in the extra-cellular medium are fully enveloped particles able to interact with the receptors of susceptible cells. Second, viral particles can directly spread from an infected cell to neighboring uninfected cells (direct cell-to-cell spread) which is advantageous because it still occurs in the presence of neutralizing antibodies in the extra-cellular medium. gB, gD, and gH/gL are required for direct cell-to-cell spread but gE and gG function independently from each other in direct cell-to-cell spread; indeed, an additive effect on plaque formation was observed in the gE/gG double deletion mutant (Muylkens *et al*., 2007).

In the host BoHV-1 may spread by viremia which results gaining access to a broader range of tissues and organs and causing other clinical manifestations such as abortion in pregnant cows and fatal systemic infection in very young seronegative calves. Regarding the mechanism of BoHV-1 systemic spread, very little information is available. Following an intranasal inoculation of a highly virulent BoHV-1 strain, virus was isolated for several days from sera of infected calves, indicating a putative cell-free viremia in some circumstances (Muylkens *et al*., 2007).

Local nerve cell endings are infected and the virus is transported to trigeminal and sacral ganglia where it establishes a lifelong latent infection (Gu and Kirkland, 2008). BoHV-1 is thought to infect neurons via nerve endings in the mucosae during the primary virus replication which occurs in the mucosal surfaces and then ascends towards the central nervous system (CNS). Trigeminus and olfactorius nerves, which are the member of six major brain nerves innervates the oronasopharyngeal mucosa, supply the nasal mucosa. The rostral parts of the nasal cavity are innervated only by the nervus trigeminus, whereas the olfactory epithelium in the caudal

parts of the nasal cavity contains both olfactory and trigeminal nerve endings. These two routes (olfactory and trigeminal) represent the major ways of viral neuroinvasion toward the CNS, BoHV-1 preferentially uses only the trigeminal way. Moreover, BoHV-1 neuroinvasion usually does not go further than the first order neuron located in the trigeminal ganglion where the latent infection is established. However BoHV-1 was isolated sporadically from cattle with central nervous system disorders. Some of these isolates were shown to be responsible for acute meningo-encephalitis occurring in adult cattle. These sporadic cases probably reflect individual host susceptibilities to CNS infection rather than BoHV-1 strain modifications giving rise to an increased neuroinvasion and/or neurovirulence (Muylkens *et al*., 2007).

2.4. Economic significance

Introduction of BoHV-1 in the cattle herd causes severe economic damages due to loss of live weight, loss of milk production (Can *et al*., 2016), abortion, loss of body condition, loss of new born calves, temporary failure of conception, insufficient feed conversion, secondary bacterial pneumonia and cost of treatment (Majumder *et al*., 2019). OIE lists IBR/IPV as a List B notifiable disease which are transmissible diseases that are of socio-economic and that are significant in international trade (Kaur and Chandra, 2016). The presence of BoHV-1 in the country can cause restrictions in the international livestock trade (Biswas *et al*., 2013). European countries restricts importation of cattle from endemically infected regions and requires IBR-free status (Armengol *et al*., 2017).

BoHV-1 is one of the causative agent which plays role of bovine mastitis due to their immunosuppressive properties (Barkema *et al*., 2009; Rola *et al*., 2015) and leads to the economic consequences due to treatment, production losses, culling, changes in product quality *(*Halasa *et al.*, 2007). Milk shows higher somatic cell count and milk composition a such fat content (Rola *et al*., 2015) and milk protein (Sayers, 2017) significantly affected.

There is a lack of reliable published disease data for economic analysis but one modelling study, a range of control strategies for Dutch dairy herds costs €99.5 million. 62.5% to vaccination, 10.5% for diagnosis, 7.8% for monitoring and 19.2% for culling and additional costs for testing

and culling was 2.7 million and 25 million euro, respectively (More *et al*., 2017). Annual losses in the United Kingdom due to the BoHV-1 effects such as milk loss, live weight loss, fertility problems, mortality /premature cull, abortion / stillbirth, condemnation carcass with cost resulted from treatment, control and monitoring were estimated at up to 7 million pound (Bennett and IJpelaar, 2005). BoHV-1 causes abortion at the late-term of pregnancy (Nettleton and Russell, 2017; Newcomer and Givens, 2016) and the estimated financial loss (average cost) estimated to be 379 United States dollar per infected cow (Can *et al*., 2016).

The cattle recovering from BoHV-1 become silent carrier for rest of their life, this serves as a source to the rest of the herd when exposed to immunosuppressive conditions which might reactivate virus replication (Biswas *et al*., 2013). Strict import restriction on cattle, semen and embryos because the reintroduction of the virus into these immunologically naive populations is likely to have serious consequences and lead to severe economic loss (Majumder *et al*., 2019).

Morbidity and mortality rates of BoHV-1 is affected by different factors including immune status of the host, virulence of the BoHV-1 strain and potential concurrent bacterial infection *(*More *et al.*, 2017). Morbidity and mortality is lower in dairy herds than in beef cattle (Majumder *et al*., 2019). Yak (*Bos grunniens*) and Mithun (*Bos frontalis*), rare cattle species located in the Asia and have high economic importance in milk, meat and wool production, highly affected by the BoHV-1 (Biswas *et al*., 2013).

2.5. Clinical signs

Route of entry of virus decides the type of the disease manifests (Kaur and Chandra, 2016). Virulence of the BoHV-1 strain, resistance factors of the host such as the age, and potential concurrent bacterial infection influences the severity of the disease caused by BoHV-1. Subclinical BoHV-1 infections are common. Weakly virulent strains are display a poor ability to induce clinical signs. This occurs by the primary infection of passively immune calves in countries where BoHV-1 is endemic (Muylkens *et al*., 2007). BoHV-1 causes three different types of infection, namely respiratory infection, genital infection, and neurological disease

which are caused by subtypes of the BoHV-1 such as BoHV-1.1, BoHV-1.2 and BoHV-1.3, respectively (Biswas *et al*., 2013).

2.5.1. Respiratory infection

A more virulent strain of infectious bovine rhinotracheitis (IBR) first appeared in the western United States in the mid-1950s (Graham, 2013). It is a highly contagious disease of cattle caused by the BoHV-1 (Kathiriya *et al*., 2018). The name IBR has been coined to it since 1955, during a conference of the US Livestock Sanitary Association (Kaur and Chandra, 2016). The disease is known by various names around the world based on the signs and symptoms, including Red Nose, Dust Pneumonia, Necrotic Rhinitis, and Necrotic Rhinotracheitis.

Respiratory disease is the most common type, and it usually affects cattle over the age of six months, but it can also affect animals younger than that. The disease is usually most severe in animals aged six months to two years (Andrews *et al*., 2004). Those cattle managed under intensive (housed) conditions are most frequently exposed for respiratory form of the disease unlike cattle under grazing conditions (Gu and Kirkland, 2008; Nettleton and Russell, 2017). The clinical signs observed in infected cattle is confused with other disease so, laboratory identification is indispensable (Gu and Kirkland, 2008). The incubation period ranges from 2-4 days (Biswas *et al.*, 2013). Seronegative calves become highly febrile (41˚C) within 4-5 days following the intranasal inoculation accompanied by apathy and anorexia (Muylkens *et al*., 2007).

The disease may be very mild or remain unnoticed with only slight serous nasal discharge and a modest rise in body temperature over one to two days. If the disease is severe there is pronounced pyrexia of 40-42ºC, which may last for several days. Declining of milk production and increment of respiratory rate are the sign of affected animal. The initial serous nasal discharge changes to muco-purulent within a few days. Muco-purulent nasal discharge, excessive salivation (some animals), reddened and shallow erosions on the mucosa of the nares, shallow erosions of the oral mucosa (uncommon) and show a drop in milk production. Some animals develop conjunctivitis which may be unilateral or bilateral and have a clear ocular dischargeconjunctivitis (Gu and Kirkland, 2008). Red appearance of pharyngeal and tracheal

mucosal epithelia and the presence of several necrotic foci recovered with dead mucosal epithelial cells embedded in fibrinous exudate are revealed by endoscopy examination (Muylkens *et al.*, 2007). There can be a severe necrotising laryngo-tracheitis and pneumonia, within the first 3-4 weeks of animals entering, which is complicated by secondary bacterial infections in intensively managed cattle so, there can be outbreaks of severe pneumonia due to BoHV-1 infection (Gu and Kirkland, 2008).

Some countries reported the abortion as a complications of the respiratory form but some countries does not report. The report of mortality is up to 5 % in cattle, and severe and prolonged drop of milk (with or without oculonasal discharges) in previously naive dairy herds (Nettleton and Russell, 2017). Systemic infection of the dam causes abortion results from fetal death. The interval between exposure and abortion can range from 15 days to several months, so fetuses may be expelled while clinical IBR is evident in the herd or as long as 100 days later (Nettleton and Russell, 2017). BoHV-1 may cause abortion storms due to breeding synchronization in cattle (Ackermann and Engels, 2006).

2.5.2. Genital infection

BoHV-1 as the cause of infectious pustular balanoposthitis (IPB) and infectious pustular vulvovaginitis (IPV) was first defined in Germany in the 19th century (Graham, 2013). Genital infection with BoHV-1 occurs in male and female and common cattle on pasture. The infection may show vesicles, pustules and erosions or ulcers in the mucosa of the vulva and vagina or on the penis and prepuce (Gu and Kirkland, 2008). The reproductive form of the disease was reported as early as 1938 and was called "*epivag*", which means causing epididymitis in males (Kaur and Chandra, 2016).

Infectious pustular vulvovaginities

A few days after mating a painful condition is observed. Repeated urination and raising of the tail are the first clinical signs. The posterior third of the vagina and vulva become hyperaemic or edematous. Small white to red ulcers changed from pustules (0.5–3 mm in diameter). There

may be a thick white or yellow mucopurulent exudate, especially in cases complicated by secondary bacterial infection (Gu and Kirkland, 2008; Nettleton and Russell, 2017).

Infectious pustular Balanopothitis

It is the disease in bulls. Hyperemia of preputial mucous membranes and penile with vesicle formation and adhesions, annular constrictions, penile distortion occurs when the bull affected with IPB (Pandey *et al*., 2014). Pustules appear on the penis and prepuce after a 2-3 day of incubation period then it progress to ulcer with mucopulurent discharge, loss of libido, and it prevent a bull from serving. A proportion of infected bulls will also excrete virus in their semen which infect susceptible females by natural or AI (Gu and Kirkland, 2008; Nettleton and Russell, 2017).

2.5.3. Neurological disease

Herpes virus-induced nervous disease caused by BoHV-5. BoHV-1 can enter the CNS of cattle without provoking any clinical signs of nervous disease following respiratory infection. Nervous and upper respiratory disease have been reported due to BoHV-1 in neonatal beef calves. Occasionally, at the same time as, or shortly after, showing signs of IBR, older cattle may develop nervous signs. Affected animals become very dull and are termed ‘sleepers’ (Nettleton and Russell, 2017)

2.6. Diagnosis

2.6.1. Virus isolation

Cell culture

The reliable diagnosis of BoHV-1 infection by virus isolation depends on the availability of cell cultures that have proven susceptibility to BoHV-1 virus. Various origins of bovine cells can be used for virus isolation such as primary or secondary bovine lungs, kidney, testis, turbinate, or

trachea and established cell lines such as Madin–Darby Bovine Kidney (MDBK) or Cell line resistant to infection with bovine viral diarrhea virus (CRIB) cells (Gu and Kirkland, 2008; OIE, 2008). It is possible to isolate virus from nasal swabs, conjunctival swabs, vaginal swabs, preputial washing, placental cotyledons of aborted fetus and similarly isolated from fetal liver, lung, spleen, kidney, lymph node, mucous membrane of the respiratory tract, tonsils, and lungs collected in virus transport medium (Gu and Kirkland, 2008; Nettleton and Russell, 2017). The virus also isolated from semen but it needs some special adaptations, because the enzymes which are found in the seminal fluid and other factors that are toxic to the cells and inhibit viral replication (OIE, 2008). CPE used to detect the presence of virus in specimens. The CPE is usually appears within 3 days of inoculation of BoHV-1 and it is typical. In cell culture, grape-like clusters of rounded cells present around a microplaque. Giant cells or syncytia are also observed. If the cells are incubated for a prolonged time the virus is cytolytic and total sloughing of the rounded cells from the plastic/glass surface of the container is observed. For 7 days should be observed the cell cultures inoculated with specimens. Before the sample is considered negative, the cell culture should be passaged at least three times (OIE, 2008; Biswas *et al*., 2013).

Fluorescent antibody test

This test is antigen detection technique and it can lead to same day diagnosis but it has lower sensitivity than virus isolation. Swabs collected from nasal, ocular or genital can be smeared on the glass cover slips, the cell deposit may be spotted on to cover-slips which are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific antiserum is conjugated to fluorescein isothiocyanate, whereas in the indirect procedure it is the anti- species immunoglobulin second antibody that is conjugated to fluorescein isothiocyanate. To obtain the best results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone within 24 hours. Smear from nasal swabs should be from serous fluid at early phase of the disease but purulent of heamoragic nasal dischares are often negative (OIE, 2008).

Polymerase Chain Reaction (PCR)

Cell cultures and fluorescent antibody tests, which are used for virus isolation has been outdated due to rapid, highly sensitive, semi quantitative PCR (Nettleton and Russell, 2017). A 'real time' PCR assay is as sensitive as virus isolation and is a practical alternative for the rapid detection of virus. As compared to virus isolation which requires 7 days the results of PCR are available in one day. For experimental infection of the cattle, virus could be detected up to 14 days from nasal swabs and the PCR assay can also detect the virus in bovine fetal serum and semen samples (Biswas *et al*., 2013). So far the PCR has been used mainly to detect BoHV-1 DNA in artificially or naturally infected semen samples (OIE, 2008) and it has been accepted by OIE as an approved test for the detection of BoHV-1 in semen (Gu and Kirkland, 2008).

One step multiplex reverse transcriptase quantitative polymerase chain reaction (mRT-qPCR) used to detect the viruses commonly implicated in BRDC. A mRT-qPCR assay is used for the simultaneous detection of BRSV, BoHV-1 and PI- 3 nucleic acids in specimens from cases of bovine respiratory disease (Thonur *et al*., 2012).

2.6.2. Serology

The identification of serologically positive animals provides a useful and reliable indicator of infection status because virus latency is a normal development to BoHV-1 infection. If the antibody is detected against the virus, the animal is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions to this are young calves that have acquired passive colostral antibody from their dam, and non-infected cattle vaccinated with inactivated vaccines. Serological tests can be used for several purposes such as to diagnose an acute infection, to demonstrate the absence of infection, to determine the prevalence of infection in seroepidemiological studies, to support eradication programs and subsequent surveillance, and for research purposes (OIE, 2008).

ELISAs and Virus neutralization tests (VNT) various are usually used for detecting antibodies against BoHV-1 in serum. A large degree on the purpose for the testing depends to the choice

of one or other test. The VNT is often requested for regulatory purposes for the certification of semen and embryo donors (Gu and Kirkland, 2008). ELISA had a higher specificity and sensitivity than VNT, thus ELISA is considered a rapid, reliable and technically superior test for detection of BoHV-1 antibodies (Kramps *et al*., 1994). The gE IBR ELISA is reported to have a lower analytical sensitivity than the gB IBR ELISA (O'Grady *et al*., 2008). Conventional and marker tests are used for serological tests of BoHV-1. BoHV-1 gE antibody blocking ELISAs is the only marker serological test available at this time. For conventional serology, VNT, BoHV-1 antibody blocking ELISAs as well as indirect ELISAs are used (OIE, 2008;Biswas *et al*., 2013).

2.6.3. Electron microscopy

The electron microscopy is the rapid method of diagnosis of BoHV-1 and used to identify the virus particles in clinical material but it should be used in the early stage of the disease BoHV-1 (Biswas *et al*., 2013).

2.7. Differential diagnosis

There are different respiratory disease causative agents that need to be differentiated from BoHV-1, particularly PI-3, BRSV, Mycoplasma bovis, Mannheimia species, *Pasteurella multocida*, *Histophilus somni* and *Trueperella pyogenes*. Some respiratory disease in adults may mimic BoHV-1 infection but most cases are not BoHV-1 related. There are Emerging common risk factors include high herd yield, poorly ventilated housing, *M.bovis* presence of in cattle, inadequate control of bovine viral diarrhea virus and a high incidence of respiratory disease in young stock. Other reports implicate lungworm (Nettleton and Russell, 2017). For the diagnosis of infectious reproductive losses, *Neospora caninum* and bovine viral diarrhea virus (BVDV) are relevant differentials (Waldner, 2005).

2.8. Treatment

Usually, the BoHV-1 are complicated with other disease causative agents as a result therapy is justified for the complication. Penicillin, streptomycin, oxytetracycline, ampicillin,

trimethoprim and sulphadiazine, sulphadimidine, sulphamethoxypyridazine and sulphapyrazole are the drug used if drugs are commenced for treating the calves for three to five days. The animal should be given good, wholesome feed and it should be encouraged to eat and drink. Acyclovir is one of the safest drug that are effective against herpes virus (Andrews *et al*., 2004).

2.9. Control and prevention

The virus is not eliminated from the infected cattle though natural immunity or induced immunity which are involved in the prevention process. The virus establishes life-long latency in the sensory ganglia after recovery, from where it may be reactivated at intervals. Therefore the detection of BoHV-1 latent carriers is a concern in the setting up of a BoHV-1 control program (Muylkens *et al*., 2007). Eradication program will always include the destruction of a great number seropositive animals which are considered to represent the virus reservoir because they are persistently infected with BoHV-1 (Ackermann and Engels, 2006).

2.9.1. Test and slaughter strategy.

Eradication of the BoHV-1 requires diligent testing and it is a time-consuming process and to be granted a disease free status. The control and eventual eradication of BoHV-1 is based on the detection and removal of infected animals (Nettleton and Russell, 2017). This has been used successfully in Scandinavian countries (Denmark, Finland, Norway, and Sweden), Austria, Switzerland (More *et al*., 2017) and some Italian and UK regions. Seropositive animal removal following vaccination using gE-deleted marker vaccines is an appropriate tool in conditions with medium/high BoHV-1 seroprevalence. This approach is called DIVA strategy (Iscaro *et al*., 2021).

2.9.2. Quarantine

The cattle which showing clinical signs shall be quarantined to prevent the transmission of the virus. As it is a latent virus and results in lifelong infection the use of quarantine in herds with BoHV-1 is not ideal control program. New animals purchased to a farm, or crossing borders,

should be quarantined while tests for the virus are being undergone. It also will help prevent the spread after an outbreak. Hygienic measures and maintaining 2-3 weeks of quarantine period before introducing new stock to the herd and precludes of virus positive animals (Majumder *et al.*, 2019).

2.9.3. Import restriction

Strict import restriction on cattle, semen and embryos because the reintroduction of the virus into these immunologically naive populations is likely to have serious consequences and lead to severe economic loss (Majumder *et al.*, 2019).

2.9.4. Vaccination

Different types of vaccines are currently available such as attenuated (modified live vaccine), inactivated (killed), subunit vaccines, and marker vaccines for BoHV-1 prevention (Biswas *et al*., 2013; Majumder *et al.*, 2019). Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response and given intramuscularly or subcutaneously (OIE, 2008). A subunit vaccine contains one or more of the antigens of the virus necessary to evoke protective immunity, and lacks nucleic acid and other components that might cause unwanted side effects. Marker vaccine is based on deletion of one or more viral proteins, which allows the distinction between vaccinated and naturally infected animals based upon respective anti-body responses. Different types of marker vaccines are available like gE-live, gE-killed, gG- killed, gC-live, gD-subunit, gB-subunit, and gD- replication-incompetent (Biswas *et al*., 2013). Marker vaccines can reduce the transmission of BoHV-1 and incidence of disease within herds. However, virus may still spread to a limited extent within a vaccinated herd, so good management in addition to a strict vaccination schedule is needed to control infection (Nettleton and Russell, 2017).

Multivalent vaccine is available for the control of co-infection associated with BoHV-1. These vaccines contain other respiratory pathogens such as PI-3, BRSV and BVDV. Sometimes it also

contains antigens of Leptospira and Campylobacter species (Biswas *et al*., 2013). To reduce the effects of the disease and risk of re-excretion of latently infected animal repeated vaccination of infected herds can be used to increase protection. A full understanding of the biology of BoHV-1, long-term planning and a commitment to management practices are required to use DIVA strategy for Eradication schemes at the herd, regional or national level (Nettleton and Russell, 2017).

Marker vaccines used for DIVA (differentiating infected from vaccinating animals) strategy

Several European countries have initiated control programs aimed at BoHV-1 eradication and based on the use of marker vaccines deleted in the gE gene. These marker vaccines, either inactivated or live attenuated, used together with a serological detection of gE-specific antibodies, allow distinction of infected from vaccinated animals (OIE, 2008).

Marker (gE-deleted) vaccines, which are considered safe and efficacious based on both experimental and field data are available and form the basis of a control strategy where initial prevalence is moderate to high. This approach should be supplemented by biosecurity measures to address risks of introduction associated with breeding, trade and husbandry activities, can be used to reduce the initial prevalence, with remaining positive animals being culled when prevalence falls to 5% (More *et al.*, 2017).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in dairy cattle herds which are located in AA, the capital city of Ethiopia. It is located at geographic coordinate 9°01′29″ N latitude and 38°44′48″ E longitude. Its average and the highest altitude (Entoto hill) are 2,400 meters and 3200 meters above sea level, respectively. AA, surrounded by mountainous landscape, has sub-tropical highland climate with average high and low temperature is 23^{o}C and 11^{o}C throughout the year, respectively. *Kiremt*, the main rainy season is from June to early October, and there is short period of rainfall, *Belg*, between early March and mid-April. The average annual rainfall is about 1,200 mm, out of which close to 80% falls during the main rainy season (Erena *et al*., 2017). The national central statistical agency that carries out national census projections put the population of Addis Ababa to be 3,774,000 (CSA, 2021). According to city farming and farmers development commission of Addis Ababa city administration in 2020, the estimated number of cattle was 109496. Similarly, the total number of registered dairy herds which are found in the city was 4783. For administrative purposes, the city is subdivided into ten sub-cities and 116 districts which is the lowest official administrative units covering a total area of 540 km^2 (Erena *et al*., 2017) but currently, the number of sub-cities are increased to eleven due to the addition of one sub-city called "Lemi Kura" as shown in Figure 2.

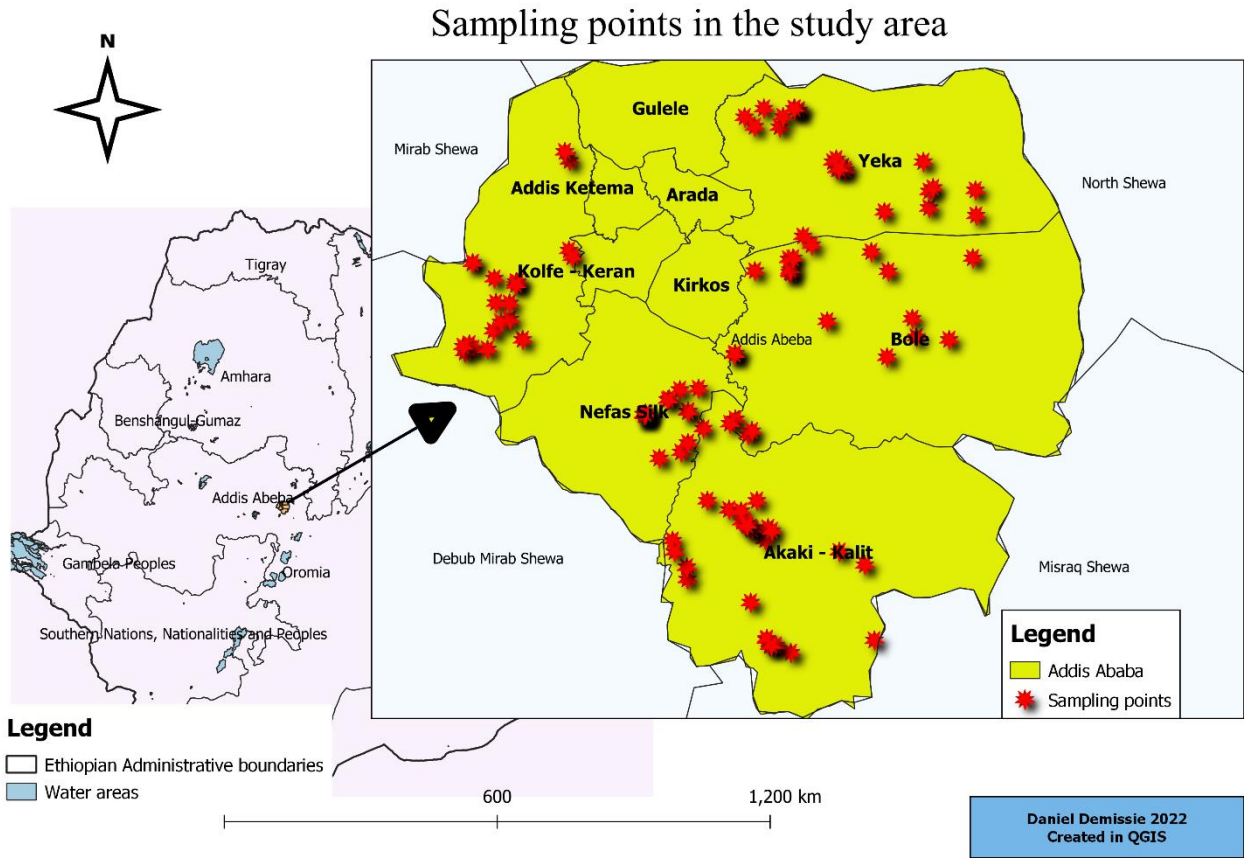

Figure 2: Map of the study area (Addis Ababa) with sampling points using QGIS Version 3.16.8-Hannover

3.2. Description of the study animals

The study animals were dairy cattle breed of Holstein-Friesians (HF), which may be pure HF or crossed with the local breed of cattle, in small-holder, medium, and large dairy herds located in Addis Ababa. Small-holder and medium-sized dairy herds are those holdings up to 10 and 10-50 dairy cattle, respectively. They produce milk for household consumption and commercial purposes. While large dairy farms had more than 50 dairy cows and produce milk basically for sale. Depending on the accessibility or preference of dairy owners, AI, natural mating (Bull) or both (AI and Bull) are used as cattle breeding systems. Vaccination against BoHV-1 has never been practiced all over Ethiopia including in Addis Ababa.

3.3. Study designs

A cross-sectional study was undertaken from November, 2021 to May, 2022 in a dairy cattle herds located in Addis Abeba. Blood samples were collected to determine the seroprevalence of the BoHV-1. Individual and farm-level data were collected from the record available in the dairy herds using a semi-structured questionnaire. Based on the questionnaire, risk factors were identified and the association of the virus with reproductive disorders such as repeat breeding, abortion, still birth, and retained fetal membrane were collected. Each animal was identified by purchase status, age, parity, herd size, herd type, breeding, controlling visitors, proximity, foot bath, and drinking water from the dairy personnel. The smallest number of samples taken was one animal, and the highest sample was 21 animals per farm.

3.4. Sample size determination

The sample size was determined based on a 32.1% animal level prevalence (Sibhat *et al*., 2018), 95% level of confidence, and 5% absolute precision (Thrusfield, 2007), requiring a minimum sample size(n) of 335 animals.

$$n = \frac{1.96^2 * \text{Pexp}(1 - \text{Pexp})}{(d)^2}$$

Where n = required sample size;
Pexp = expected prevalence;
d = desired absolute precision

10 % of the sample was added in order to adjust non-response. Accordingly, a total of 369 blood sample were collected.

3.5. Sampling method

A stratified random sampling methods was employed to divide the study population based on smallholder farms (<10 animals), medium farms (10 to 50 animals), and large farms with more

than 50 animals (Megersa *et al*., 2011). From each stratum sampling units was selected based Proportionate stratified random sampling according to Thrusfield. (2007), $S_k = (n/N)* Ss$, where S_k is sample size for the n^{th} stratum, n is required sample size, N is the entire sample size, and Ss is stratum size, based on the number of cattle which obtained from city farming and farmers development commission of Addis Ababa city administration.

3.6. Blood sample and data collection

Blood samples

Blood samples were collected from the jugular vein or coccygeal vein of dairy cattle using plain vacutainer tubes (Annex 1). About 10 ml of blood were drawn from each randomly selected animal and kept overnight in an upright position then the clotted blood was centrifuged to allow the separation of blood serum. The serum samples were decanted into sterile cryovials, labeled and transported to Animal Health Institute (AHI) serology laboratory in an icebox with the aid of an ice pack where preserved at −20 °C until screened.

Questionnaire survey data collection

Consent was obtained from the respondents, and the objective of the survey were explained to the dairy herd owner before start of the interview (Annex 2). Then, 369 semi-structured questionnaire was used to collect data in parallel to collection of samples from dairy herd. The potential risk factors that could contribute to the occurrence of BoHV-1 and the association of the virus with reproductive disorders was included in the questionnaire (Annex 3).

3.7. Serological assay

IBRgB ELISA (HerdChek® Infectious Bovine Rhinotracheitis [IBR]/ Bovine Herpesvirus-1 [BHV-1] gB Antibody ELISA Test Kit, IDEXX Europe BV, The Netherlands), a diagnostic sensitivity and diagnostic specificity of 99% and 99.7% (Kramps *et al*., 1994; O'Grady *et al*., 2008), was used for the detection of specific antibodies to the glycoprotein B (gB)of virus in

serum samples according to the manufacturer's protocol. Briefly, 50 µ1 reconstituted wash were added to all wells and then 50 µ1 (test samples, negative and positive control) were added as per plate layout into BoHV-1 antigen pre-coated plate. Samples and controls in seal plate was evaluated and was incubated for 2 hour at 37ºC. The plate were washed 5 times according to the procedure. Then 100 µl of the conjugate were added to each well and were incubated for 2 hour at 18-26ºC. The plate were washed 5 times according to the procedure. The plate will be sealed and incubated for 30 min at +37ºC and washed 5 times following the procedure. Finally, 100 µl of the substrate solution were added, to each well. The plate were kept for 10 min at 10-18 ºC away from direct light and were stopped by 100 µl /well of the stop solution. Measured and recorded the absorbance of the samples and controls at 450 nm or using a dual wavelength of 450 nm and 650 nm. In the presence of enzyme, substrate was converted into a product which reacts with the chromogen to generate a blue color. Upon addition of stop solution, a yellow color is generated. The absorbance at a single wavelength of 450 nm [A (450)] or a dual wavelength of 450 nm and 650 nm [A (450/650)] were measured using a spectrophotometer. The blocking percentage of the samples is calculated by using the absorbance [A (450)] or [A (450/650)] obtained with the test sample and the negative control containing no specific antibodies. The blocking percentage were <45 interpreted as "Negative", if the blocking percentage is in between 45 ≤ Blocking % < 55 interpreted as "suspect" and if the blocking percentage were g % ≥ 55 interpreted as "Positive"(Annex 4)

3.8. Data management and analysis

For statistical analysis, paper-based serological and questionnaire data were coded, entered, and managed by the Microsoft Office Excel® spreadsheet and analyzed. Herd and individual animal level data were organized categorically as described in Table 5. Following coding and editing, and cleaning, the data were transferred for further analysis to R_studio software (RStudio 2022.02.1+461 "Prairie Trillium" Release for Windows). Animal level BoHV-1 seroprevalence (Apparent Prevalence (AP)) was calculated by dividing the number of gB ELISA positive animals by the total number of animals tested. True prevalence (TP) of BoHV-1 seroprevalence was calculated by adjusting the AP for specificity and sensitivity of the test using the formula, TP = AP +(Specificity-1)/sensitivity + (specificity-1), (Thrusfield, 2007) where specificity

(99.7%), sensitivity (99%) of the gB ELISA test used. Herd level seroprevalence was calculated by dividing the number of herds with at least one seropositive cows/heifers in gB ELISA by the number of all herds tested. Considering the number of samples per herd, multivariable mixed-effects logistic regression model was developed, where the unique herd-name identification was considered as the random-effect variable. Importance of random effect structure was tested by Akaike's Information Criteria (AIC) and Model Likelihood Ratio test by comparing the glm model and glmer model. In the development of the model, all the non-collinear predictors, namely age, purchase status, herd size, breeding type, proximity and controlling visitors were considered and the final model was developed using manual step-wise step-up, forward elimination procedure based on AIC, Bayesian Information Criteria (BIC) and likelihood ratio test statistics ($p < 0.05$) (Annex 5). Herd type, parity, drinking water, and footbath were excluded in the process of model fitting because these predictors was not explain significant quantity of variance. History of abortion, repeat breeding, still birth, and retain placenta are reproductive abnormalities which were considered from the first pregnancy until time of sampling. The final model fit analysis was assessed using plot (predicted values against the residuals), the Hosmer-Lemeshow goodness of fit test and the receiver operating curve (ROC) (Annex 6). A map showing the study area and the corresponding dairy herd from which the sample was taken was generated using QGIS software (QGIS Version: 3.16.8-Hannover).

To assess the association of the virus with the reproduction problem, univariate mixed effect logistic regression analysis were conducted by considering BoHV-1 serostatus as dependent and each of the reproductive disorders as independent variables. Variables with p-values < 0.05 in the univariable logistic regression analyses were then separately analyzed using multivariable mixed effects logistic regression analyses by considering BoHV-1 serostatus as an dependent and each reproductive disorders as independent variables and the herd- name as a random variable.

Table 5:Variables and their categories for which data were collected during field work

Variables	**Categories**
Result	**0** (BoHV-1 negative animal following gB ELISA test)

	1 (BoHV-1 positive animal following gB ELISA test)
Age	**Young** (< 17 months)
	Adult (≥17 months)
Purchase_Status	**Yes** (Purchased from other dairy herd), or
	No (Born in the dairy herd/ Homebred)
Herd_Size	**Small sized herd** (≤10 animals),
	Medium sized herd (10 to 50 animals), and
	Large sized herd (≥ 50 animals)
Breeding	**AI** (Those dairy herd uses AI),
	Bull (Those dairy herd uses Bull), and
	Both (Those dairy herd uses AI and Bull depending on the accessibility)
Control Visitors	**Yes**, or
	No
	(The term "Control visitor" in this study was used to describe monitoring all people who came into contact with dairy herd including professionals (Veterinarian, Artificial insemination technicians) or occasional visitors (relatives, cattle traders or a temporary worker who also worked on other farms) and also customers of dairy products.
Parity	**Nulliparous** (for heifers or cows that was not gave birth)
	Uniparous (for cows/heifers having a single calf)
	Multiparous (for cows/heifers having two or more pregnancies and resulting offsprings). far from next herd at least by 100 meters
Proximity	**Far** (gap between herds at least by 100 meters), and
	Closer (a gap between herds <100 meters)
Foot bath	**Yes** (Those dairy cattle farms having foot bath in the entry)
	No (Those dairy cattle herds do not having foot bath in the entry)
Abortion History	**Yes** (if abortion encountered at least one time)
	No (if abortion was not encountered at least one time)
	(Abortion is as a loss of the fetus between the age of 42 days and 260 days. Pregnancies lost <42 days are usually referred to as early embryonic deaths, and a calf that is born dead between 260 days and full term is defined a stillbirth)

RB History (Repeat breeding)	**Yes** (if Repeat breeding encountered at least one time) , or **No** (if Repeat breeding was not encountered at least one time) (A cow is called as repeat breeding when it has failed to conceive even after three number of services, has normal estrus cycle length, no abnormality in the vaginal discharge, no palpable abnormality in the reproductive tract, has calved at least once before and less than ten year of age)
SB History (Still birth)	**Yes** (if Still birth encountered at least one time) , or **No** (if Still birth was not encountered at least one time) (Stillborn calves include full-term calves that are born dead or die in the first 24 to 48 hours after birth)
RP History (Retain placenta)	**Yes** (If placenta retains after birth at list once), or **No** (If Retain placenta was not encountered at least one time)
Herd- name	Dairy cattle herds where samples were taken and used as a random variable.

3.9. Ethical considerations

Ethical clearance certificate for this study with certificate Ref.no:VM/ERC/04/01/14/2022 was obtained from the the animal research Ethics Review Committee of the College of Veterinary Medicine and Agriculture (CVMA) at Addis Ababa University (AAU) evidencing that humble handling of the sampled animals was done to minimize unnecessary suffering during sample collection (Annex 7).

4. RESULTS

Seroprevalence of BoHV-1

The seroprevalence of BoHV-1 at animal level and herd level in the study areas are presented in table 6. Among the 369 dairy cattle tested, 77 (21%; 95% CI: 17% - 25%) were found antibody positive against BoHV-1. The TP adjusted for sensitivity and specificity of the test was 20.97% which was almost equal to AP. The herd level seroprevalence of BoHV-1 of the present study was 32% (95% CI: 24% - 42%).

Table 6: Individual animal and herd level seroprevalence

Descriptions	Animal level	Herd level
Total number	369	115
Number of Positive	77	37
Seroprevalence in percent	21%	32%
Confidence interval	17% - 25%	24% - 42%

4.1. Socio-demographic characteristics of the respondents

One hundred fifteen dairy cattle herd owners interviewed during survey, of which 69% were males and rest 31% were females. The majority of the respondents age were categorized above 46 years which accounts 60 % and the rest 35% and 3.5% were accounted by 36-45 years of age and 18-35% age, respectively. Based on education level, 31% of respondents were found between high school (9-10) and the rest 4.3%, 27%, 7.8%, 10%, and 19% of respondents were found in the group of illiterate, primary (1-8), preparatory (11-12), diploma and, first degree and above levels, respectively. However, these sociodemographic factors included into the present study were not showed significant association with herd seroprevalence of BoHV-1 ($p > 0.05$) as shown in (table 7).

Table 7:Socio-demographic characteristics of the respondents and their association with BoHV-1

Characteristic		Sampled herd(N=115)(%[1])	Seropositive(%[2])	p-value
Gender	Female	36 (31%)	13 (35%)	0.6929[3]
	Male	79 (69%)	24 (65%)	
Age	18-35 Year	6 (5.2%)	1(2.7%)	0.5937[4]
	36-45 Years	40 (35%)	15 (41%)	
	Over 46 Year	69 (60%)	21 (57%)	
Education level	Illiterate	5 (4.3%)	1 (2.7%)	0.935[4]
	Primary (1-8)	31 (27%)	11 (30%)	
	High School (9-10)	36 (31%)	11 (30%)	
	Preparatory (11-12)	9 (7.8%)	4 (11%)	
	Diploma	12 (10%)	3 (8.1%)	
	BA Degree and Above	22 (19%)	7 (19%)	

1= proportions from total number 2= proportions from number of positives

3= based on chi-square test 4= based on fisher exact test

4.2. BoHV-1 seroprevalence and associated risks factors

4.2.1. Univariate logistic mixed model

A univariate logistic mixed model were fitted to predict result (BoHV-1 seropositivity) with age, purchase status, herd size, herd type, breeding, control visitors, proximity**,** parity, foot bath, and drinking water separately (Table 8). The model included herd-name as random effect (formula: ~1 | herd-name). The models total explanatory power for age, purchase status, herd size, herd type, breeding, control visitors, proximity**,** parity, foot-bath, and drinking water was substantial (conditional R^2 = 0.74, 0.74, 0.70, 0.74, 0.66, 0.89, 0.82, 0.75, 0.76,0.70) and the part related to the fixed effects alone (marginal R^2) is of 0.11, 0.06, 0.20, 0.04, 0.18, 0.15, 0.10, 0.06, 9.01^{e-04},0.08, respectively.

Within this model: Adult cattle had a significantly higher seroprevalence (26%; 95% CI: 21–32%; P<0.001) than young cattle (3%; 95% CI: 0–7%).

Purchased cattle had significantly higher seroprevalence (34%; 95% CI: 26-42%; P=0.001) than home-bred cattle (13%; 95% CI: 9-17%).

In comparison to large herd size, small herd size had significantly lower seroprevalence (4%; 95% CI: 1-8%; P=0.009), while medium herd size had insignificantly lower seroprevalence (21%; 95% CI: 16-27%; P=0.232).

Insignificantly higher seroprevalence was observed in mixed farms (dairy with fattening) (49%; 95% CI: 33-64%; P=0.108) than in dairy cattle only (17%; 95% CI: 13-21%).

Compared to those herds using only AI for breeding, a significantly higher seroprevalence was observed in dairy herds using bulls for breeding (84%; 95% CI: 69-99%, P=0.001). Additionally, herds that used both (AI and bull) had significantly higher seroprevalence (21%; 95% CI: 15-27%; P=0.011).

Dairy farms that did not control visitors had a significantly higher seroprevalence (40 %; 95 % CI: 3-49 %; P=0.001) than dairy farms that did.

Seroprevalence was found to be significantly higher in cattle herds less than 100 meters apart (29%; 95% CI: 23-35%; P=0.006) than in cattle herds more than 100 meters apart (8%; 95% CI: 3-21%).

When compared to multiparous cows, nulliparous (3%; 95% CI: 3-8%; P=0.014) and uniparous (19%; 95% CI: 9-24%; P=0.018) cows had significantly lower seroprevalence.

Equal seroprevalence was observed on farms that had footbaths (21%; 95% CI: 3-38%; P=0.784) and no footbaths (21%; 95% CI: 17-25%) and statistically insignificant.

Individual water consumption resulted in significantly lower seroprevalence (12%; 95% CI: 8-16%; P=0.013) than communal water consumption (42%; 95% CI: 33-52%).

Table 8: Univariable mixed-effects binomial logistic regression analyses of risk factor in relation to BoHV1 exposure status

Predictors		Total # sampled (%[1])	Seropositive (%[2])	Prevalence [95%CI]	OR [95%CI]	P-value
Age	Adult	280 (76%)	74 (96%)	26% [21-32%]	15.71[3.54 – 69.63]	<0.001
	Young	89 (24%)	3 (3.9%)	3% [0-7%]	1	
Purchase Status						
	Yes	137 (37%)	47(61%)	34% [26- 42%]	5.81[2.11 – 15.98]	0.001
	No	232 (63%)	30 (39%)	13% [9-17%]	1	
Herd Size						
	Medium herd	234 (63%)	50(65%)	21% [16-27%]	0.14[0.01 – 3.45]	0.232
	Small herd	80 (22%)	3 (3.9%)	4% [1-8%]	0.01[0.00 – 0.30]	0.009
	Large herd	55 (15%)	24(31%)	44% [30-57%]	1	
Herd type						
	Mixed with fattening	43 (12%)	21(27%)	49% [33-64%]	10.11[0.60 – 169.74]	0.108
	Dairy	326 (88%)	56(73%)	17% [13-21%]	1	
Breeding						
	Both	168 (46%)	35(45%)	21% [15-27%]	5.34[1.46 – 19.50]	0.011
	Bull	25 (6.8%)	21(27%)	84% [69-99%]	136.20[7.67 – 2419.06]	0.001
	AI	176 (48%)	21(27%)	12% [7-17%]	1	
Control visitors						
	No	123 (33%)	49 (64%)	40% [31- 49%]	86.96[6.65 – 1137.08]	0.001
	Yes	246 (67%)	28(36%)	11% [7-15%]	1	
Proximity						
	Closer	225 (61%)	66(86%)	29% [23-35%]	15.45[2.15 – 110.85]	0.006
	Far	144 (39%)	11(14%)	8% [3-21%]	1	
Parity	Nulliparous	36 (9.8%)	1 (1.3%)	3% [-3- 8%]	0.05[0.01 – 0.56]	0.014
	Uniparous	98 (27%)	16 (21%)	19% [9-24%]	0.31[0.12 – 0.82]	0.018
	Multiparous	235 (64%)	60 (78%)	26% [20- 31%]	1	
Foot bath						
	Yes	24 (6.5%)	5 (6.5%)	21% [3- 38%]	1.56[0.06 – 37.82]	0.784
	No	345 (93.5%)	72 (93.5%)	21% [17- 25%]	1	
Drinking water						
	Individual	265 (72%)	33 (43%)	12% [8- 16%]	0.12[0.02 – 0.64]	0.013
	Common	104 (28%)	44 (57%)	42% [33- 52%]	1	

1= proportions from total number) 2= proportions from number of positives

4.2.2. Multivariable logistic mixed model

A logistic mixed model was fitted to predict the result (BoHV-1 seropositivity) with age, purchase status, herd size, breeding, control visitors, and proximity (formula: result ~ age + purchase status + herd size + breeding + control visitors + proximity) (Table 9). The model included herd-name as a random effect (formula: ~1 | herd-name). The model's total explanatory power is substantial (conditional R^2 = 0.85) and the part related to the fixed effects alone (marginal R^2) is 0.62. The model's intercept, corresponding to age = young, purchase status = no, herd size = large herd, breeding = AI, control visitors = yes, and proximity = far, was at 0.00 (95% CI: 0.00–0.03; p = 0.002).

Within this model, adult cattle were fourteen times more likely to increase the risk of being BoHV-1 seropositive (OR = 14.32; 95% CI: 2.53–81.5; P = 0.003) than young cattle.

Purchased cattle were four times more likely to increase the risk of being BoHV-1 seropositive (OR = 3.19; 95% CI: 0.08–132.69; P = 0.542) than homebred cattle.

When compared to large herd size, medium herd size was more likely to increase the risk of being BoHV-1 seropositive and statistically non-significant (OR = 3.19; 95% CI: 0.08–132.69; P = 0.542), and small herd size was more likely to decrease the risk of being BoHV-1 seropositive and statistically non-significant (OR = 0.02; 95% CI: 0.00–1.56; P = 0.079).

The risk of being BoHV-1 seropositive was 195 times higher in herds using bulls (OR = 195.51; 95% CI: 3.62–1056.51; P = 0.010) and five times higher in herds using both (Bull and AI) (OR = 5.68; 95% CI: 1.05–30.84; P = 0.044) than in herds using breeding AI only for breeding, and it was statistically significant.

Less than 100m distance between cattle herds were eleven times more likely to increase the risk of being BoHV-1 seropositive than beyond 100m distance between cattle herds (OR = 10.75; 95% CI: 1.47–78.30; P = 0.019), and the difference was statistically significant.

The risk of being BoHV-1 seropositive was twenty-four times higher on farms that did not control visitors (OR = 23.92; 95% CI: 1.93–296.33; P = 0.013) than on farms that did, and it was statistically significant.

Table 9: Multivariable mixed-effects binomial logistic regression analyses of risk factor in relation to BoHV1 exposure status

Predictors		OR	95% CI	P-value
Intercept		0.00	0.00 – 0.03	0.002
Age	Adult	14.32	2.53 – 81.15	0.003
	Young	1		
Purchase Status	Yes	4.15	1.36 – 12.66	0.012
	No	1		
Herd Size	Medium herd	3.19	0.08 – 132.69	0.542
	Small herd	0.02	0.00 – 1.56	0.079
	Large herd	1		
Breeding	Both	5.68	1.05 – 30.84	0.044
	Bull	195.51	3.62 – 10567.51	0.010
	AI	1		
Control visitors	No	23.92	1.93 – 296.33	0.013
	Yes			
Proximity	Closer	10.75	1.47 – 78.30	0.019
	Far	1		

AIC=230.37, BIC= 269.47, Log Likelihood=-105.18, Residual Deviance=210.37, X^2= 12.2

Hosmer Lemeshow p-value = 0.4129, AUC value =0.9827

4.3. Association of reproductive problems and seroprevalence of BoHV-1

4.3.1. Univariate logistic mixed model for selection of variables based on p-values

A univariate logistic mixed model was fitted to predict Result (dependent variables) with Abortion_History, RB History, RP History, and SB History, respectively (formula: Result ~ factor (Abortion_History), Result ~ factor (RB History), Result ~ factor (RP History) and Result ~ factor (SB History)). The model included Herd_name as random effect (formula: ~1 | Herd_name). The model's total explanatory power was substantial (conditional R^2 = 0.77, 0.74, 0.72,0.78) and the part related to the fixed effects alone (marginal R^2) is of 0.04, 0.02, 0.04, 5.73^{e-03}, respectably.

Within this model: Among the 60 dairy cattle with a history of abortion, 23 (38%; 95% CI: 26–51%) were found antibody positive against BoHV-1, while 54 (17%; 95% CI: 13-22%) of the 309 dairy cattle with no history of abortion tested positive for BoHV-1. The risk of being BoHV-1 seropositive was seven times higher in cattle with a history of abortion than in those without (OR = 7.13, 95% CI 2.13–23.86, P = 0.001) and it was statistically significant.

Among the 61 dairy cattle with a history of retained placenta, 22 (36%; 95% CI: 24–48%) were found antibody positive against BoHV-1, while 55 (18%; 95% CI: 14 –22%) of the 308 dairy cattle with no history of retained placenta tested positive for BoHV-1. The risk of being BoHV-1 seropositive was four times higher in cattle with a history of retained placenta than in those without (OR = 3.83; 95% CI: 1.27–11.56; P = 0.017) and it was statistically significant.

Among the 188 dairy cattle with a history of repeat breeders, 52 (28%; 95% CI: 21–34%) were found antibody positive against BoHV-1, while 25 (14%; 95% CI: 9 –19%) of the 181 dairy cattle with no history of repeat breeders tested positive for BoHV-1. The risk of being BoHV-1 seropositive was four times higher in cattle with a history of repeat breeders than in those without (OR = 3.80, 95% CI:1.61–9.00, P = 0.002) and it was statistically significant.

Among the 26 dairy cattle with a history of still birth, 5 (19%; 95% CI: 3–35%) were found antibody positive against BoHV-1, while 72 (21%; 95% CI: 17 –25%) of the 343 dairy cattle with no history of still birth tested positive for BoHV-1. The risk of being BoHV-1 seropositive

was three times higher in cattle with a history of still birth than in those without (OR = 3.13, 95% CI: 0.44–21.97, P = 0.252) and it was statistically insignificant (Table 10).

Table 10:Univariable mixed-effects binomial logistic regression analyses of reproductive disorders in relation to BoHV1 exposure status and their prevalence

Predictor's		Total # sampled (%[1])	Seropositive (%[2])	Prevalence [95%CI]	OR [95%CI]	P-value
Abortion History	Yes	60 (16%)	23 (30%)	38% [26 –51%]	7.13 [2.13 – 23.86]	0.001
	No	309 (84%)	54 (70%)	17% [13 –22%]	1	
RP History	Yes	61 (17%)	22 (29%)	36% [24 – 48%]	3.83 [1.27 – 11.56]	0.017
	No	308 (83%)	55 (71%)	18% [14 –22%]	1	
RB History	Yes	188 (51%)	52 (49%)	28% [21– 34%]	3.80 [1.61 – 9.00]	0.002
	No	181 (49%)	25 (51%)	14% [9 –19%]	1	
SB History	Yes	26 (7.0%)	5 (6.5%)	19% [3–35%]	3.13 [0.44 – 21.97]	0.252
	No	343 (93%)	72 (93.5%)	21% [17–25%]	1	

1= proportions from total number) 2= proportions from number of positives

4.3.2. Multivariable a logistic mixed model

Multivariable a logistic mixed model were fitted to predict the association of seropositivity of BoHV-1 with Abortion History, RB_History and RP_History (formula: Result ~ factor (Abortion_History) + factor (RB_History) + factor (RP_History)). The model included Herd_name as random effect (formula: ~1 | Herd_name). The model's total explanatory power is substantial (conditional $R^2 = 0.76$) and the part related to the fixed effects alone (marginal R^2) is of 0.09. The model's intercept, corresponding to Abortion_History = no, RB_History = no and RP_History = no, was (OR=0.01; 95% CI: 0.00 – 0.08; p < .001).

Within this model: The risk of being BoHV-1 seropositive was seven times higher in cows with a history of abortion than in those without (OR = 6.69; 95% CI: 1.97–22.76; P = 0.002) and it was statistically significant.

The risk of being BoHV-1 seropositive was three times higher in cattle with a history of retained placenta than in those without (OR = 3.26; 95% CI:1.32–8.07; P = 0.010) and it was statistically significant.

The risk of being BoHV-1 seropositive was four times higher in cattle with a history of repeat breeders than in those without (OR = 3.64; 95% CI:1.08–12.18; P = 0.036) and it was statistically significant (Table 11).

Table 11:Association of reproductive problems with the seroprevalence of BoHV-1

Predictors		OR	CI [95%CI]	p-value
Intercept		0.01	0.00 – 0.08	<0.001
Abortion History	Yes	6.69	1.97 – 22.76	0.002
	No	1		
RP History	Yes	3.26	1.32 – 8.07	0.010
	No	1		
RB History	Yes	3.64	1.08 – 12.18	0.036
	No	1		

5. DISCUSSION

BoHV-1 is not a fatal disease, but it can result in serious financial losses due to abortion, loss of body condition, loss of milk yield, loss of new born calves, temporary failure of conception, insufficient feed conversion, and cost of treatment. It is also one of the most common causes of BRDC, which is a multifactorial disease caused by multiple deleterious management practices and interactions among viral and bacterial pathogen (Majumder *et al*., 2019).

In this study, the seroprevalence of BoHV-1 antibodies was 21% (95% CI: 17-25%). The presence of BoHV-1 infection has been confirmed through seroprevalence of BoHV-1 antibodies among local and exotic breeds of cattle reared in different parts of Ethiopia. The seroprevalence ranges from 5.9% in Sebeta (Sibhat *et al*., 2018) to 81% Ghibe (river valley between Shoa and Jimma) (Bekele *et al*., 1989). Comparing this result with previously conducted research in Ethiopia, the seroprevalence result was lower than Bekele *et al*., (1989) who studied in the local breed (Zebu) of cattle in Ghibe and Gobe of central Ethiopia; Sibhat *et al*. (2018) who studied in the exotic and cross dairy cattle breeds of central, western, and southern Ethiopia; Wedajo *et al*. (2021) who studied the exotic and local breeds of cattle in Kombolcha and Dessie districts of north Ethiopia; and Zewde *et al*. (2021) who studied in indigenous zebus (*bos indicus*) cattle in north western part of Ethiopia, and their overall seroprevalence were 67%, 41.0%, 25.6%, and 77.6%, respectively. These antibody response induced against the virus were from natural infection because vaccination against the virus was not practiced in Ethiopia. Similarly, the seroprevalence of current studies was lower than that of other countries, such as South Africa (Njiro *et al*., 2011), Egypt (Hekal *et al*., 2019), Mexico (Segura-Correa *et al*., 2016), Colombia (León *et al*., 2019), and the UK (Williams and Winden, 2014), with seroprevalence of 74.47%, 93.75%, 64.4%, 94.7%, and 69.2%, respectively. However, the seroprevalence of this study was higher than other previous reports, such as in Sudan (8.75%) (Elhassan *et al*., 2015), Kenya (17.4%) (Kipyego *et al*., 2020), Cameroon (16.1 ± 1.6%), and Algeria (14.16%) (Kaddour *et al*., 2019).

As mentioned above, the seroprevalence of BoHV-1 in the current study was lower as compared with Bekele *et al*. (1989) and Zewde *et al*. (2021), who studied the BoHV-1 seroprevalence in local breeds of cattle managed extensively and semi-intensively. This could have been due to differences in the breeds of cattle and the production system because open grazing and cattle grazed at other farms are a risk factor for BoHV–1 infection (Van Schaik *et al*., 2002; Gonzalez-Garcia *et al*., 2009). Different factors such as dairy herd management, sample size, number of herd sizes involved, disease control system, type of breeding, animal age (Ackermann and Engels, 2006), and bias (Dohoo *et al*., 2003) could explain the differences of seroprevalences.

Although the seroprevalence of BoHV-1 antibody is lower than in prior studies, this does not undermine the significance of the virus. This is for different reasons. First, the virus has unique characteristics of establishing latency in the trigeminal or sacral ganglia (Biswas *et al*., 2013). When cattle get infected with the virus, they become lifelong carriers and result in a possible source of infection for vulnerable animals when the virus is reactivated by different stress factors. Once the virus has reactivated, it spreads widely on the dairy farm to cause secondary infections which is called reproduction ratio. BoHV-1 has a reproduction ratio (R_0) of greater than nine for calves and greater than seven for adult cattle (More *et al*., 2017). The other important point that makes this seroprevalence significant is that the virus is not considered a serious disease-causing agent at the country level. As a result, there is no prevention and control strategy implemented by the government to combat the virus. Besides, the farmers are not aware of the diseases due to unnoticeable clinical signs. All of these contributes to the virus's ease of propagation.

The final model of this study revealed that a number of risk factors were associated with seroprevalence of BHV-1 antibody, such as age of the cattle, purchase status of the cattle, controlling visitors, proximity of herds, and breeding type.

The prevalence of the virus was found to be higher in adult aged cattle (26%; 95% CI: 21–32%) than in the young age groups (3%; 95% CI: 0-7%). Age was a risk factor in this research, and it was found to be significantly associated with BoHV-1 infection. The age related seroprevalence of BoHV-1 infection indicated that the incidence of infection increased as the age increases.

Adult aged cattle were fourteen times more likely to increase the risk of being BoHV-1 seropositive than young aged cattle and the difference was statistically significant (OR = 14.32; 95% CI: 2.53–81.5%; P = 0.003). This result is consistent with other studies (Boelaert *et al*., 2005; León *et al*., 2019; Kipyego *et al*., 2020). Age, on the other hand, does not appear to be linked to BoHV-1 seropositivity (Hussein *et al*., 2005; O'Grady *et al*., 2008). The association between BoHV-1 and adult age was expected. The rationale for the link is that the chances of being exposed increase dramatically with age. In adult cattle, exposure potential may give more vulnerable contacts for an infectious disease than in younger cattle. Breeding and contact with animals and humans are the most important factors that increase the exposure. Insemination is an easy way to deliver the virus to the heifer (Nuotio *et al*., 2007).

In this study, the introduction of new cattle through purchase was found to be a risk factor for BoHV-1 seropositivity. The seroprevalence of the BoHV-1 infection was significantly associated with the purchase of the cattle. Purchased cattle (34%; 95% CI: 29-42%) were four times more likely to increase the risk of being BoHV-1 seropositive than homebred cattle (13%; 95% CI: 9-17%) and it was statistically significant (OR = 4.15; 95% CI: 1.36–12.66; P = 0.012). Similar trends were reported in studies by Boelaert *et al*. (2005), Williams and Winden. (2014), and Zewde *et al*. (2021). However, the result was different from O'Grady *et al*. (2008). This study documented a large number of animal movements, as 37 percent of the animals examined were purchased cattle for expansion and stock replacement. Reproductive problems, such as repeated breeding, are one of the most important factors that influence calving interval, open days (calving to conception), first service conception rate, and calving to first service interval, all of which influence the owner's decision to purchase the cattle to offset the trend. When latently infected cattle are introduced into such herds, the virus will most likely reactivate as a result of stress, resulting in a high seroprevalence.

The seroprevalence of BoHV-1 was not associated with herd size in this study, and there was no statistically significant difference between the herd sizes. When compared with large herd size (44%; 95% CI: 30-57%), medium herd size (21%; 95% CI: 16-27%) was more likely to increase the risk of being BoHV-1 seropositive but statistically non-significant (OR = 3.19; 95% CI: 0.08–132.69; P = 0.542), and small herd size (4%; 95% CI: 1-8%) was more likely to

decrease the risk of being BoHV-1 seropositive but statistically non-significant (OR = 0.02, 95% CI: 0.00–1.56, P = 0.079). As a result, the size of the herd was not discovered to be a risk factor for BoHV-1 seropositivity. Studies by Van Schaik *et al*. (1998) and O'Grady *et al*. (2008) revealed similar results, but the number of animals considered in each herd was not the same. However, herd size and BoHV-1 seropositivity of cattle have been linked in several studies. Boelaert *et al*., (2005), Williams and Winden. (2014), and Segura-Correa *et al*. (2016) found that seropositivity increased with herd size increment, whereas Van Wuijckhuise *et al*. (1998) found that seropositivity decreased with herd size increment.

In this study, herd size was determined to be significant during univariate analysis but not in the final model. This could be due to confounding with other risk variables related to herd size. This research also closely linked herd size to a number of other risk factors. The importance of herd size for BoHV-1 Seropositivity is influenced by the proximity of herds. Because of the ease with which the disease can be spread by sharing materials, visiting neighborhood youngsters, cattle interaction with surrounding dairy herds, and other animal connections on neighboring farms, proximity may play a role in disease transmission. The number of customers who came to buy dairy products, as well as professional visitors like veterinarians and AI technicians, was largely determined by the number of cows on the farm. As a result, several risk factors use 'herd size' as a cluster variable.

The seroprevalence of BoHV-1 was associated with controlling visitors in the herd. In this study, the risk of being BoHV-1 seropositive was twenty-three times higher (OR =23.92; % CI: 1.93–296.33%; P=0.013) on farms that did not control visitors (40%; 95% CI: 31-49%) than on farms that did (11%; 95% CI: 7-15%) and it was statistically significant. Similar trends were found by Van Schaik *et al*. (1998) and Van Schaik *et al*. (2001), indicted that not only direct animal contacts are important but also visitors and workers on the farm and the infection pressure from other farms in the neighborhood are important. Visitors, including professionals (veterinarians, artificial inseminators), infrequent visitors (relatives, cattle traders, or a temporary worker who has also worked on other farms), and dairy product customers, are important sources of indirect transmission of virus. In the study areas, most dairy herds do not have a special place to sell

their dairy products; rather, they sell them on the spot at the dairy farm. These all causes indirect transmission of infection to the farm from other farms resulting increments of seropositivity.

The seroprevalence of BoHV-1 was associated with the proximity of the herd. It was statistically significant that less than 100m distance between cattle herds (29%; 95% CI: 23–35%) was ten times more likely to increase the risk of being BoHV-1 seropositive (OR = 10.75; 95% CI: 1.47–78.30; P =0.019) than greater than 100m distance between cattle herds (8%; 95% CI: 3-21%). Similar trends were found by studies by Van Schaik *et al*. (1998). The distance between the farms is a factor for the farmers because they may contribute to the transmission of the disease easily by sharing materials, having neighbor farmers' children visit, contact of cattle with neighboring dairy herd, or other animal contacts like dogs, cats, rats, *etc.* of neighboring farms. So, the probability of transmitting the virus may be very high.

In this study, the seroprevalence of the BoHV-1 infection was significantly associated with the breeding of the cattle. The risk of being BoHV-1 seropositive was 195 times higher in herds using bulls (84%; 95% CI: 36-99%) (OR = 195.5, 95% CI: 3.62–1056.51, P = 0.010) and five times higher in herds using both (Bull and AI) (21%; 95% CI: 15-27%) (OR = 5.68; 95% CI: 1.05–30.84%; P = 0.044) than in herds using AI only (12%; 95% CI: 7-17%) for breeding and it was statistically significant. The presence of a bull in a dairy herd for breeding purposes was found to be significantly increased risk for BoHV-1 seropositivity, which is consistent with other study by Williams and Winden. (2014).

There is still a high proportion of dairy farms in Ethiopia that rely on the use of a bull for breeding cattle even though AI, routinely examined for diseases including BoHV-1, is produced. This is for different reasons. First, the availability and accessibility of AI on a timely basis was a problem. Second, some artificial inseminators are inefficient and have limited technical skills, resulting in an increase in service per consumption. As a result, the dairy herd owner was obliged to either raise a bull from home or purchase one from other herds. Frequently, bulls were purchased without evaluating the health status, particularly for BoHV-1. This increases the chances of the BoHV-1 infection during mating and contact. Purchased bulls are more likely to be seropositive for BoHV-1 than homebred bulls (Martinez-Ibeas *et al*., 2015). Another relevant

scenario is that the herd owner/manager may not have their own bull due to space constraints and the costs that are incurred for bull management. As a result, they choose to employ the bull of a neighbor. As a result, the likelihood of transmission is greatly increased.

In this study, cattle having a history of abortion were seven times associated than (OR = 6.9; 95% CI: 1.97–22.76; P = 0.002) those cattle that did not have a history of abortion. Similarly, cattle having a history of retained placenta were three times more associated than (OR = 6.9; 95% CI: 1.97–22.76; P = 0.002) those cattle that did not have a history of retained placenta. Seroprevalence was found to be significantly higher in cattle having a history of abortion (38%; 95% CI: 26–51%) and a history of retained fetal membranes (36%; 95% CI: 24–48%) than in cattle not having a history of abortion (17%; 95% CI: 13–22%) and retained fetal membranes (18%; 95% CI: 14–22%), respectively. The majority of cases of fetal membrane retention are linked to abortion (Patel and Parmar, 2016). The association of abortion and retention of the placenta with the seropositivity of BoHV-1 was comparable to that seen in studies by Sibhat *et al*. (2018) and Wedajo *et al*. (2021). However, there was no observed evidence of an association between BoHV-1 serological status and reproductive abnormalities in Canadian beef herds (Waldner, 2005). BoHV-1 is the most common infectious agent in cattle, and it's associated with abortions (Jones, 2019) and more typically linked to late-term abortions and infertility (Yamini *et al*., 2004). Miller *et al*. (1991) indicated that heifers given BoHV-1 subtype aborted between 17 and 85 days after inoculation.

This research revealed a significant association between serological evidence of BoHV-1 infection and history repeated breeding. The risk of being BoHV-1 seropositive was four times higher in cattle with a history of repeat breeders than in those without a history of repeat breeders (OR = 3.64; 95% CI:1.08–12.18; P = 0.036) and it was statistically significant. This finding is consistent with prior research undertaken in Ethiopia by Wedajo *et al*. (2021) and in Ireland's commercial dairy herds by Geraghty and O'Grady. (2012). Repeat breeding is caused by fertilization failure and early embryonic mortality (Gustafsson and Emanuelson, 2002). Increases in service for conception are caused by BoHV-1 infected semen. This is due to its negative impact on the ovaries, which disturbs the estrus cycle and the virus has been associated with early embryonic mortality (Graham, 2013).

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that the seroprevalence of BoHV-1 at animal and herd level was high in dairy cattle of Addis Ababa, Ethiopia. Obviously, vaccinations of BoHV-1 are not available in Ethiopia, the seroprevalence was due to naturally circulating virus. Seroprevalence studies have an important role to play in highlighting disease control and eradication schemes. Older age, purchasing animals, breeding the cattle with bull, closer herds, and allowing of visitor to the herd significantly increases the risk of BoHV-1 seropositivity. Similarly, the virus was associated with reproductive problems such as repeated breeding, abortion and retained placenta in the study. This virus has a negative impact on production, productivity, and international trade, resulting in significant economic losses in the dairy sector on a national level. The latency nature of the BoHV-1 makes proper detection mandatory to set up an effective control program in the country. Therefore, based on the present findings the following recommendations are worth mentioning:

- Those risk factors should be used as a reference guide to educate, create awareness and behavioral change of dairy farmers including veterinarians and concerning bodies.
- Artificial insemination method is recommended and if bull are used for breeding, it should be free from BoHV-1 and not be shared with other dairy herds.
- Planned biosecurity measures should be a primarily considered due to the latency nature of the virus.
- BoHV-1 should be given emphasis as other reproductive disease causing agents during prevention and control strategy of the country.
- Further isolation and characterization of BoHV-1 should be needed to introduce strategic intervention.
- Vaccine development in the country should be considered.

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8. ANNEXES

Annex 1: Procedures of Blood collection

- Hygiene and Personal Protective Equipment (PPE)
  - Ensure that adequate PPE is worn to protect the handler from unintentional harm or exposure to blood and other body fluids, such as: Scrubs or coveralls, Steel-toed shoes or boots, Disposable gloves, other PPE as required by protocol/facility
  - Hands should be washed and/or gloves changed between animals; if vacutainer hubs used, ideally, they should be disinfected between barns or other animal holding areas.
  - Promptly dispose of used sharps in the provided leak-proof, puncture resistant sharps container

Supply List

- ✓ Vacutainer tubes
- ✓ Vacationer needles and needle holder
- ✓ Restraint
- ✓ Clippers (optional)
- ✓ Antiseptic
- ✓ Gauze

- Blood collection in cattle from **jugular vein**

Cattle Restraint

Handlers should be vigilant at all times so as to avoid injury to animals or themselves.

- Head butting and arc of swing
- Being caught between animal and solid structure, (e.g., a wall, fence, chute)
- Avoid being kicked or stepped on:
  - Front foot pawing
  - Hind foot swings forward and back out to the side (“cow kick”)

Detailed Procedure

- ✓ Restrain animal with head elevated and jugular vein exposed.
- ✓ Swipe with antiseptic gauze to remove superficial dirt and debris. This may also assist in visualizing raised vein.
- ✓ Occlude jugular vein by applying pressure at the base of the jugular groove and visualize raised vein
- ✓ With bevel up, insert needle firmly into skin and into vein at 20° angle
- ✓ If using vacutainer, once needle inserted, stabilize needle and push the vacutainer tube into hub. If you have hit the vein, blood will flow freely into tube (**NOTE**: Do not pull needle out of vein with vacutainer tube still attached as this will release vacuum in vacutainer)
- ✓ If you have missed the vein, carefully reposition needle, with vacutainer attached, until vessel penetrated. Vessel is fairly deep and may roll away from needle. Typically no more than two to three attempts should be made at a time to minimize distress to the animal and potential damage to the vein.
- ✓ Once collection complete, remove vacutainer tube, and then, applying pressure over injection site, remove needle.
- ✓ Dispose of needle in approved Sharps container.
- ✓ In order to ensure adequate hemostasis, apply pressure with gauze for 30 to 60 seconds.

- Blood collection in cattle from **coccygeal vein**
  - ✓ Restrain animal.
  - ✓ Raise the tail vertically until it is horizontal to the ground.
  - ✓ Locate the groove lying in the ventral midline of the tail.
  - ✓ Swab the site with antiseptic.
  - ✓ Midway along the body of a coccygeal vertebra, insert the needle perpendicularly to the surface of the skin to a depth of a few millimeters.
  - ✓ Withdraw blood sample and remove needle.
  - ✓ Dispose of needle in approved sharps container.
  - ✓ In order to ensure adequate hemostasis, apply pressure with gauze for 30 to 60 seconds

- Potential Adverse Effects, Mitigation, or Treatment
  - Hematoma or thrombus
    - ✓ Enter vessel at an angle of 30 degrees or less
    - ✓ Use a gauge of needle smaller than the vein
    - ✓ Apply pressure until bleeding has stopped (1+ minutes)
  - Pain at blood collection site
    - ✓ Use a needle of smaller gauge than the vein
    - ✓ Practice on vein models prior to live animal
  - Infection at blood collection site
    - ✓ Use sterile single-use devices only
    - ✓ Clean work surfaces with disinfectant

Annex 2: Consent form

The objective of the questionnaire is to identify the risk factor and the reproductive disorder which are associated with BoHV-1. The information you provide is totally required for research purpose and shall be kept strictly secrete and confidential. Please feel free to provide every information concerning the questionnaire.

Thank you in advance for your kind cooperation.

I confirm that I have read and understand all the information written above. My participation in this survey s voluntary and I am willing to share necessary information for this survey

Name of participant ----------

Signature ----------

Date----------

Annex 3:Questionnaire format used to determine risk factors and association

Survey Questionnaire

Remember: Put √ mark on the box you choose.

1. PERSONAL DATA OF THE RESPONDENTS

1.1. Full Name ---------- Sex--------- Phone no----------

1.2. Region---------- Kifle ketema ---------- Wereda----------

1.3. Location of the Herd in GPS Long---------- Lat---------- Altitude-------

1.4. Age Breakup: A.18-35 Year ☐ B. 36-45 Years☐ C. Over 46 Year☐

1.5. Education level: A. Illiterate ☐ B. Primary (1-8) ☐ C. High School (9-10) ☐

D. Preparatory(11-12) ☐ E. Diploma ☐ F. BA Degree and Above ☐

2. INDIVIDUAL LEVEL INFORMATION

2.1. Cattle Id---------- Cattle name---------- Sex------ Heart Girth------

2.2. Breed---------- Exotic blood Level---------- Birth date/Age ---------- Parity----------

2.3. Purchased Yes ☐ No ☐

2.4. Clinical signs

Nasal discharge ☐ Dyspnea ☐ Ocular discharge ☐ Reddened and shallow erosions on the mucosa of the nares ☐ Shallow erosions of the oral mucosa ☐ Fever ☐ Diarrhea ☐

Repeated urination ☐ Raising of the tail ☐ Hyperaemic or edematous of the vulva and vagina ☐ Small white to red ulcers/pustules of the vulva and vagina ☐

2.5. Reproductive disorder history Yes ☐ No ☐ If yes

2.5.1. Abortion history? Yes☐ No ☐ Month of abortion(if) -------- frequency----

2.5.2. Still birth history? Yes☐ No ☐ frequency--------

2.5.3. Retained placenta history? Yes☐ No ☐ frequency--------

2.5.4. Uterine problem history? Yes☐ No ☐ frequency--------

2.5.5. Repeat breeder history (>3 times)? Yes☐ No ☐

2.5.6. Defective calf history Yes☐ No ☐

2.5.7. Age at first calving in years---------- Calving interval in days------- Average open days---

3. HERD LEVEL INFORMATION

3.1. Herd type Dairy☐ Mixed with fattening ☐ Number of Cattle/Herd size---------

3.2. Production system Intensive ☐ Extensive☐

3.3. Proximity to other cattle herd Closer ☐ Far ☐

3.4. Ventilation system Good ☐ Fair ☐ Poor ☐

3.5. Farm hygiene? Good ☐ Fair ☐ Poor ☐

3.6. Breeding method AI ☐ Bull ☐ Both ☐

3.7. Water drinking system Per individual ☐ common ☐

3.8. Do you control visitors? Yes ☐ No ☐

3.9. Professionals wearing protective clothes when handling cattle Yes☐ No☐

3.10. Foot bath for visitors Yes ☐ No ☐

Thank you very much for your cooperation

Annex 4: IBRgB antibody test Procedures

- Descriptions and principles

IDEXX IBR gB X3 is an enzyme immunoassay designed to detect the presence of antibodies to IBR/IPV in individual bovine serum, plasma and milk samples. In addition, antibody responses induced by vaccines which contain the glycogen B(gB) of BHV-1 are detected by this ELISA. A microtitration format has been configured by immobilizing IBR viral antigens on the plate. Upon incubation of the test sample in the antigen- coated wall, antibody specific to IBR forms a complex with the immobilized viral antigens. After washing away unbound materials from the wells, a gB-specific monoclonal antibody Horseradish Peroxidase Conjugate is added which will not bind to BHV1 antigen when the antigenic determinant, recognized by the monoclonal antibody, has been occupied(blocked) previously by antibodies in the test sample. Next, unbound conjugate is washed away and a substrate solution is added. In the presence of enzyme, substrate is converted into a product which reacts with the chromogen to generate a blue color. Upon addition of stop solution, a yellow color is generated. The absorbance at a single wavelength of 450nm [A(450)] or dual wavelength of 450nm and 650nm[A(450/650)] is

measured using spectrophotometer. The blocking percentage of the sample is calculated by using the absorbance [A (450)] OR [A (450/650)] obtained with the test sample and the negative control containing no specific antibodies.

- Test procedure

All reagents must be allowed to come to 18-26^{o}c before use. Reagents should be mixed by gentle inverting and swirling.

1. Obtain coated plate(s) and record the sample position. If using partial plates, remove only those wells sufficient for samples to be tested. Place the remaining wells, along with the desiccant, in the extra zip lock bag provided and return to 2-8^{o}c.

2. Add 50µL of reconstituted wash solution to each well.

3. Dispense 50 µL of Negative control (NC) into duplicate wells.

4. Dispense 50 µL of positive control (PC) into duplicate wells.

5. Dispense 50 µL of samples into the remaining wells.

6. Mix the content of the microwells by gently tapping the plate or use a microplate shaker.

7. Cover the wells and incubate for 2 hours ($\pm$5 min.) at 37^{o}C ($\pm$3^{o}C) or overnight (12-18hours) at 2-8^{o}C. With either option, plates should be hermetically sealed or covered and incubated in a humid chamber.

8. Remove the solution and wash each well with approximately 300 µL of wash solution 5 times. Avoid plate drying between plate washing and prior to the addition of the next reagent. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

9. Dispense 100µL of conjugate into each well.

10. Incubate for 1 hour ($\pm$5 min.) at 18-26 ^{o}C.

11. Remove the solution and wash each well with approximately 300 µL of wash solution 5 times. Avoid plate drying between plate washing and prior to the addition of the next reagent. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

12. Dispense 100µL of TMB substrate N.12 into each well.

13. Incubate for 10 minutes (±1min) at 18-26°C away from direct light.

14. Dispense 100 µL of stop solution N.3 into each well

15. Measure and record the absorbance of the samples and controls at 450nm or using a dual wavelength of 450 nm and 650nm.

16. Calculations

Controls

$$\text{NC } \bar{X} = \frac{\text{NC1 A(450)+NC2 A(450)}}{2} \qquad \text{PC } \bar{X} = \frac{\text{PC1 A(450)+PC2 A(450)}}{2}$$

Validity criteria

NC $\bar{X} \geq 0.500$ PC $\bar{X}$ Blocking % > 80

For invalid assays, techniques may be suspect and the assay should be repeated following a thorough review of the package insert.

Samples

$$\text{Blocking \%} = 100\ x\ \frac{NC\ \bar{X}\ \text{A(450)} - \text{sample A(450)}}{NC\ \bar{X}\ A(450)}$$

The presence or absence of antibody to IBR-gB is determined by blocking percentage for each sample.

17. Interpretation:

Negative	Suspect	Positive
Blocking %< 45	45≤ Blocking % < 55	Blocking % ≥ 55

Note: IDEXX has instrument and software systems available which calculate means and % value and provide data summaries

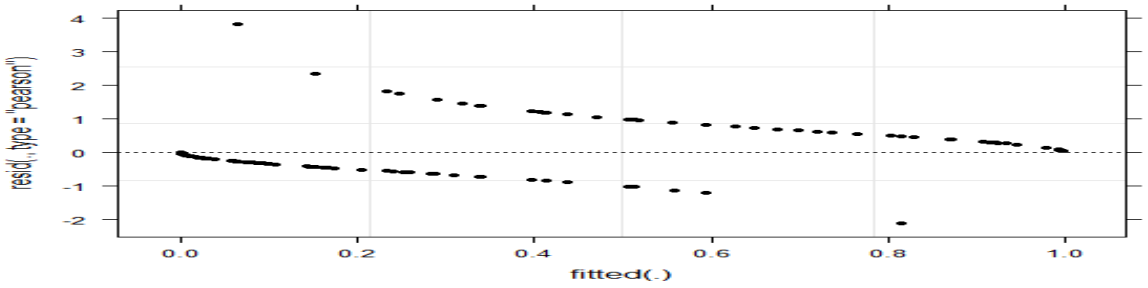
Annex 5: Manual step-wise step-up, forward elimination procedure

	Model	Formula
1	m1.glmer	Result~ (1|Herd_name) +Age
2	m2.glmer	Result~ (1|Herd_name) +Age+ Purchase Status
3	m3.glmer	Result~ (1|Herd_name) +Age+ Purchase Status + Herd Size
4	m4.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Herd Type
5	m5.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size +Breeding
6	m6.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Breeding+ Control Visitors
7	m7.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Breeding+ Control Visitors+ Proximity
8	m8.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Breeding+ Control Visitors+ Proximity+ Drinking Water
9	m9.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Breeding+ Control Visitors+ Proximity+ Parity
10	m10.glmer	Result~ (1|Herd_name) +Age+ Purchase Status+ Herd Size+ Breeding+ Control Visitors+ Proximity+ Footbath
11	m11.glmer	Result~ (1|Herd_name) +Age+ Purchase Status +Herd Size+ Breeding+ Control Visitors+ Proximity+ Age: Purchase Status

	Term Added	Compared to	DF	AIC	BIC	Log Likelihood	Residual Deviance	X2	X2DF	p-value	Significance
1	1+Age	m0.glmer	3	288.06	299.79	-141.03	282.06	19.99	1	1e-05	p < .001***
2	Purchase Status	m1.glmer	4	282.18	297.83	-137.09	274.18	7.87	1	0.00502	p < .01 **
3	Herd Size	m2.glmer	6	268.14	291.61	-128.07	256.14	18.04	2	0.00012	p < .001***
4	Herd Type	m3.glmer	7	268.76	296.13	-127.38	254.76	1.39	1	0.23907	n.s.
5	Breeding	m3.glmer	8	261.77	293.06	-122.89	245.77	10.37	2	0.00559	p < .01 **
6	Control Visitors	m5.glmer	9	240.57	275.76	-111.28	222.57	23.2	1	0	p < .001**
7	Proximity	m6.glmer	10	**230.37**	**269.47**	**-105.18**	**210.37**	**12.2**	1	**0.00048**	**p < .001*** **
8	Drinking Water	m7.glmer	11	230.48	273.5	-104.24	208.48	1.89	1	0.1692	n.s.
9	Parity	m7.glmer	12	230.12	277.04	-103.06	206.12	1.41	2	0.49405	n.s.
10	Footbath	m7.glmer	11	229.26	272.27	-103.63	207.26	0.27	1	0.60342	n.s.
11	Age: Purchase Status	m7.glmer	11	229.47	272.49	-103.74	207.47	0.05	1	0.81521	n.s

Annex 6: Model diagnosis for final model

- Model diagnostics by generating a diagnostic that plots the predicted values against the residuals.



- Hosmer-Lemeshow Goodness of fit

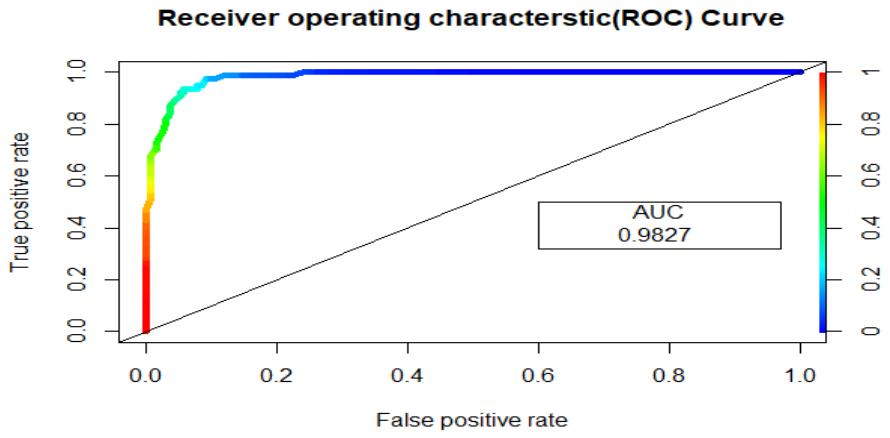
Tells us how well the model fits depending on the difference between the model and the observed data. Hosmer and Lemeshow goodness of fit (GOF) test

X-squared = 8.2134, df = 8, p-value = 0.4129

Interpretation: our model appears fit because we have a insignificant difference between the model and the observed data (*i.e*. the p-value is greater than 0.05)

- Receiver Operating Characteristic (ROC) curve

Both the AUC value (0.9827) and the ROC curve indicate a relatively good fit



Annex 7: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/01/14/2022

Name of Applicant: **Daniel Demissie (DVM, MSc fellow)**

Address: Department of clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Sero-prevalence and risk factors of bovine herpes virus-1 in dairy cattle herds of Addis Ababa, Ethiopia*

Date of application:	**November, 2021**
Nature of the project:	**Mildly invasive (little stress)**
Target animal species:	**Dairy cattle**
Number of animals involved:	**369**
Study area:	**Addis Ababa, Ethiopia**

Minutes No. and date of review: **VM/ERC/01/14/022, 22/02/2022**

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

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

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

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

መልሱን በሚጽፉልን ጊዜ እባክዎን የኛን ደብዳቤ ቁጥር ይጥቀሱልን
Please quote Our Ref. No. When replying

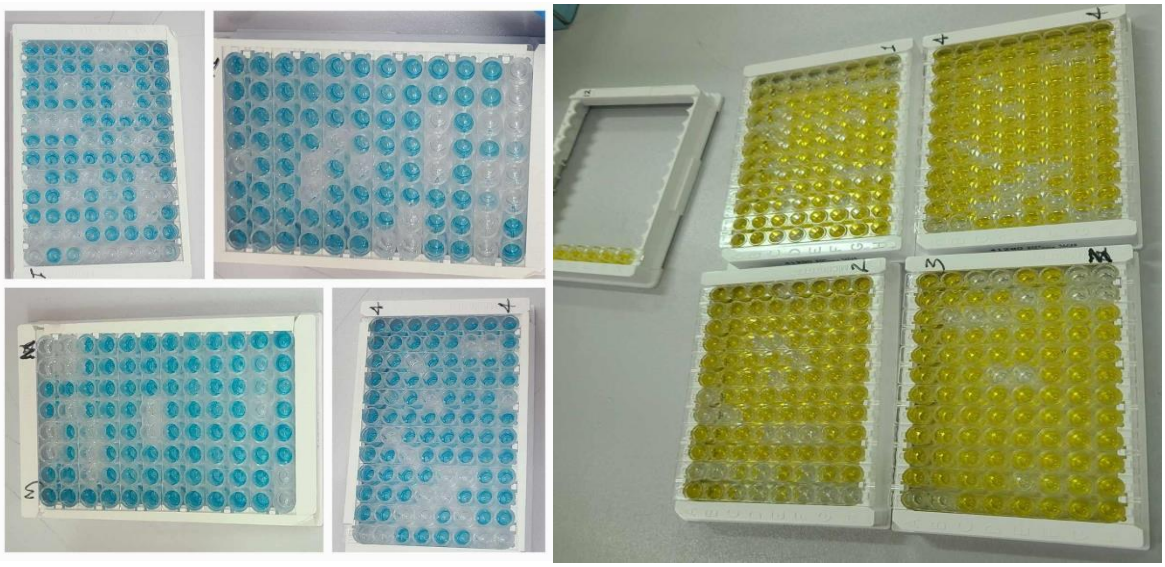
ፋክስ} ስልክ } ፖ.ሣ.ቁ ቢሾፍቱ፤ ኢትዮጵያ
Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34 Bishoftu, Ethiopia

Annex 8: Pictures during blood sample collection

A. Blood sample collection from coccygeal vein

Annex 9: Pictures of laboratory process and result

A. Laboratory work



B. Laboratory result

Annex 10: Pictures of different dairy farms

A. Good hygiene and good ventilation

B. Poor hygiene and poor ventilation