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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

SEROEPIDEMIOLOGY OF INFECTIOUS BOVINE RHINOTRACHEITIS AND BOVINE BRUCELLOSIS, AND MAJOR REPRODUCTIVE PROBLEMS IN COMMERCIAL AND SMALLHOLDER DAIRY FARMS IN NORTH SHEWA, CENTRAL HIGHLANDS OF ETHIOPIA

MVSc THESIS

BY: AWEKE ENGDAWORK

DEPARTMENT OF CLINICAL STUDIES

MASTERS OF SCIENCE IN VETERINARY EPIDEMIOLOGY

JUNE, 2023

BISHOFTU, ETHIOPIA

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BY: AWEKE ENGDAWORK

A Thesis Submitted to Addis Ababa University, College of Veterinary Medicine and Agriculture in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Science in Veterinary Epidemiology

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Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As MVSc research advisors, we hereby confirm that we have read and reviewed the Thesis written under our supervision by **Aweke Engdawork,** entitled "**Seroepidemiology of Infectious Bovine Rhinotracheitis and Bovine Brucellosis, and Major Reproductive Problems in Commercial and Smallholder Dairy Farms in North Shewa, Central Highlands of Ethiopia**". We recommend that this research can be submitted as fulfilling the MVSc Thesis requirement.

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STATEMENT OF THE AUTHOR

First, I declare that this thesis research is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MVSc degree in Veterinary Epidemiology at Addis Ababa University, College of Veterinary Medicine and Agriculture. The thesis is stored in the University/College Library to be made available to borrowers in accordance with the rules of the library. I further solemnly declare that this thesis has not been submitted to any other institution anywhere for the purpose of receiving any academic degree, diploma or certificate.

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LIST OF ABBREVIATIONS

AHI	Animal Health Institute
AI	Artificial Insemination
BAPA	Buffered Acidified Plate Antigen
BoHV-1	Bovine Herpes Virus-1
CFT	Complement Fixation Test
CSA	Central Statistical Agency
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FPA	Fluorescence Polarization Assay
gE	glycoprotein-E
HRP	Horseradish Peroxidase
IBR	Infectious Bovine Rhinotracheitis
IPB	Infectious Pustular Balanoposthitis
IPV	Infectious Pustular Vulvovaginitis
KAP	Knowledge Attitude and Practices
LPS	Lipopolysaccharide
MHD	Minimum Hemolytic Dose
OD	Optical Density
OIE	Office International des Epizooties
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RB	Repeat Breeding
RBPT	Rose Bengal Plate Test
RFM	Retained Fetal Membrane
SRBC	Sheep Red Blood Cell
VNT	Virus Neutralization Test
WHO	World Health Organization

ABSTRACT

A cross-sectional study was conducted from November 2022 to May 2023 to determine the seroprevalence of infectious bovine rhinotracheitis and bovine brucellosis, and to identify the major reproductive problems and potential risk factors in commercial and smallholder dairy farms in North Shewa, the central highlands of Ethiopia. Stratified random sampling technique was employed to sample animals from the respective farm types. A total of 511 blood samples were collected from crossbred and local cattle from 142 herds. The serological investigations were conducted using competitive gE-ELISA for IBR, RBPT and CFT for bovine brucellosis. The overall seroprevalence of IBR was 61.84% (95% CI: 57.53-65.97%) at animal level and 85.21% (95% CI: 78.28-90.21%) at the herd level; while the prevalence of bovine brucellosis was 0.98% (95% CI: 0.41-2.34%) at animal level and 3.52% (95% CI: 1.46-8.26%) in the herds based on combined RBPT and CFT tests. The univariable logistic regression analysis revealed that age, breed, parity, history of abortion, retained fetal membrane and ocular/nasal discharges were significantly associated with IBR seropositivity. The final adjusted model for potential risk factors indicated that animals above 6 years old were 9.16 (95% CI: 3.09-27.16; p = 0.000) times at higher risk, while animals with abortion were 4.51 (95% CI: 1.23-16.53; p = 0.019) times and nasal discharges were nearly 3 (95% CI: 1.86-9.31; p = 0.030) times more at risk for IBR seropositivity. The univariable firth's bias reduced logistic regression analysis indicated that district, age, breed, parity, history of abortion, retained placenta and dystocia were found to be significant factors for bovine brucellosis seropositivity in dairy cattle. The odds of brucellosis were higher in animals above 6 years old (OR = 3.82; 95% CI: 1.71-7.97; p = 0.004), local breeds (OR = 6.2; 95% CI: 2.08-8.21; p = 0.016), and aborted cows (OR = 22.35; 95% CI: 3.90-107.91; p = 0.002). Semi-structured questionnaire was administered to 120 respondents to determine major reproductive problems, and assess the knowledge, attitude and practices of farmers. The most common reproductive problems in dairy herds were repeat breeding (47.5%), anoestrus (44.17%), retained placenta (40%), abortion (32.5%) and dystocia (22.5%). Most of the farmers (55.83%) reported assisting a cow during parturition and 75% of them did not use protective glove or personal protection. The practices of raw milk (26.67%) and raw meat (64.17%) consumption were reported. However, only 14.17% of the respondents were found to have the awareness on zoonotic transmission of brucellosis. In conclusion, the results of the present study showed that IBR was widely distributed in commercial and smallholder dairy farms; whereas, the prevalence of bovine brucellosis was found to be very low among dairy cattle. Therefore, the initiation of vaccination for *Bovine Herpes Virus-1* with marker vaccines in Ethiopia, regular herd testing, isolation of animal with predictive signs, and implementation of strict farm biosecurity measures are forwarded to minimize impacts of the diseases on the growing dairy industry, and public health impact of brucellosis.

Keywords: *Dairy Cattle; Bovine Brucellosis; North Shewa; Infectious Bovine Rhinotracheitis; Reproductive Problems; Seroprevalence*

1. INTRODUCTION

Infectious reproductive diseases of cattle are caused by pathogenic infectious organisms such as viruses, bacteria, fungi, and protozoa parasites (Yoo, 2010). Reproductive diseases of cattle are responsible for early embryonic death, abortion, stillbirth, neonatal calve mortality, dystocia, endometritis, retained placental membranes, and infertility (Mathew *et al*., 2017). The most common infectious reproductive diseases of cattle are bovine brucellosis, leptospirosis, campylobacteriosis, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), and trichomoniasis (Messele *et al*., 2021). Reproductive diseases are major constraints of the dairy industry worldwide, which cause huge economic losses due to the loss of replacement herds, reduced milk production, anoestrus and repeat breeding, culling of mature animals, and costs for veterinary inputs (Khan *et al*., 2016).

Infectious bovine rhinotracheitis and bovine brucellosis are the most important reproductive diseases of cattle which cause various reproductive disorders and infertility problems, particularly in dairy cattle (Trangadia *et al*., 2010). The diseases are limiting the reproductive performance and productivity of cattle, and responsible for substantial economic impacts on dairy productions (Tadeg *et al*., 2021). Infectious bovine rhinotracheitis also called infectious pustular vulvovaginitis (IPV) in cows, and infectious pustular balanoposthitis (IPB) in bulls, is a highly contagious and infectious viral disease of the respiratory and reproductive systems. The disease is caused by bovine herpesvirus-1 (BoHV-1), and affect cattle of all age groups. IBR is a globally distributed disease of cattle that mainly characterized by rhinotracheitis, conjunctivitis, vulvovaginitis in cows, balanoposthitis, necrotic rhinitis, neonatal infections, epididymitis, abortion, metritis, and infertility (Graham, 2013; Sibhat *et al*., 2018).

Bovine brucellosis is a contagious bacterial disease that affect livestock, wildlife and human-beings worldwide (Mfune *et al*., 2021). It is an infectious disease of cattle mostly caused by *Brucella abortus*, and occasionally by *Brucella melitensis* and *Brucella suis* (Getahun *et al*., 2023). The disease is characterized by late-term abortion, infertility, endometritis, retained placenta, reduced milk production, calves' mortality, hygroma, epididymitis and orchitis

(Ducrotoy *et al*., 2017). Brucellosis causes serious economic consequences in dairy cattle production, and the livestock industry as a whole due to poor reproductive performances. The disease also possesses major public health threats through occupational exposure and the consumption of unpasteurized milk and dairy products in endemic areas (Gulati *et al*., 2021).

Infectious bovine rhinotracheitis is transmitted within and between herds through the respiratory and genital routes. Bovine herpes virus is well-known for latent infection and periodic reactivation in immunosuppressant conditions (Farooq *et al*., 2019). The transmission of bovine brucellosis is mainly by ingestion of the organism though contaminated feeds and water (Aparicio, 2013; Tulu, 2022). The reproductive form of infectious bovine rhinotracheitis (IPV/IPB) and bovine brucellosis are more common in sexually mature and productive animals. The susceptibility productive animals to these diseases causes higher impact on the livestock production, particularly in the dairy industry (Yoo, 2010; Bifo *et al*., 2020).

Infectious bovine rhinotracheitis and bovine brucellosis are infectious diseases that are capable of transmitting through bovine semen and embryos/ova (Cardenas *et al*., 2019). The diseases are major challenges to the breeding technologies such as artificial insemination (AI), multi-ovulation and embryo transfer due to transmission through fresh and frozen semen and embryo (Givens, 2018). The infectious agents have the potential to shed in the germplasms and genital tracts of the bulls and cows. The diseases restrict the collection, preservation and utilization of valued animal genetic resources (Van Soom *et al*., 2007). The subclinical nature of IBR and brucellosis allows them to develop inapparent carriers from which genetic materials can be collected and transferred to health animals (Tadeg *et al*., 2021).

Reproductive problems cause substantial and immediate impacts on reproductive performance and productivity of dairy cattle. The major reproductive problems in dairy cows are abortion, dystocia, stillbirth, retained fetal membrane, metritis, pyometra, prolapse (uterine and vaginal), anoestrus, and repeated breeding (Misebo *et al*., 2018; Tulu and Negera, 2022). The occurrence of most of the reproductive problems are highly associated with infectious reproductive diseases, mainly infectious bovine rhinotracheitis and bovine brucellosis

(Głowacka *et al*., 2018). The reproductive problems can be classified into three categories according to their phase of occurrence. The reproductive problems which occur before gestation as anoestrus and repeat breeding; during gestation which include abortion, dystocia and stillbirth; and after gestation like neonatal calve mortality, retained fetal membrane, uterine/vaginal prolapses, and metritis (Lobago *et al*., 2006; Amanuel and Tekalign, 2020).

Infectious reproductive diseases are widely distributed in Ethiopia, and are major constraints of smallholder and commercial dairy productions in the country (Tulu *et al*., 2018; Sibhat *et al*., 2018). In Ethiopia, the presence of infectious bovine rhinotracheitis has been evidenced since the mid-1970s and late-1980s, when preliminary serological surveys were conducted in some geographical areas of the country to demonstrate the serological evidence for the presence of BoHV-1 (Lefevre, 1975; Bekele *et al*., 1989). Recently, few studies have been conducted to determine the status of infectious bovine rhinotracheitis in different parts of the country. Accordingly, the seroprevalence of IBR that ranges from 25.6-79.1% was reported in Ethiopia (Tadeg *et al*., 2021; Messele *et al*., 2021).

Bovine brucellosis is a widespread and endemic animal disease in Ethiopia. The disease has been identified as one of the most important livestock diseases having significant impacts on livestock production, since first reported in the country in 1970s (Domenech, 1977; Mayer, 1980). Studies conducted on bovine brucellosis in the last two decades in Ethiopia indicated that the seroprevalence of the disease was varied up to 22.5% at the individual level and up to 68.8% at the herd level (Megersa *et al*., 2011; Ararsa *et al*., 2021). The pooled seroprevalence estimate of brucellosis between 2000 and 2020 at the national level was 2.6% in cattle (Sibhat *et al*., 2022). Thus, studies indicated a high prevalence of IBR and bovine brucellosis in various production systems of the country.

In the North Shewa of the central highlands of Ethiopia, particularly in Angolela Tera and Kimbibit districts, there is rapid growth and expansion of commercial dairy industries. Accordingly, smallholder dairy productions are very potential and well-known for the production of Sheno dairy butter and other dairy products. The areas have intensely attracted

dairy farming, particularly in the last decade, and stated as one of the major milk-shed areas of the country. Infectious bovine rhinotracheitis and bovine brucellosis are major diseases in both commercial and smallholder dairy farms in different parts of the country. Nowadays, IBR has progressively become a major challenge to the growing dairy industry. Despite the significance of IBR, there are only a few studies that are mainly conducted on commercial dairy farms in Ethiopia. Besides, bovine brucellosis remains a major challenge that causes poor animal health and productivity, and zoonotic infections. However, there is little information on the status of bovine brucellosis in the current study areas, and updating the current status of the disease is of paramount importance for local and national disease control. Moreover, the identification of major reproductive disorders has critical importance for improving productivity of commercial and smallholder dairy production. Herewith, assessing public awareness on brucellosis has great significance for intervening with appropriate measures to control zoonotic brucellosis.

General Objective

- To determine the seroprevalence of Infectious Bovine Rhinotracheitis (IBR) and Bovine Brucellosis, and to identify the major reproductive problems and associated risk factors in commercial and smallholder dairy farms in North Shewa, the central highlands of Ethiopia.

Specific Objectives

- To determine the current seroprevalence of infectious bovine rhinotracheitis (IBR) and bovine brucellosis in the study areas.
- To describe the potential animal and herd level risk factors associated with IBR and bovine brucellosis.
- To identify the major reproductive problems and associated factors in dairy cattle in commercial and smallholder dairy farms.
- To assess the knowledge, attitude and practices (KAP) of the farmers on handling of reproductive disorders and zoonotic brucellosis.

2. LITERATURE REVIEW

2.1. Diseases Definition

Infectious bovine rhinotracheitis (IBR) is a highly contagious viral disease of cattle that causes respiratory and reproductive infections. IBR is an extremely infectious disease with substantial economic losses throughout the world, particularly in the dairy industry (Bosco *et al*., 2011). The reproductive forms of the disease are known as infectious pustular vulvovaginitis (IPV) in cows and infectious pustular balanoposthitis (IPB) in the bulls (Gould *et al*., 2013). IBR is an important disease of cattle causing abortion, retained placenta, metritis, infertility, and various respiratory or genital infections (Graham, 2013). The most important epidemiological feature of the disease is that infections usually produce latency after clinical or subclinical infection, which can be reactivated later on following immunosuppressing factors (OIE, 2017).

Brucellosis is an infectious bacterial disease of many domestic and wild animals with great zoonotic importance. Bovine brucellosis is an economically important disease of livestock mainly characterized by causing several reproductive problems and loss of productivity (Scacchia *et al*., 2013). The disease affects sexually mature and productive animals causing abortions in late pregnancy, followed by retention of the fetal membrane and infertility in succeeding pregnancies in the cattle (Mariana *et al*., 2010). Bovine brucellosis is a major constraint of dairy productions in many countries due to its higher economic and public health consequences. In humans, zoonotic transmission of brucellosis causes severe febrile disease and joint pain (Habtamu *et al*., 2015).

2.2. Etiology

Infectious bovine rhinotracheitis (IBR) is caused by bovine herpesvirus-1 (BoHV-1). Bovine herpesvirus-1 is also the causative agent of IPV and IPB in cows and bulls, respectively. BoHV-1 is belongs to the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Muylkens *et al*., 2007a). It is a large (150-200 nm), enveloped virus,

consisting of double-stranded DNA that is approximately 140 kilobase (kb) pairs in length. Basically, BoHV-1 has three serologically indistinguishable strains (BoHV-1.1, BoHV-1.2a, and BoHV-1.2b). BoHV-1.1 is primarily causing respiratory infections (IBR), and also reproductive infections. BoHV-1.2a cause respiratory and genital infections, and is frequently isolated from aborted fetuses; while strain BoHV-1.2b is associated with reproductive and neurologic infections (Graham, 2013).

Bovine brucellosis is primarily caused by the bacterium *Brucella abortus*, while cattle are also infected with *B. suis* and *B. melitensis* when they are in close contact or feed together with infected pigs or small ruminants (Radostits *et al*., 2007). *Brucella* is a genus of Gram-negative bacteria belonging to the family *Brucellaceae,* in the class *Alphaproteobacteria*. *Brucella* is non-spore forming, non-motile, aerobic, and facultative intracellular bacteria. The bacterium is coccobacillus of 0.5 to 0.7 μm in diameter, and 0.5 to 1.5 μm in length (Godfroid *et al*., 2013). Currently, about eight biotypes (1-7, 9) as well as several variants of *Brucella abortus* are recognized. *B. suis* and *B. melitensis* also contain five and three biovars, respectively. Most *Brucella* species are associated with certain hosts, while there is also infection to other hosts and human beings (Mizak *et al*., 2014).

2.3. Epidemiology of Infectious Bovine Rhinotracheitis (IBR)

Infectious bovine rhinotracheitis is a contagious disease that causes substantial consequences in cattle production throughout the world. The disease is accountable for significant economic losses in both dairy and beef cattle industries (Raaperi *et al*., 2014). IBR commonly occurs when an infected animal is introduced into a herd, or when several animals are confined in a crowded space in feedlot situations. Domestic ruminants such as goats, and wild ruminants like deer are also susceptible to the disease and can transmit the virus to cattle (Balakrishnan, 2017). Epidemiologically, the most important characteristic of BoHV-1 is that infections usually produce latency. IBR is frequently characterized by synergism with other viral infections like bovine viral diarrhea (BVD), and bacterial diseases particularly with pasteurellosis to induce case fatalities (Lopez and Martinson, 2017).

2.3.1. Global distribution of infectious bovine rhinotracheitis

Infectious bovine rhinotracheitis was first recognized in the United States of America as a respiratory disease of cattle in the 1950s. Currently, the disease has been distributed throughout the world, except in some countries that proclaimed freedom from the disease. According to OIE, Austria, Denmark, Finland, Sweden, Switzerland, and Norway have eradicated the disease (Ackermann and Engels, 2006); whereas, IBR control programs are implemented in Australia, Belgium, Canada, India, Poland, Turkey, and the USA (Boelaert *et al*., 2005; OIE, 2017). Globally, several studies have been conducted to determine the seroprevalence of IBR in cattle (Table 1).

Table 1: Seroprevalence of infectious bovine rhinotracheitis in different parts of the world

Countries	Study population	Prevalence (%)	References
Sudan	Sudanese cattle	38.00	(Husayn *et al*., 2005)
Turkey	Dairy cattle	19.50	(Tan *et al*., 2006)
Egypt	Female buffalos	43.30	(Ghazy *et al*., 2007)
Lithuania	Dairy cows	33.86	(Eugenijus *et al*., 2008)
Brazil	Bulls	82.40	(Ferreira *et al*., 2010)
South Africa	Cattle	74.47	(Njiro *et al*., 2011)
India	Cattle	46.51	(Verma et al., 2014)
	Buffalo	35.28	
Mexico	Cattle	64.40	(Segura-Correa *et al*., 2016)
France	Cattle	4.60	(Valas *et al*., 2019)
Algeria	Dairy cattle	24.19	(Kaddour *et al*., 2019)
Kenya	Cattle (Farm level)	30.90	(Kipyego *et al*., 2020)
	Animal level	17.40	
Colombia	Dairy herds	57.50	(Ortiz-González *et al*., 2022)

Even though the disease is worldwide distributed, there is a significant difference in the incidence and prevalence among different regions. Currently, infectious bovine rhinotracheitis is highly prevalent in Africa, Latin America, and Asian countries (Table 1). Geographical location, disease management strategies, and breeding systems are the main influential factors determining the disease distribution (Muylkens *et al*., 2007b; Ortiz-González *et al*., 2022). The latent characterization of the virus creates difficulty in the disease control and eradication from the countries (Farooq *et al*., 2019).

2.3.2. Status of infectious bovine rhinotracheitis (IBR) in Ethiopia

Infectious bovine rhinotracheitis becomes an important and widely distributed disease in various parts of Ethiopia. The presence of infectious bovine rhinotracheitis has been evidenced since the mid-1970s and late-1980s in the country (Table 2). In Ethiopia, the preliminary serological surveys conducted in limited geographic areas were demonstrated serological evidence for the presence of BoHV-1 (Lefevre, 1975; Bekele *et al*., 1989). The importation of exotic cattle breeds from European countries for increasing milk production and genetic improvement of the local cattle have been primarily implicated in the introduction of infectious bovine rhinotracheitis into the country (Asgedom and Mekonnen, 2021).

The first serological survey in the country revealed 41.8% seroprevalence of IBR in Harar and Sidamo provinces (Lefevre, 1975). In the late-1980s, the seroprevalence study conducted in Gobe and Ghibe areas determined 67% prevalence of the disease (Bekele *et al*., 1989). In recent years, some studies have been conducted to determine the status of IBR in different parts of the country. The seroprevalence of IBR was found 41% in the main milk shed areas of the central, southern, and southwestern parts of Ethiopia (Sibhat *et al*., 2018). In Dessie and Kombolcha towns in south Wollo zone, the seroprevalence of IBR was 25.6% in selected dairy cattle (Tadeg *et al*., 2021). An overall animal-level seroprevalence of BoHV-1 antibodies was found 77.6% in a study conducted in northwestern, Ethiopia (Zewde *et al*., 2021). The highest prevalence of IBR in the country was reported to be 79.1% in central, Ethiopia (Messele *et al*., 2021), indicating the endemic nature of the disease in the country (Table 2).

Table 2: Seroprevalence of infectious bovine rhinotracheitis in different parts of Ethiopia

Study areas	Study population	Prevalence (%)	References
Harar and Sidama	Cattle	41.8	(Lefevre, 1975)
Gobe and Ghibe	Cattle	67.0	(Bekele *et al*., 1989)
Central, Southern and Southwestern, Ethiopia	Dairy cattle	41.0	(Sibhat *et al*., 2018)
Central, Ethiopia	Dairy cattle	79.1	(Messele *et al*., 2021)
Dessie and Kombolcha towns	Dairy cattle	25.6	(Tadeg *et al*., 2021)
Northwestern, Ethiopia	Indigenous Zebu	77.6	(Zewde *et al*., 2021)

2.3.3. Risk factors

The occurrence and distribution of infectious bovine rhinotracheitis is influenced by several host, pathogen, environmental and management factors. The effects of the risk factors are variable among regions, farms, or herds because of variations in microclimatic conditions, management practices, and stocking densities, along with other factors (Almeida *et al.*, 2021). Farm management systems significantly affect the epidemiology of the disease. The introduction of latently infected animals into the farm can expose the entire herd to IBR infection. High stocking densities in confined pens increase the transmission of the virus through respiratory routes. Herd size and housing strategy have a higher impact on the transmission of the disease. The transmission and survival of the virus is relatively high in seasons with low ambient temperature (González-Garcia *et al.*, 2009).

The incidence and severity of infectious bovine rhinotracheitis is variable depending on associated risk factors such as animal species, age, vaccination status, and physiological conditions. The naturally susceptible species of animals are domestic and wild bovines. Sheep, goats, and pigs may also be infected with the virus (Boelaert *et al*., 2005). Domestic and wild ruminants may serve as a reservoir of the disease and can transmit the virus to cattle.

However, the common sources of infection are latently infected cattle from IBR endemic areas which are considered as carriers of the virus. Animals in sufficient stressful conditions and those having concurrent viral or bacterial infections are highly susceptible to the disease (Chothe *et al*., 2018). Immune-compromised animals are also vulnerable to reactivated infection that has attained latency during the previous infection. Commonly, infections occur after six months of age after waning of the maternal immunity (Muylkens *et al*., 2007b).

2.3.4. Transmission and maintenance of bovine herpes virus-1 (BoHV-1)

The transmission of infectious bovine rhinotracheitis is usually by direct contact of a susceptible animal with an infected animal excreting the virus in oronasal or genital secretions. The primary route of entry for the respiratory form of IBR is the nasal cavity (Majumder *et al*., 2015). Aerosols laden with the virus are inhaled and can initiate respiratory infections. Aerosol transmission is the major means of BoHV-1 transmission in confined herds and during the introduction of infected animals to susceptible herds (Segura-Correa *et al*., 2016). The other important means of transmission is through genital routes, most importantly the route via infected semen and vertical transmission may occur in the uterus. BoHV-1 is shed in semen following a primary or reactivated infection, with the potential of transmission following either artificial or natural insemination. Embryo transfer may also result in the transmission of BoHV-1 adsorbed to the zona pellucida (EFSA, 2006; Foster, 2017).

The transmission of the virus is influenced by several environmental and climatic factors. The bovine herpes virus is fairly resistant to environmental influences. Environmental survival depends on temperature, pH, light, humidity, and the medium harbouring the virus (Biswas *et al*., 2013). Virus infectivity is stable for up to one month at 4 °C, but higher temperatures inactivated the virus more rapidly. BoHV-1 can survive for 30 days during cold periods when the relative humidity is above 90%. In warmer environments such as in buildings, survival between 5 to 13 days has been recorded (Kelling *et al*., 2007). In favorable environmental conditions, the virus may survive for more than 30 days in feedstuffs. The virus is sensitive to

many disinfectants, such as the routinely used phenol derivates, quaternary ammonium bases, and formalin (Nandi *et al*., 2009).

2.3.5. Viral latency and reactivation

The most important epidemiological feature of infectious bovine rhinotracheitis is that the cattle become latent carriers of the virus after clinical infections (Figure 1). The virus travels from the sites of initial/local infections by axonal migration to the sites of latency. After replications in the mucosal epithelium, it establishes lifelong latency in the peripheral nervous system such as trigeminal and sacral ganglia. Latency can also occur in lymphoid tissue and peripheral blood lymphocyte (Henderson *et al*., 2004).

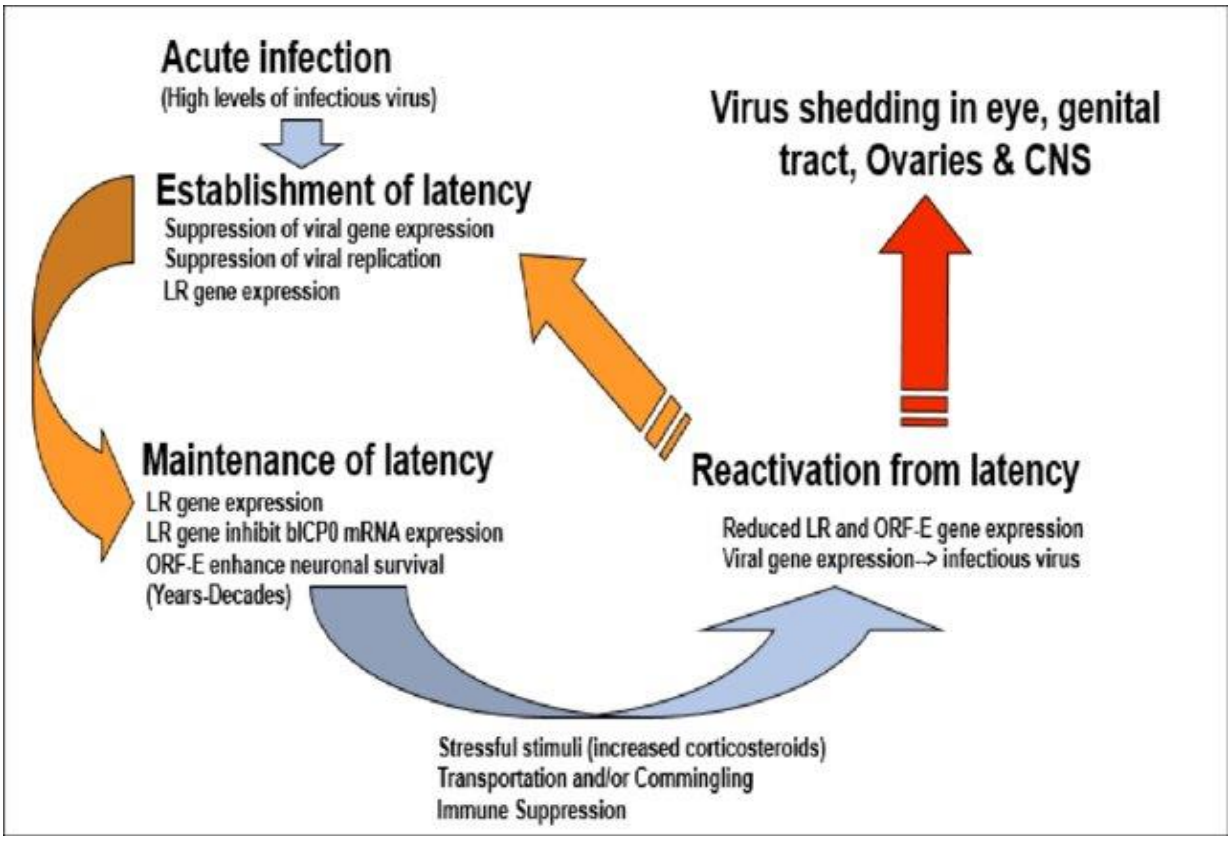

Figure 1: Bovine Herpes Virus-1 (BoHV-1) latency and reactivation in cattle (**Source**: Farooq *et al*., 2019)

There are various factors contributing to BoHV-1 latency (Figure 1). The weakness in the host immune system to clear the virus has been implicated in regulating the establishment of viral latency. The infectious virus did not present during the course of latent infections (Farooq *et al*., 2019). However, the latent infection can be reactivated periodically, particularly when the host got an infection with synergistic pathogens, stress and other immunosuppressant conditions. The virus travels to the initial site of infection and shed with potential transmission to other animals. The reactivation may be with or without clinical signs, and most seropositive animals are latently infected (Xingnian and Kirkland, 2008).

2.4. Epidemiology of Bovine Brucellosis

Brucellosis is a globally distributed disease with significant animal health, public health, and international trade consequences. Bovine brucellosis is a serious disease of livestock causing huge production losses, particularly in developing countries (Głowacka *et al*., 2018). The disease is a major challenge to the dairy sector in particular due to reproductive problems and loss of milk production. Brucellosis affects wider range of hosts including domestic animals, wild animals and human beings. Bovine brucellosis which is mostly caused by *B.abortus* is endemic in most Sub-Saharan countries (Godfroid *et al*., 2011). The disease has substantial socio-economic consequences, especially in developing countries in which rural income relies largely on livestock production. Brucellosis affects people having exposed to contaminated tissue or consumption of unsafe animal products (Smirnova *et al*., 2013).

2.4.1. Global distribution of bovine brucellosis

Bovine brucellosis is a globally distributed disease that has been reported in Africa, Asia, South and Central America, Mediterranean basin, and Caribbeans; where livestock production is highly important and the basis of livelihoods (Franc *et al*., 2018). Irrespective of the disease eradication, brucellosis infected or exposed animals are also reported in some European and North American countries. The incidence and prevalence of the disease is variable from region to region and the production systems (Table 3). Higher prevalence of the disease reported in

many countries (Abubakar *et al*., 2012). Even if there are variations in the occurrence of the disease, bovine brucellosis is widespread in Africa where it is endemic and major constraint of livestock production in most Sub-Saharan countries. The absence of strict disease control strategies contributing for higher impact of the disease in these countries (Hussen *et al*., 2020).

Table 3: The prevalence of bovine brucellosis in different parts the world

Countries	Sample type	Tests	Prevalence (%)	References
Egypt	Blood	BAPA	5.44	(Samaha *et al*., 2008)
Argentina	Blood	CFT	1.80	(Aznar *et al*., 2015)
Ghana	Blood	c-ELISA	22.90	(Tasiame *et al*., 2016)
Nigeria	Blood	c-ELISA	7.80	(Ayoola *et al*., 2017)
Pakistan	Blood/Milk	RBPT	6.30	(Ali *et al*., 2017)
Kenya	Milk	RBPT	19.00	(Njuguna *et al*., 2017)
Tanzania	Blood	c-ELISA	9.30	(Sagamiko *et al*., 2018)
Turkey	Milk	RBPT	17.32	(Babaoglu *et al*., 2018)
South Sudan	Blood	c-ELISA	31.00	(Madut *et al*., 2018)
Uganda	Blood	c-ELISA	1.20	(Nguna *et al*., 2019)
India	Blood	c-ELISA	8.30	(Shome *et al*., 2019)
South Africa	Blood	CFT	3.18	(McCrindle *et al*., 2020)
Brazil	Blood	FPA	4.87	(Rodrigues *et al*., 2021)
India	Milk	c-ELISA	15.10	(Holt *et al*., 2021)
Zambia	Blood	c-ELISA	7.53	(Mfune *et al*., 2021)

Most developed countries have successfully controlled bovine brucellosis to eradicate, while some European countries have eradicated through intensive disease control and eradication programs. Trade in animal and animal products in the global trend intensify livestock productions, thereby allows the transmission of the disease from endemic countries to other areas (Dean *et al*., 2012). The higher burden of bovine brucellosis in low and medium-income countries brings extensive impact on livestock production. In developing countries, the lack of

sufficient and efficient infrastructures and socio-economic situations regarding the disease aggravates the economic and public health impacts of the disease (McDermott *et al*., 2013).

2.4.2. Status of bovine brucellosis in Ethiopia

In Ethiopia, bovine brucellosis is a widespread and endemic animal disease that has substantial economic and public health impacts. The presence of brucellosis in the country was evidenced in 1970s for the first time (Domenech, 1977; Mayer, 1980). Bovine brucellosis becomes one of the most important livestock diseases in the country, that have serious impacts on livestock production and productivity (Kebede *et al*., 2008). In the last two decades, several studies have been conducted to determine the status and distribution of bovine brucellosis in various parts of the country (Table 4). Some studies indicated lower prevalence of the disease, while others reported very high animal and herd level prevalence of bovine brucellosis in studies conducted in different parts of Ethiopia (Asmare *et al*., 2013; Yilma *et al*., 2016; Tulu, 2022).

The seroprevalence of bovine brucellosis ranges up to 22.5% in individual animal and 68.8% at the herd-level (Megersa *et al*., 2011; Ararsa *et al*., 2021). Recent studies indicated that the prevalence of bovine brucellosis was 2.4% in cattle in Borena zone, southern Ethiopia (Edao *et al*., 2020), 5.7% in Afar pastoralist community (Negash and Dubie, 2021), 3.3% in Jimma zone (Etefa *et al*., 2022), and 0.6% in central highlands of Ethiopia (Getahun *et al*., 2023). In cattle, 2.6% pooled seroprevalence estimate of brucellosis between 2000 and 2020 was reported at national level (Sibhat *et al*., 2022). Bovine brucellosis is also a significant threat of public health in Ethiopia, where occupational hazards and livestock keepers were exposed to the disease (Table 4). The seroprevalence of human brucellosis was 10.6% in southern, Ethiopia (Workalemahu *et al*., 2017), 15.8% in districts of Afar region (Mehari *et al*., 2021), 34.9% in Somalia region (Tschopp *et al*., 2021), and 1.2% in a study conducted in and around Holeta town (Getahun *et al*., 2023).

Table 4: The prevalence of bovine and human brucellosis in various parts of Ethiopia

Study areas	Test used	Prevalence in Cattle (%)	Prevalence in Human (%)	References
Southern, Ethiopia	CFT	-	10.6	(Workalemahu *et al*., 2017)
Addis Ababa	RBPT/CFT	2.77/0.06	-	(Edao *et al*., 2018)
Sendafa	CFT	0.40	-	(Bifo *et al*., 2020)
Afar region	RBPT/CFT	11.9/5.7	-	(Negash and Dubie, 2021)
Western, Ethiopia	CFT	1.2	-	(Sima *et al*., 2021)
Somalia region	CFT	1.5	2.8	(Ibrahim *et al*., 2021)
Awra and Gulina districts of Afar	RBPT/CFT	-	31.5/15.8	(Mehari *et al*., 2021)
Amibara district of Afar	RBPT/CFT	10.5/2.2	-	(Gutema *et al*., 2021)
South Omo zone	CFT	5.26	-	(Mekonnen *et al*., 2021)
South Wollo zone	RBPT	5.4	-	(Tadeg *et al*., 2021)
Somalia region	RBPT	8.6	34.9	(Tschopp *et al*., 2021)
Central highlands	RBPT/CFT	1.2/0.6	4.2/1.2	(Getahun *et al*., 2023)

2.4.3. Public health significance of brucellosis

Brucellosis is a serious zoonotic disease that affects several people throughout the world (Table 5). Annually, about 830,000 peoples are infected with brucellosis. Human brucellosis is most commonly caused by *B. melitensis*, *B. abortus* and *B. suis* (WHO, 2015). Bovine brucellosis is an important zoonotic disease that considered as one of the essential occupational diseases in cattle farms, livestock keepers, slaughterhouses, leather industries and microbiology laboratories (Guerrier *et al*., 2011). In humans, the disease is characterized by

severe febrile symptoms accompanied by muscle and bone pain; and in most case, it remained under-diagnosed worldwide. The disease in human is confused symptomatically with several febrile diseases, such as malaria, leptospirosis and typhoid fever (Mufinda *et al*., 2017).

Table 5: The prevalence of human brucellosis in different parts of the world

Countries	Study Population	Prevalence (%)	References
Algeria	Pastoralists	11.11	(Hamiroune *et al*., 2020)
Saudi Arabia	Febrile patients	12.80	(Alkahtani *et al*., 2020)
Tanzania	Pregnant women	10.90	(Makala *et al*., 2020)
Egypt	Resident	23.90	(Diab *et al*., 2020)
Kenya	Pastoralist	1.70	Munyua *et al*., 2021
Turkey	Resident	3.60	(Bozlak and Celebi, 2021)
Zambia	Occupationally exposed	20.30	(Mubanga *et al*., 2021)
India	Rural population	1.83	(Ghugey *et al*., 2021)
Kenya	Livestock keepers	0.60	(Lokamar *et al*., 2022)
Brazil	Resident	12.34	(Bernardi *et al*., 2022)
Morocco	Farmers and patients	33.20	(Faddane *et al*., 2022)
Oman	Hospital patients	11.00	(Ani *et al*., 2023)

Human brucellosis is most commonly occurring in developing countries, while rarely reported in developed nations (Table 5). Humans usually become infected by ingesting organisms or through mucous membranes and abraded skin. In laboratory and in some instances in abattoirs aerosol transmission can also occur (WHO, 2006). The most common sources of brucellosis in humans, particularly in developing countries are consumption of unpasteurized dairy products and uncooked meat, contact with aborted materials, tissue and discharges, and laboratory specimen (Habtamu *et al*., 2015). Human to human transmission is unusual, but congenital infection can occur through placental transmission and breast feeding. Human infections with *Brucella* are also reported following exposed to *Brucella* vaccine (Hussen *et al*., 2020).

2.4.4. Risk factors

The epidemiology of bovine brucellosis is affected by several factors related to management system, host, and environmental factors. The occurrence of the disease is variable with age, sex, and breed of cattle, herd size and type, and production system (Godfroid *et al*., 2011). The understanding of essential risk factors associated with bovine brucellosis is very important in mitigating the socioeconomic and public health impacts. The most important host risk factors include age, sex and reproductive status of the particular animal. The susceptibility for bovine brucellosis increases in sexually mature and pregnant cattle. They are more prone to the disease than sexually immature cattle. The occurrence of brucellosis in females is higher than males due to the presence of *Brucella* growth factor and tropism to the fetal tissues, whereas it is usually subclinical in non-pregnant animals (Borba *et al*., 2013).

Brucella abortus is capable of multiplication and survival inside the host phagocytic cells. Polymorpho-nuclear leukocytes phagocytized the bacteria but unable to kill it, and the bacteria does survive and further multiplies. The bacterium is also able to survive within macrophages (Ducrotoy *et al*., 2017). As the host defense system cannot effectively clear the bacteria at the primary route of infection, the bacteria are then disseminated to the regional lymph nodes, reticulo-endothelial system and the reproductive tracts. *Brucella abortus* mostly prefers fetal tissue, placenta, and the reproductive tracts of the animal; and preferentially use erythritol (a four-carbon sugar) in the placenta as a growth factor (Radostits *et al*., 2007).

Furthermore, the occurrence and transmission of brucellosis is significantly influenced with management, macro and micro-climatic conditions. The movement of infected animals from one region to the other areas is the main means of disease spread. Management factors influencing disease transmission include herd size, housing, calving pens, handling of aborted fetus and retained placenta, sanitation and disinfections (Robi and Gelalcha, 2020). The most important feature of brucellosis is that the ability of the bacteria to survive longer outside the hosts in relative with most non-spore forming pathogenic bacteria. The bacteria can retain infectivity for several months in water, aborted fetuses and fetal membranes under suitable

environmental conditions (pH, temperature and humidity). *Brucella* survival is prolonged at pH of 4 and lower temperature (Głowacka *et al*., 2018).

2.4.5. Mode of transmission and maintenance of Brucella

Brucellosis is typically transmitted to susceptible cattle by direct or indirect contact with infected animals and their discharges. Cattle become infected with brucellosis through the ingestion of contaminated feed and water. Feedstuffs and drinking water can be contaminated by the bacteria from reproductive discharges and fluids (Jergefa *et al*., 2009). The habit of cattle to lick new born calves significantly predispose to the disease. The major routes of infection are through the oral and nasal cavities, mucous membranes of the conjunctiva, and apparently through vertical transmission or colostrum (Aparicio, 2013). Brucella may also enter to the body through mucosal layers and intact skin, and spread via the blood and lymphatic system to any other organ where it causes localized infection (Lapaque *et al*., 2005).

The most significant epidemiological feature of bovine brucellosis is that it shed large number of organisms during few days following abortion or calving of infected cows. Brucella becomes a herd problem, as it is primarily spread by contact and ingestion of contaminated materials. The spread between herds is facilitated by the introduction of asymptomatic animals (Tukana and Gummow, 2017). The peak epidemics are associated with higher number of abortions in pregnant cows. Infected animals release infectious discharges such as gravid uterine tissue, vaginal secretions, urine, milk, semen, and suppuration matter; which serve as potential source of environmental contamination. Brucellosis is rarely spread through sexual contact in cattle; however, the use of artificial insemination can potentially transmit the disease from infected bulls to health cows (Khurana *et al*., 2021).

2.5. Pathogenesis and Clinical Signs of Infectious Bovine Rhinotracheitis

The pathogenesis of bovine herpes virus-1 starts with the replication of the virus in the mucosal epithelial surfaces of the upper respiratory tract and genital mucosa. The disease is

characterized by invasion and inflammation of the respiratory and reproductive tracts. Then the virus infects the local nerve cells and transported to trigeminal and sacral ganglia where it establishes a lifelong latent infection (Xingnian and Kirkland, 2008). The latency nature of the herpes virus makes the infected animals to become carrier of the virus throughout life. After infection, the animal sheds the virus in nasal and genital secretions. Semen and vaginal discharges can be contaminated with the virus from the reproductive tracts. The virus may not be detected during the period of latent infections (Farooq *et al*., 2019).

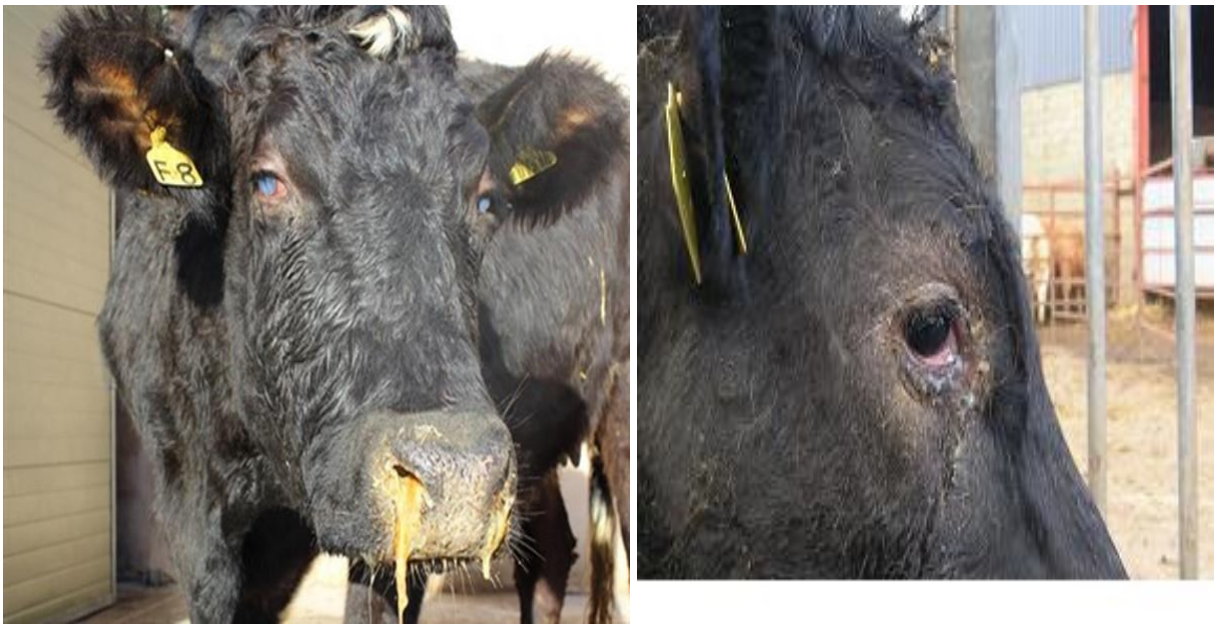

Figure 2: Infectious Bovine Rhinotracheitis induced nasal and ocular discharges and corneal opacity (**Source**: Bastawecy and Abouzeid, 2016)

Infectious bovine rhinotracheitis (IBR) has an incubation period of 10-12 days under natural conditions. The disease commonly occurs as a subclinical infection. The clinical cases of the disease are characterized by fever, coughing, in appetence, depression, tachypnea, decreased milk production, keratoconjunctivitis, serous nasal and ocular discharges which become mucopurulent in later stages (Figure 2). The reproductive forms of the disease cause abortion, retained placenta, metritis, vulvovaginitis, and balanoposthitis (Jones and Chowdhury, 2007). The clinical disease usually occurs following immunosuppressant and stressful conditions

such as transportation, crowding and housing. The morbidity rate may reach 100% during outbreak situations, but the mortality rate is generally less than 2%. The death of infected animals is from severe damage and necrosis of the trachea, and accompanied by secondary bacterial infections (Loi *et al*., 2013).

2.6. Pathogenesis and Clinical Signs of Bovine Brucellosis

The evasion of host defense systems and mode of intracellular survival is the main mechanism and virulence factor of *Brucella* (Gopalakrishnan *et al*., 2016). The pathogenesis of brucellosis begins when the bacteria penetrate intact mucosal surfaces, thereby multiplies and survives inside phagocytic polymorpho-nuclear leukocytes. Then, the bacteria transported within the phagocytic cells to regional lymph nodes and the reticuloendothelial system. The bacteremic phase is mostly subclinical that can go through few weeks to several months (Figueiredo *et al*., 2015). The localization of *Brucella* in reproductive tracts such as uterus and udder of cow, and testicles and accessory glands of bulls is the most important pathogenic stage of the disease. The presence of erythritol favor bacterial growth in pregnant cow, and the fetal membrane and placenta become inflamed and infection spread via the blood to fetus. (Poester *et al*., 2013).

The clinical manifestations of brucellosis depend on age, reproductive and immunological status of the animal, and virulence and dose of the *Brucella* strain. The incubation period of the disease varies between 14 and 120 days. The predominant clinical signs of brucellosis in cattle are abortions in second half of gestation, stillbirths, and the birth of weak calves, retained fetal membranes, metritis and marked reduction in milk yield (Radostits *et al*., 2007; Dougherty *et al*., 2013). In the bulls, brucellosis causes epididymitis, seminal vesiculitis, orchitis or testicular abscesses. Brucellosis also causes joint infections such as hygroma and arthritis (Figure 3). The disease causes infertility or poor performance in both sexes, due to metritis or orchitis. In the infected cows, abortion usually occurs only once after brucellosis infection, but the cows continued to excrete a huge number of organisms in the subsequent calving (Abernethy *et al*., 2006).

Figure 3: Joint swelling (hygroma) due to brucellosis (Left) and licking of cattle with placental membrane (Right) (**Source**: Mantur *et al*., 2019)

2.7. Diagnosis of Infectious Bovine Rhinotracheitis

The diagnosis of infectious bovine rhinotracheitis is through direct agent identification and indirectly through the detection of host immune responses. Whole blood, swabs of upper respiratory and genital tracts, and lung tissue samples are used for viral isolation and identification. Antibody detections are preformed from the serum and milk samples (Divers, 2008). The most commonly applied diagnostic techniques of IBR are Virus neutralization test (VNT), indirect ELISA, blocking ELISA, and polymerase chain reaction (PCR). The virus isolation and polymerase chain reaction are considered to have a high sensitivity and specificity. The specificity of all ELISA tests and VNT is higher, although nonspecific reactions can occur due to different factors such as suboptimal quality of the sample, early testing after collection, recent vaccination and batch variation of the kits (Kramps *et al*., 2004; OIE, 2017). The presence of cross-reactivity with related bovine herpes viruses leads to epidemiologically non-feasible singleton serological reactors (Bottcher *et al*., 2012).

2.7.1. Serological tests

The serological diagnosis of infectious bovine rhinotracheitis is conducted on serum and milk samples. The most frequently used serological tests for serum samples of animals are indirect ELISA, blocking ELISA, and the Virus Neutralization Test (Mahajan *et al*., 2013). The serological tests are very convenient for screening large number of samples as the tests require a short time to provide the results. Most importantly, detecting the virus is very difficult in latently infected animals. Therefore, serological tests are able to identifying serologically positive and healthy animals that serves as a good predictor of infection level in a herd. Animals positive on serological tests should be considered as infected with bovine herpes virus-1 except in the case of serological responses caused by inactivated vaccine immunization or colostral antibodies (Chatterjee *et al*., 2016).

Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

Indirect ELISA is the commonly utilized diagnostic technique of IBR for the detection of antibodies in the serum and milk samples. The ELISA kits are coated with BoHV-1 antigens into the wells of a polystyrene plate. The antibodies in the sample bind with the coated antigen, which are identified using enzyme-labeled anti-bovine immunoglobulins (Zeedan *et al*., 2018). The quantity of antibodies in the sample are indicated by the level of optical densities. The greater Corrected Optical Density (COD) indicated the higher quantity of antibodies in the sample (Peter and George, 2017). Indirect ELISAs have high diagnostic specificity (Sp > 95%) and diagnostic sensitivity (Se > 99%) commonly used to identify anti-BoHV-1 antibodies in serum (Righi *et al*., 2022).

Blocking Enzyme-Linked Immunosorbent Assay (Blocking ELISA)

The commercial blocking gE-ELISA is the only serological test that allows differentiation of wildly infected animal from those immunized with gE-deleted marker vaccine. IBR-gE ELISA test is commonly used as differentiating infected from vaccinated animals (DIVA test) in IBR

free farms or marker-vaccinated herds (Colitti *et al*., 2018). The animals immunized with marker vaccines and exposed to a field strain of BoHV-1 can be differentiated using enzyme-labeled monoclonal antibodies specific for gB or gE. Bovine herpesvirus type-1 antigen coated microwells on a polystyrene plate are used for detection of the antibodies from field infection. The serum samples are mixed with enzyme-labeled anti-BoHV-1 antibody and incubated. The quantity of antibodies in the samples reduce the color development resulting from the addition of substrate (chromogen) solution (Peter and George, 2017; Righi *et al*., 2022).

2.7.2. Virus isolation and neutralization test (VNT)

The virus neutralization test is a very sensitive and specific test that employed to identify antibodies against BoHV-1 in samples. VNT is the gold standard method to measure neutralizing antibodies in the serum to bovine herpesviruses (Holz *et al*., 2010). About 50µl of the serum sample and equal volume viral suspension are added in two wells of the tissue culture plates. The mixture is incubated for 2 hours at 37 °C in the presence of 5% CO_2. Then, 50µl of cell suspension is added and cultured for 24–48 hours. The neutralization index is calculated after incubation of the mixture. The isolate can be classified as BoHV-1 when the neutralization index is more than 1.5. However, definite confirmations require molecular detection to differentiate the virus from associated bovine alpha herpes viruses (OIE, 2018).

2.7.3. Polymerase chain reaction (PCR)

The molecular detection of the virus is a confirmatory technique for the identification of bovine herpes virus-1. Polymerase chain reaction is a molecular technique that used to directly detect the virus genetic material in the specimens. The DNA of the herpes virus can be recovered from supernatant of infected cells culture using viral DNA extraction kits, and the extracted DNA is used as a template for copy DNA synthesis (Peili *et al*., 2017). The PCR assay is able to detect the virus in nasal swaps, fetal serum and bovine semen. There is a good correlation between PCR and the virus isolation for the detection of BoHV-1 in clinical field samples. Bovine herpes virus can also be diagnosed using real time polymerase chain reaction

(RT-PCR). Real time PCR is the most successful technique for testing BoHV-1 in aborted and autolyzed fetuses. The virus is detected directly from swab samples or propagated monolayer cell lines (Crook *et al*., 2012).

2.8. Diagnosis of Bovine Brucellosis

The clinical diagnosis of bovine brucellosis is based on epidemiological patterns and clinical manifestations such as abortion during the second trimester and birth of weak offspring in cows, and orchitis and epididymitis in bulls. However, there is no pathognomonic symptoms for brucellosis in animals and human. Therefore, the diagnosis of brucellosis is based on bacteriology or serology (Godfroid *et al*., 2013). The testing of blood and milk samples, and the culture of *Brucella* from swabs of fetal membranes, aborted fetus and vaginal discharges of infected cows forms the definitive laboratory diagnosis of brucellosis. The suspected cases of brucellosis should be confirmed using different laboratory methods including isolation and identification of the organism, *Brucella* specific antigen and antibody detection methods, and molecular detection of the organism (Karthik *et al*., 2014).

The diagnosis of brucellosis is based on several laboratory techniques that include serological assay, bacteriological isolation and identification, and molecular methods. The serological techniques are mostly based on agglutination reactions between antigens and *Brucella* antibody found in the serum or milk samples (Simsek *et al*., 2004). The most important diagnostic method of brucellosis is the isolation and further identification of the bacteria. In the convectional serological tests, it is difficult to distinguish between infected and vaccinated animals. Therefore, bacteriological isolation and identification of biotypes of the *Brucella* organism is the most critical procedure in planning national brucellosis control and eradication programs. However, due to the zoonotic importance of brucellosis handling and working with the live agent is hazardous to laboratory personnel. The available diagnostic methods, the choice of a particular method is depending on the prevailing epidemiological situation, laboratory facilities and safeties (Godfroid *et al*., 2013).

2.8.1. Bacteriological isolation and identification

The isolation of bacterial pathogen is the gold standard and confirmatory diagnosis of brucellosis. The most reliable samples for the isolation of the bacteria are body fluids such as fetal fluids, vaginal discharges and blood; and tissues including aborted fetus, spleen and lymph nodes of the infected animals (Smirnova *et al*., 2013). The viability of the organism in clinical samples is highly essential for the isolation of the bacteria. Even if, bacteriological isolation is the gold standard and highly specific, the culturing of *Brucella* is challenging. *Brucella* organisms are fastidious bacteria which requires enriched media for the primary cultures. Moreover, this technique is long and time taking that required weeks for definitive identification of the bacteria (Seleem *et al*., 2010).

The direct isolation and culture of *Brucella* requires solid media, whereas liquid media are recommended for the purpose of enrichment. The most commonly used media for culturing *Brucella* species include Tryptose Soya Agar (TSA), Blood Agar Base (Oxoid), Columbia Agar, Serum Dextrose Agar (SDA) and Glycerol Dextrose Agar (Poester *et al*., 2010). The colonies of *Brucella* species are appeared in few days to weeks. *Brucella* colonies are elevated, transparent, smooth, and convex with intact borders and brilliant surfaces. The identification of the etiological agent can be performed using colony characteristics, staining and biochemical tests. *Brucella* organisms are identified through growth characteristics, Gram stain, Modified Ziehl-Neelsen stain, oxidase and urease activity, H_2S production, Dye tolerance (basic fuchsin and thionin) and sero-agglutination (Mantur *et al*., 2019).

2.8.2. Polymerase chain reaction (PCR) assay

The PCR technique is used for molecular characterization of the infectious agent, epidemiological interpretations, and taxonomic classifications. It provides a rapid diagnostic method and can be used on samples of lower quality. PCR identifies the DNA of *Brucella* and classify at genus, species and even biovar levels, and it has become a very essential diagnostic technique (Alamian *et al*., 2017). The polymerase chain reaction method can be applied for

range of purposes from the diagnosis of the disease to characterization of field isolates. The number of sequences has been recognized as target for genus-specific PCR assays for confirmation of *Brucella* species (Habtamu *et al*., 2013). PCR is proven to be useful in diagnosing relapsing infections, assessing the efficacy of treatments, identification and differentiation of biovars and biotypes, respectively (Christopher *et al*., 2010).

A real-time PCR has been developed for the authentic diagnosis of *B. abortus*, *B. melitensis* and *B. suis*. The primers utilized by PCR are derived from different polymorphic regions in the genomes of *Brucella* species. The specific primers are able to detect specific *Brucella* species authentically (Neha *et al*., 2017). Multiplex PCR typing is more effective method that can identify the bacteria up to biovar level using different primer combinations. Generally, PCR methods are cost effective, rapid and safe ways of brucellosis diagnosis (Smirnova *et al*., 2013). The major drawback of PCR is that some uncertainties to distinguish the field strain from the vaccine strains. However, a novel real-time PCR-based method which targeting protein of the outer membrane is able to differentiate between the virulent strain and vaccine strain of the bacteria in endemic regions (Onurdag *et al*., 2016).

2.8.3. Serological tests

Serological tests are sensitive and rapid screening techniques that are used to detect the presence of antibodies against *Brucella* infection in the animal. Serological diagnosis is very important for monitoring, surveillance, control, and eradication of the disease. Indirect diagnosis of brucellosis is based on that antibodies start to appear in the blood or milk after infection of the animal with *Brucella* (Lucero *et al*., 2003). The IgM appears first followed by the appearance of IgG. However, antibodies against smooth *Brucella* species such as *B. abortus*, *B. melitensis*, and *B. suis* are possessing cross-reactivity with antigen preparations from *B. abortus*. The most commonly employed serological techniques for the diagnosis of brucellosis include Rose Bengal Plate Test (RBPT), Milk Ring Test (MRT), Complement Fixation Test (CFT), Serum Agglutination Test (SAT), Immune Capture Agglutination (ICA), Lateral Flow Assay (LFA), and ELISA (Ezama *et al*., 2018).

Rose Bengal Plate Test (RBPT)

Rose Bengal plate test (RBPT) is a rapid screening method used to detect *Brucella* antibodies in serum using a plate agglutination test. RBPT detects antibodies against *B. abortus, B. melitensis* and *B. suis* in serum samples (OIE, 2009). It is an ideal *Brucella* diagnostic technique that is simple and easy to perform, and does not require special laboratory facilities. The sensitivity of RBPT is very high and useful in the quick confirmation of clinical manifestations such as reproductive failures (Mantur *et al*., 2019). RBPT is based on the detection of specific *Brucella* antibodies such as the IgM and IgG types. The pH and temperature of the antigen, and the ambient room temperature influences the sensitivity and specificity of the test. Appropriate pH level (3.65) improves the specificity of the RBPT by inhibiting nonspecific agglutinations. The overall sensitivity of RBPT is 92.9%, that RBPT can be effectively used in individuals exposed to brucellosis and those having history of *Brucella* infection; but relatively low specificity (Díaz *et al*., 2011).

Complement Fixation Test (CFT)

Complement fixation test (CFT) is a very specific diagnostic technique that can detect IgM and IgG1 *Brucella* antibodies. CFT is highly specific test that used as confirmatory test for *B. abortus*, *B. melitensis*, and *B. ovis* infections. The test is the reference test recommended by the OIE due to its higher accuracy (OIE, 2009). Complement fixation test is majorly based on quantitative measurements of the IgG1 type antibodies due to the partial destructions of IgM antibodies during the inactivation processes. The minimum hemolytic dose of the complement (MHD) is used to read the result of the procedure. CFT test is usually recommended and considered as better for brucellosis control and surveillance programs (Khurana *et al*., 2021). The major disadvantages of CFT method include high cost, complexity of procedures, requirement of special equipment and laboratory facilities, and trained laboratory technicians. The test also presents limitations when used with hemolyzed serum samples or serum with anticomplement activity (Pfukenyi *et al*., 2020).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA methods are suitable alternatives for the diagnosis of brucellosis that can screen large number of samples under high clinical suspicions. ELISA tests can provide the total and individual specific immunoglobulins (IgG, IgA and IgM) within few hours with high sensitivity and specificity (Peeridogaheh *et al*., 2013). Enzyme-linked immunosorbent assay technique further classified into indirect ELISA (iELISA) and competitive ELISA (c-ELISA). The indirect ELISA tests are based on specific antibodies in the serum sample that will bind with the immobilized antigens in the ELISA kits. The antibody-antigen conjugation is visualized using chemically or enzymatically derived fluorescent or luminescent reaction. It used for smooth LPS *Brucella* species (*B. abortus* or *B. melitensis*). The sensitivity of iELISA varies from 96 to 100%, and its specificity from 93.8% and 100% (Poester *et al*., 2010).

Competitive ELISA test can be used as confirmatory test for further testing of the RBPT screening result. c-ELISA is based on the detection of *Brucella* antibodies in the sample using antigens from smooth *Brucella* lipopolysaccharides (LPS). Competitive ELISA test is capable of differentiating the field infection from antibody responses due to vaccination (Perrett *et al*., 2010). The test has sensitivity that varies from 92 to 100%, and specificity which ranges from 90 and 99%. The development of c-ELISA is associated with the vulnerability of indirect ELISA to nonspecific reactions, particularly with Yersinia enterocolitica serotype O9 (YO9) infection. The specific epitopes of *Brucella* O-polysaccharides that are not shared with the LPS of YO9 are used as antigens (Nielsen *et al*., 2008).

2.9. Treatment, Control and Prevention of Infectious Bovine Rhinotracheitis

Infectious bovine rhinotracheitis has no specific drug treatments. The use of broad-spectrum antibiotics prevents secondary bacterial complications and severity of pneumonia during outbreaks. Anti-inflammatory and nonsteroidal drugs can be used to relieve pyrexia and respiratory symptoms (Iscaro *et al*., 2021). Therefore, prevention and control strategies are the most important and effective techniques for avoiding or minimizing the risk of IBR. The

prevention and control of IBR is mainly based on biosecurity measures which minimize the risk of viral introduction, and transmission of infection from carrier animals to the rest of herds (Nandi *et al*., 2009). Quarantine of the newly purchased animals for 2-3 weeks should be implemented before introducing to the herd. Immunization of the susceptible herds, strict import restriction on animals and potential genetic materials (semen and embryo) are the basis for control and eradication of IBR (Majumder *et al*., 2015).

2.9.1. Stress management

Stress and any immunosuppressant conditions are the main predisposing factors for infection with bovine herpes virus-1. Most animals that recovered from IBR infection remains latently infected throughout life. The typical epidemiological feature of bovine herpes virus is reactivated infection in latently carrier animals following stressful conditions, and spread to cause more cases (Raaperi *et al*., 2014). The triggering factors include movement of cattle, poor husbandry system, feed or water shortage, co-infections, treatments with corticosteroids, and unfavorable weather conditions. Managing stressful conditions is very important in the control and prevention of IBR. Animals should be housed in well-ventilated rooms with appropriate stocking densities. The control of co-infections with other diseases, the use of prophylaxis treatment before transporting animals, and access to sufficient and good quality feed and water significantly minimizes the risk of IBR infection (Iscaro *et al*., 2021).

2.9.2. Vaccination

Immunization of susceptible animals plays a significant role in the control and prevention of IBR. There are different types of vaccines that both live attenuated and killed vaccines are available, where the choice depends on the efficacy of the vaccine and disease status in the country (Mars *et al*., 2001). Vaccination of latently infected animals also reduces the amount of infectious virus excreted following reactivations. Traditional and marker vaccines are commercially available for the control of infectious bovine rhinotracheitis. The traditional vaccines prevent severe clinical disease and minimizes the quantity of virus shedding after infections (Iscaro *et al*., 2021). The new generation vaccines are developed by removing the

non-essential viral glycoproteins, mainly glycoprotein-E (gE) of BoHV-1. This vaccine allows the serological differentiation of antibodies from animals with field infection and those vaccinated with the gE-deleted marker vaccine (Wernike *et al*., 2011).

2.9.3. Biosecurity measures

The implementation of biosecurity measures is the most important strategy for the prevention of IBR in herds. The countries as a whole and farms in particular should implement strict biosecurity measures to control bovine herpes virus-1 infection. The farm boundaries should be secured enough to prevent close contact with neighboring animals (Waldeck *et al*., 2021). The replacement cattle must be purchased from farms that have officially IBR-free herds. Animals brought from endemic areas, purchased from other farms, or in contact with untested animals must be quarantined for 21 days before being introduced into the herds. The quarantined animal should be tested for the presence of antibodies to BoHV-1 (Majumder *et al*., 2015). Regular serological investigation of animals in the herd is essential for the timely detection of the disease's introduction. Herd surveillance enables segregation of serologically positive individuals to avoid further dissemination of the disease in the herds (Mandelik *et al*., 2021) and for the successful control and gradual eradication of IBR (Ackermann and Engels, 2006).

2.10. Treatment, Control and Prevention of Bovine Brucellosis

The treatment of brucellosis in animals is not indicated and is usually unsuccessful due to the intracellular survival of the bacteria. The combinations of antibiotics such as doxycycline and rifampicin are found to be relatively effective against brucellosis. However, due to the presence of a high rate of treatment failures and higher costs, the treatment is not attempted in animals (Seleem *et al*., 2010). Control and prevention of brucellosis is the most important methods of mitigating the risk of the disease. The control and prevention of brucellosis is through immunization of the herd, quarantine of newly arrived animals, and disease surveillance. Advanced disease control strategies with herd testing and removal of positives are applied in developed countries (Moriyon *et al*., 2004).

The treatment of human brucellosis is performed using combinations of different antibiotics. Monotherapy antimicrobials have low success rates in the treatment of brucellosis and relapse of infection is common in humans. The combination of antibiotics must be wisely selected to prevent side effects and the emergence of bacterial resistance (Villate and Casallas, 2020). Currently, the combination of doxycycline with streptomycin is the best therapeutic option due to fewer side effects and less relapses, particularly in cases of acute and localized cases of brucellosis. Treatment for brucellosis is typically taking long period at least up to one month and there could be several associated side effects (Singh *et al*., 2014).

2.10.1. Surveillance and biosafety measures

Surveillance provides efficient system for the control of bovine brucellosis which enables the identification of infected animals, prevention of further spreads between animals and herds, and the removal of carrier animals from the herd (Gwida *et al*., 2010). Regular herd testing is essential in early identification of cases of brucellosis, while testing of newly purchased animals is very crucial in preventing the introduction of the disease. Robust surveillance mechanism enables the successful prevention of the disease from introduction and spread into the herd (Hull and Schumaker 2018). Biosafety measures are very important to reduce the risk of infection in humans and animals. *Brucella* is one of environmental contaminant bacteria. Aborted fetus, fetal membranes, and genital discharges including semen can contaminate the housing and utensils. Therefore, personal hygiene, proper disposal of aborted fetus and fetal membranes, environmental and food hygiene provide very significant roles in the control and prevention of both human and animal brucellosis (WHO, 2006; Cardenas *et al*., 2019).

2.10.2. Vaccination

Vaccination of susceptible animals is the best method for the prevention of brucellosis in animals. The successful control and eradication of bovine brucellosis can be achieved through testing, elimination of positive animals, and vaccination of susceptible herds (Beauvais *et al*., 2016). There are different types of vaccines available for bovine brucellosis. The live vaccines

include *B. abortus* S-19 and *B. abortus* strain RB51, while the killed type of the vaccine is *B. abortus* 45/20. The most efficient and widely used vaccine for control of bovine brucellosis in many countries is RB51 attenuated live vaccine. It also does not interfere with serological diagnostic results. In endemic regions, vaccination of cattle with the most efficient vaccine is utmost essential in control and prevention of brucellosis (Gheibi *et al*., 2018). There is no effective vaccine approved for use in the prevention of human brucellosis, despite of several trials. However, vaccines for animal brucellosis provide significant results in the management of the disease in animals, thereby reducing the risk in humans (Marzetti *et al*., 2013).

2.11. Major Reproductive Problems and Public Awarnesss on Zoonotic Brucellosis

The reproductive performance of dairy cattle is one of the most important determinants of dairy production. Reproductive problems are disorders of the reproductive system that prevent or restrict estrus, conception, pregnancy, calving and productive efficiency. The most frequent reproductive problems are abortion, retained placenta, dystocia, pyometra, metritis, uterine and vaginal prolapse, anoestrus and repeat breeding (Tulu and Negera, 2022). In Ethiopia, reproductive problems are widely occurring in commercial and smallholder dairy production. The prevalence of abortion was 28.30% (Weldegebriel, 2015) and 27% (Lakew *et al*., 2022) in dairy herds. Higher infertility problems are reported in dairy cattle in the country; Eshete *et al*. (2023) indicated 38.4% of repeat breeding, and Kidusan (2009) reported 38.6% of anoestrus.

Brucellosis is a neglected zoonosis with great public health significance in Ethiopia and most developing countries. The risk of zoonotic brucellosis is higher in the country due to livestock production, close interactions with animals, higher practices that can expose people to brucellosis, and lower public awareness of zoonotic diseases. Assisted parturition and contact with fetal membranes were 59.3% in Debrebirhan and 94.62% in Addis Ababa (Edao *et al*., 2018; Lakew *et al*., 2022). In Ethiopia, the practice of raw milk consumption is higher, particularly in pastoral areas; raw milk consumption was 65.53% in central Ethiopia (Getahun *et al*., 2023) and 84.16% in Borena zone (Edao *et al*., 2020). The pooled awareness of zoonotic brucellosis in the country was 18.4% (Sibhat *et al*., 2022).

3. MATERIALS AND METHODS

3.1. Description of the Study Areas

The study was carried out on commercial dairy farms and smallholder farmers located in North Shewa, in the central highlands of Ethiopia. The study areas were represented in North Shewa zones of Amhara and Oromia regional states, where the major focus of the study was the areas in the way from Addis Ababa to Debre Birhan (Figure 4). Angolela Tera and Kimbibit districts were purposely selected based on their potential dairy production, where the dairy industries are rapidly growing due to their climatic suitability and proximity to the main cities such as Addis Ababa and Debre Birhan. Commercial dairy farms are widely expanded in both districts, while smallholder dairy productions have been progressively increasing with the introduction of improved and crossbred dairy cattle into the mixed farming system (Tadesse, 2015). Agriculture is the main means of livelihood in both Angolela Tera and Kimbibit districts. The major livestock populations in the districts include cattle, sheep, goats, chicken and equines. Mixed farming system which is the integration of crop-livestock production is the major agricultural system. Beyond agriculture, trade in agricultural products mainly butter and dairy products are dominant. The districts are well-known and potential for milk production, and are familiar for production of the organic Sheno dairy butter (Idahe and Zemedu, 2017).

Angolela Tera district is located in the North Shewa zone of Amhara regional state. The administrative center of the district is Chacha town, which is the second largest market place in Angolela Tera following Kotu Gebeya. Chacha town is situated 110 km to the Northeast of Addis Ababa and 20 km to the South of Debre Birhan. Angolela Tera is geographically situated between 9°23'–9°60' N latitude and 39°26'–39°64' E longitude (Figure 4). The district consists of 19 rural and 3 urban kebeles (smallest administrative units). Angolela Tera is bordered on the South by Hagere Mariam, on the North by Basona Werana, on the Southeast by Asagirt district, and on the West by the Oromia region (Fikre, 2020). The area was characterized by mean annual minimum and maximum temperature of 6.7 °C and 19.9 °C, respectively. The annual rainfall in the district ranges from 800-1500 mm. The district is

majorly represented by highland and midland agroecologies with the altitude that ranges from 1700-3400 m above sea level (Lakew *et al*., 2022). The district in particular, and North Shewa zone of the Amhara region is one of the highest livestock-producing areas with 1,704,407 heads of cattle, 1,941,024 sheep, 823,550 goats, 704,128 equines, and 2,000,196 poultry (CSA, 2021).

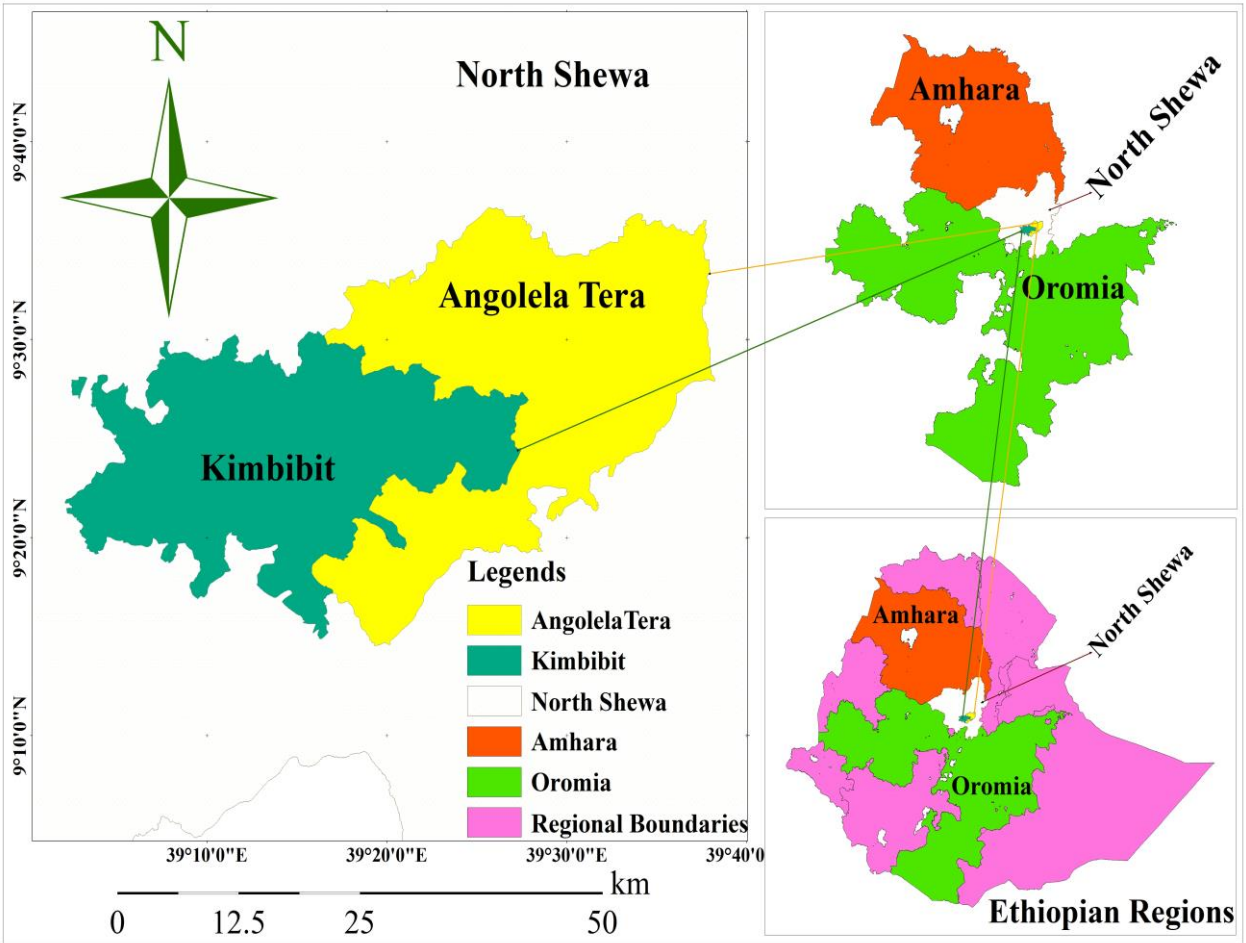

Figure 4: Map of the study areas in North Shewa zones of Amhara and Oromia regional states, central Ethiopia (Projected using ArcGIS software)

Kimbibit district is found in the North Shewa zone of Oromia regional state. The administrative center of Kimbibit is Sheno town, which is located 78 km from Addis Ababa on the way to Debre Birhan (Figure 4). The district is geographically extending between 9°12'–9°32' N latitude and 39°21'–39°33' E longitudes with an elevation ranges form 1390-2980 m above sea

level. Kimbibit has 31 kebeles from which 29 are rural and 2 are urban kebeles. The district is bordered on the South by Aleltu, on the West by Jido, on the North by Abichuna Gne'a district, and on the East by the Amhara region (Bezabih and Tesfaye, 2013). The climatic condition of the district is predominantly Dega (semi-arid climate). The mean annual rainfall is 913 mm, and the mean annual minimum and maximum temperature is 13 ^{o}C and 19 ^{o}C, respectively (Abera *et al*., 2021). Livestock production is one of the main components in the mixed farming activities in Kimbibit district, where 1,323,045 cattle, 945,409 sheep 158,116 goats, 402,907 equines, and 656,314 poultry were found in the North Shewa zone of the Oromia region (CSA, 2021).

3.2. Study Animal Population

In this study, the cattle population located in North Shewa, particularly in Angolela Tera and Kimbibit districts were used as study animals. Cattle in both commercial and smallholder dairy farms were considered in the study. There are many small and large-scale dairy farms in the areas that supply milk and other dairy products to Addis Ababa, Debre Birhan, and its surroundings. The study animal population were constituted different breeds of cattle, age categories, number of parities, lactation and pregnancy status, and both sexes in the dairy farming. The study was conducted on sexually mature dairy animals, as reproductive disorders and the reproductive forms of IBR and bovine brucellosis are more common in adult cattle than younger animals (Głowacka *et al*., 2018).

The study population were included indigenous cattle breeds and their crossbreds with Jersey and Holstein Friesians breeds in the districts. Cattle were kept in different management systems that commercial dairy farms were used intensive and semi-intensive management systems, while smallholder farmers were largely depending on extensive production system. The age of the animal was classified in to three categories that animals below 3 years old, animals with age of between 3-6 years old, and animals above 6 years old (Sima *et al*., 2021; Lakew *et al*., 2022). The number of parities in the cows were categorized as non-parous (no calving), monoparous (given calving once), and multiparous (given 2 or more calving) (Abera,

2018; Etefa *et al*., 2022). The classification of the study herds was based on slight modification of (Alehegn *et al*., 2016) that very small (≤5 cattle), small (6-10 cattle), medium (11-20 cattle) and large (>20 cattle) herd size.

3.3. Study Design

A cross-sectional study on infectious bovine rhinotracheitis and bovine brucellosis was conducted from November 2022 to May 2023 in the selected districts of North Shewa. Different types of research approaches were employed to determine the status of the diseases, identify major reproductive problems, and associated animal and herd-level risk factors. Blood samples were collected from each selected animal to determine the seroprevalence of the diseases. A Questionnaire survey was conducted to determine the occurrence of major reproductive problems in study areas, and to assess the farmers' knowledge, attitude and practices. Retrospective information was recorded along with blood sample collection to determine the history of reproductive disorders such as cases of abortion, dystocia, and retained fetal membranes, and respiratory signs. The potential individual animals and herd-level risk factors including sex, breed, age, lactation status, parity, herd size, mating system used, management system and herd replacement strategies were properly recorded.

3.4. Sampling Methodology

The study districts were purposefully selected based on their potential dairy productions. Angolela Tera and Kimbibit districts are the major milkshed areas in the North Shewa zones of Amhara and Oromia regions, respectively (Idahe and Zemedu, 2017; Lakew *et al*., 2022). There is productive expansion of commercial dairy farming; accordingly, smallholder dairy productions are very potential with the introduction of improved dairy cattle and are well-known areas for the production and marketing of the highly demanded Sheno butter and other dairy products. The farms/herds were randomly selected from the commercial dairy farms and smallholder dairy herds in both districts.

This study employed stratified random sampling techniques to select the constituents of the study animals. The study animal population were including cattle kept in different farming environments, and were divided into subgroups (strata). The variable farm type was used for the stratification of the study animal populations. Based on the farm types, the study animals were stratified into commercial (intensive and semi-intensive) dairy farms and smallholder dairy productions that are mainly based on extensive management systems. A simple random sampling technique was used to sample individual cattle from each stratum. Male animals above six months of age and female animals with ages of 2 years and above that were maintained for breeding and production purposes were selected from the herds.

3.5. Sample Size Determination

The study on the seroprevalence of IBR and bovine brucellosis was conducted at 95% of level of confidence and 5% desired absolute precision. The target population were categorized into different strata, and appropriate sample size was drawn from each farm type in both districts. The seroprevalence investigation of infectious bovine rhinotracheitis has not been conducted before in Angolela Tera and Kimbibit districts, or the surrounding districts of North Shewa. Therefore, as there was no baseline information on the status of the disease in the current study areas, the number of animals to be sampled were determined using 50% expected prevalence (Thrusfield *et al*., 2018).

$$n = \frac{Z^2 * P_{exp}(1-P_{exp})}{d^2}$$

$$= \frac{(1.96)^2 * 0.5 * (0.5)}{(0.05)^2}$$

Where: n = required sample size (number of cattle),

P_{exp} = expected prevalence,

d = desired absolute precision (5%), and

1.96 = Z value of 95% confidence interval (CI)

In accordance with the above formula, the minimum sample size required for the current study was 384 animals. The number of herds required for this study were computed based on 6.2% herd prevalence of bovine brucellosis reported on dairy farms in Debre Birhan (Lakew *et al*., 2022). Therefore, the number of herds required for this study were 90 herds (Humphry *et al*., 2004). However, 511 animals (276 animals from Angolela Tera and 235 animals from Kimbibit districts) from 142 herds were sampled to increase the precision, thereby reducing the standard error of the study. Accordingly, 250 animals were sampled from the commercial dairy farms, while the remaining 261 samples were selected from smallholder dairy productions. The number of animals sampled was limited to a maximum of 30% of the herd/farm (Neumann *et al*., 2018).

$$\mathbf{HN = (1.96/L)^2 \times HTP(1\text{-}HTP)}$$

Where: HN = sample size (number of herds),
HTP = estimated herd prevalence, and
L = tolerance around the prevalence (5%)

The sample size required for the questionnaire survey was determined using 5% (0.05) standard error, and calculated with the formula provided by (Arsham, 2005). $N = 0.25/SE^2$, where N = number of samples (respondents), and SE = standard error of the estimate. Accordingly, 100 respondents were required, however the survey was conducted on 120 respondents to improve the precision of the study.

3.6. Sample Collection

3.6.1. Blood sample collection

The foremost aim of the current study was serological investigation of IBR and bovine brucellosis. Therefore, collection of blood samples was one of the major procedures during the research activities. Blood samples were collected from the jugular vein using plain vacutainer tube and vacutainer needles following proper disinfection of the collection site. During sample

collection, the animal was properly restrained in the crush to avoid injury to the animal and study personnel. Fresh needles were used for each animal to eliminate the risk of cross contamination. About 7-10 ml of blood sample was drawn from each study animal. Each vacutainer tube was labeled with identification codes with farm type, sampling site and date of collection. The blood samples were kept at room temperature overnight in a slanting position to allow clotting and separation of the serum (OIE, 2009). The serum samples were then gently decanted into 1.8 ml sterile cryovials, labeled and transported to laboratory on ice, and preserved at -20 °C until serologically screened for IBR and bovine brucellosis at Animal Health Institute (AHI) in Sebeta, Ethiopia.

3.6.2. Retrospective and clinical data collection

The presence of reproductive disorders such as abortion, retained placenta, dystocia and orchitis, and respiratory clinical signs including coughing, ocular and/or nasal discharges were collected from the farmers, record books, and through clinical observations to determine their association with the seropositivity of the animals for infectious bovine rhinotracheitis and bovine brucellosis. Besides, several animal level (sex, age, cattle breed, number of parities and lactation/pregnancy status) and herd level (herd size, production systems, breeding methods and herd replacement strategies) risk factors were properly recorded along with blood sample collection to identify the potential risk factors for the diseases (Annex 4).

3.6.3. Questionnaire survey

The questionnaire survey was conducted on dairy farm owners, attendants and local farmers to determine the major reproductive problems and potential risk factors in commercial and smallholder dairy farms and to assess the farmers' knowledge, attitude and practice on handling of reproductive problems and zoonotic infections of brucellosis. Information regarding cattle production, herd size, management systems, breeding methods and occurrence of reproductive problems was collected using a semi-structured questionnaire (Annex 6). The dairy cattle production systems were categorized into intensive, semi-intensive and extensive systems.

Herd size was classified into very small, small, medium and large (Alehegn *et al*., 2016). The strategy of breeding was characterized by service types as artificial insemination (AI) or natural mating or both natural mating and AI.

The occurrences of abortion, stillbirth, retained fetal membranes, dystocia, cervical/vaginal prolapses, repeat breeding, anoestrus and neonatal calves' mortality in the herds/farms were recorded. Cattle production systems and practices such as cattle breeds in the herd, herd size, management systems, breeding methods and the presence of group mating were taken as the risk factors for the presence of reproductive problems. The perception of farmers on handling of reproductive disorders, disposal of fetal membranes, stillbirth and aborted fetus, the use of protective materials during assisted parturition, practices of raw milk and meat consumptions, and zoonotic transmission of bovine brucellosis was noted (Annex 6).

3.7. Serological Laboratory Test

The serological investigation of infectious bovine rhinotracheitis was conducted using competitive IBR-gE ELISA, while the investigation of bovine brucellosis was performed using RBPT and CFT tests. All the laboratory procedures were conducted at Animal Health Institute (AHI), Sebeta, Ethiopia.

3.7.1. Competitive gE-ELISA test for infectious bovine rhinotracheitis

A competitive enzyme-linked immunosorbent assay (ELISA) test was conducted to detect antibodies against glycoprotein-E (gE) of *Bovine alphaherpesvirus-1* (BoHV-1) in the serum samples. The IBR-gE competitive ELISA is a commercially available product (IDvet, Idexx, and CIV-HIPRA) that was primarily developed for detection of anti-gE antibodies in serum, and milk samples. The IBR-gE ELISA has the potential for serological differentiation of infected and vaccinated animals (DIVA) with IBR gE-deleted marker vaccine. Moreover, the gE-ELISA test has higher specificity and low serological cross-reactivity with related alpha herpes viruses as compared to gB-ELISA (Petrini *et al*., 2020). The diagnostic sensitivity and

specificity of IBR-gE competition ELISA was 93-96% and >99%, respectively. The IDvet competitive gE-ELISA is based on the antigen-antibody complexes using anti-gE antibodies that masks gE epitopes in the field virus. The anti-gE horseradish peroxidase (HRP) conjugate fixes remaining free gE epitopes, and forming antigen-conjugate-HRP complex that resulting in coloration depending on quantity of specific antibodies present in the test sample (Annex 1).

The serological analysis of the serum samples was performed according to the instructions of the manufacturer (Annex 1). For each sample, the competition percentage (S/N%) was calculated that S/N% equals to optical density (OD) of the sample divided by the OD of negative controls multiplied by 100. Hence, with regards to the manufacturer's guideline the serum samples with ≤60% competition percentage were considered as positives (antibodies present). The serum samples with competition percentage of greater than 60% were taken as negative for IBR.

3.7.2. Rose Bengal plate test (RBPT) for bovine brucellosis

All the serum samples were screened with Rose Bengal plate test (RBPT) for bovine brucellosis. RBPT is a simple screening test that detects antibodies against *B. abortus*, *B. melitensis* and *B. suis* in serum samples. The sensitivity of the RBPT was on average 92.9% (89.7-97.6%), while it had a lower specificity (77.6%). The plate agglutination tests were conducted using an antigen prepared from *Brucella abortus* (S99) stained with Rose Bengal dye and suspended in acid buffer (pH 3.65). The RBPT test was performed according to the World Organization for Animal Health (OIE, 2009) and the manufacturer's procedure (Annex 2). The results were read by recording agglutinations as 0 (absence of agglutination), + (barely visible agglutination), ++ (fine agglutination), and +++ (coarse agglutination). Hence, samples with no agglutination were recorded as negative, while those with visible agglutination were considered as positive (OIE, 2012).

3.7.3. Complement fixation test (CFT) for bovine brucellosis

Complement fixation test (CFT) was conducted on those samples which were positive on RBPT test. CFT is the most commonly used test for the serological confirmation of *Brucella* infection in cattle, and is recommended by the World Organization for Animal Health (OIE, 2009). The sensitivity of complement fixation test is on average 81% and its specificity ranges from 95 to 100%. The preparation of reagents and CFT procedures was performed according to the OIE protocols (Annex 3). Titration of hemolysin and antigen was performed for the test. The positive reactions were indicated by sedimentation of Sheep Red Blood Cells (SRBC) and the absence of hemolysis, while negative reactions were revealed by the lack of fixation and hemolysis of SRBC, and the result was read by recoding MHD. The sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:10, and at least with 50% fixation of complement (2+) at a dilution of 1:5 were classified as positive.

3.8. Data Management and Analysis

The data collected from the field and laboratory investigations were entered into a Microsoft Excel spread sheet. The many attribute data that were imported to the database system include results of the questionnaire survey, animal and herd-level factors, and the laboratory result of the diseases status. The imported data were then cleaned, checked and coded properly. Then, the data were transferred to R-statistical software version 4.1.2 for statistical analysis. The data were summarized using descriptive statistics, and cross-tabulations and frequency tables were constructed. The Chi-square ($\chi 2$) test was used to analyze the questionnaire survey results and the relationship of categorical variables with the outcomes.

Univariable logistic regression analysis was used to determine the association between various animal and herd-level risk factors with IBR seropositivity. Whereas, univariable Firth's Bias-Reduced logistic regression analysis was employed to analyze the association between individual as well as group risk factors against bovine brucellosis infection. Then after, the presence of multicollinearity was checked for all predictor variables using Pearson chi-square

and Goodman and Kruskal's Gamma statistics, and variables with strong correlation (age with parity; gamma=0.89 and RFM with dystocia; p=0.004) were dropped from the final model. Multivariable logistic regression models were built for variables with a p-value less than 0.25 on univariable logistic regression analyses.

Further selection of variables in the final model was based on backward stepwise selection procedure that less significant variables were dropped. The goodness of fit in the logistic regression models were evaluated using the Receiver Operator Curve (ROC) analysis. The model with a value of area under the curve (AUC) of the ROC greater than 0.7 was accepted. Mixed (multilevel) regression models were run using farm type and district as random effect variables. However, mixed models found insignificant for both IBR and bovine brucellosis due to weak intraclass correlation (ICC<0.3), indicated lower clustering effects of the districts and farm types. The predictive factors were considered as statistically significant factors of the diseases when p-value is less than 0.05 at a 95% CI.

3.9. Ethical Clearance

Ethical clearance certificate was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture animal research ethics and review committee (Ref. No: VM/ERC/14 /03/15/2023) following the necessary request by explaining the purpose of the study (Annex 7). The appropriate ethical principles and conducts was applied both in animal and human study participants. Herewith, informed consents were obtained from human study participants, livestock and dairy farm owners (Annex5).

4. RESULTS

4.1. Seroprevalence of Infectious Bovine Rhinotracheitis (IBR)

4.1.1. Seroprevalence of infectious bovine rhinotracheitis at the animal level

Among 511 serum samples tested, a total of 316 animals were found positive with competitive IBR-gE ELISA test that revealed an overall 61.84% (95% CI: 57.53-65.97%) seroprevalence of infectious bovine rhinotracheitis in the study areas. The seroprevalence of IBR was 57.97% (160/276) in Angolela Tera district, and 66.38% (156/235) in Kimbibit district. The result indicated that the seroprevalence of IBR was higher in Kimbibit district, and animals in Kimbibit district were 1.43 times more at risk for IBR seropositivity than animals in Angolela Tera district. However, there was no significant difference ($p = 0.051$) in the prevalence of IBR between the districts. The seroprevalence of infectious bovine rhinotracheitis was relatively higher in smallholder farms (64.37%) than in commercial dairy farms (59.20%). Though, there was no significant association (OR = 1.24; 95% CI: 0.87-1.78; $p = 0.230$) in the prevalence of the disease with the farm types (Table 6).

The present study revealed that the seroprevalence of IBR was 59.90% (245/409) in female animals and 69.61% (71/102) in male animals. Even though, males were 1.53 times more at risk of IBR seropositivity than female animals, there was no significant association between IBR seroprevalence and sex groups ($p = 0.072$). Animals less than 3 years old had a seroprevalence of 40.23% of IBR, while the prevalence was 57.26% in animals between 3-6 years old and 78.98% in animals above 6 years old. The study showed that there was a significant association in the seroprevalence of IBR among age groups; the prevalence was very high in animals above 6 years old and low in animals below 3 years old. Animals with age of 3-6 years old were 2 (OR = 1.99; 95% CI: 1.21-3.27; $p = 0.007$) times more likely to be IBR seropositive, while animals above 6 years old were 5.58 (95% CI: 3.18-9.78; $p = 0.000$) times more at risk for IBR infection as compared to animals below 3 years old (Table 6).

Table 6: Univariable logistic regression analysis of IBR seroprevalence at the animal level and associated risk factors

Predictive variables	Categories	Total no. of animals	Positive (%)	OR (95% CI)	P-value
District	Angolela Tera	276	160 (57.97)	1	
	Kimbibit	235	156 (66.38)	1.43 (0.998-2.054)	0.051
	Total	511	316 (61.84)		
Farm types	Commercial	250	148 (59.20)	1	
	Smallholder	261	168 (64.37)	1.24 (0.871-1.780)	0.230
Sex	Female	409	245 (59.90)	1	
	Male	102	71 (69.61)	1.53 (0.962-2.443)	0.072
Age	<3	87	35 (40.23)	1	
	3-6	248	142 (57.26)	1.99 (1.211-3.271)	0.007
	>6	176	139 (78.98)	5.58 (3.184-9.784)	0.000
Breed	Cross	385	249 (64.68)	1	
	Local	126	67 (53.17)	0.62 (0.413-0.932)	0.022
Status of the cow	Dry	33	19 (57.58)	1	
	Heifer	90	34 (37.78)	0.45 (0.199-0.692)	0.047
	Lactating	177	110 (62.15)	1.21 (0.569-2.572)	0.621
	Pregnant	109	82 (75.23)	2.24 (1.990-5.060)	0.048
Parity	0	108	48 (44.44)	1	
	1	164	93 (56.71)	1.64 (1.004-2.671)	0.048
	≥2	137	104 (75.91)	3.94 (2.283-6.796)	0.000

The result revealed that there was a significant association in the seroprevalence of IBR between the cattle breeds; the prevalence was higher in crossbred cattle (64.68%; 249/385) than in local cattle breeds (53.17%; 67/126). The odds of IBR seropositivity were significantly lower in local cattle breeds as compared to crossbred cattle (OR = 0.62; 95% CI: 0.41-0.93; p = 0.022). The prevalence of IBR was 37.78% in heifers, whereas it was 57.58% in dry cows, 62.15% in lactating cows, and 75.23% in pregnant cows. There was a significant association between IBR seroprevalence and the lactation and pregnancy status of the cow, where the

prevalence was very high in pregnant and lactating cows as compared to dry cows and heifers. The study indicated that pregnant cows were 2 (OR = 2.24, 95% CI: 1.99-5.06; p = 0.048) times at higher risk of IBR seropositivity as compared to dry cows, while heifers were less (OR = 0.45; 95% CI: 0.20-0.69; p = 0.047) at risk of the disease (Table 6).

The seroprevalence of IBR was 44.44% (48/108) in non-parous cows (no calving), 56.71% (93/164) in monoparous cows, and 75.91% (104/137) in multiparous cows. There was a statistically significant difference in the prevalence of infectious bovine rhinotracheitis among the number of parities in the cows. The seroprevalence of the disease was significantly higher (OR = 1.64; 95% CI: 1.004-2.671; p = 0.048) in monoparous cows, whereas multiparous cows were 4 (OR = 3.94; 95% CI: 2.28-6.80; p = 0.000) times at higher risk of IBR seropositivity as compared to non-parous cows. The result indicated that the seroprevalence of IBR was higher in older animals and multiparous cows (Table 6).

4.1.2. Association of IBR seropositivity with reproductive and respiratory clinical signs

The results of the present study indicated that IBR seroprevalence was significantly associated with a history of abortion, RFM, and the presence of ocular or nasal discharge. The prevalence of IBR was 60.22% in cows with no history of abortion and 88.89% in cows with a history of abortion. The cows with a history of abortion were 5 (OR = 5.28; 95% CI: 1.55-17.98; p = 0.008) times more at risk of IBR seropositivity than the cows without a history of abortion. The study revealed that the seroprevalence of IBR was higher in cows with a history of RFM (79.49%; 31/39) as compared to cows with no history of retained placenta (60.31%; 158/262). The odds of IBR seropositivity were 2.55 (95% CI: 1.13-5.77; p = 0.024) times higher in cows with a history of RFM than in cows without retained placenta. The results indicated that cows with a history of abortion and RFM had higher odds of IBR seropositivity (Table 7).

The current study showed that the seroprevalence of IBR was 62.86% (176/280) in cows with no history of dystocia, while the prevalence was 61.90% (13/21) in cows with a history of dystocia. Bulls with a history of orchitis had IBR seroprevalence of 50%, and the prevalence

of the disease in bulls without orchitis was 70.41%. There were no significant associations between the seroprevalence of IBR with the history of dystocia (p = 0.931) in cows, and orchitis (p = 0.397) in bulls (Table 7).

Table 7: Univariable logistic regression analysis of IBR seroprevalence in association with reproductive and respiratory clinical signs

Predictive variables	Groups	Total no. of animals	Positive (%)	OR (95% CI)	P-value
Abortion history	Absent	274	165 (60.22)	1	
	Present	27	24 (88.89)	5.28 (1.553-17.979)	0.008
RFM	Absent	262	158 (60.31)	1	
	Present	39	31 (79.49)	2.55 (1.128-5.766)	0.024
Dystocia	Absent	280	176 (62.86)	1	
	Present	21	13 (61.90)	0.96 (0.385-2.394)	0.931
Orchitis	Absent	98	69 (70.41)	1	
	Present	4	2 (50.00)	0.42 (0.056-3.129)	0.397
Ocular/nasal discharges	Absent	478	290 (60.67)	1	
	Present	33	26 (78.79)	2.41 (1.025-5.659)	0.044
Respiratory problems	Absent	447	277 (61.97)	1	
	Present	64	39 (60.94)	0.96 (0.559-1.638)	0.874

Animals with the clinical presence of ocular and/or nasal discharges had a seroprevalence of 78.79% of IBR, which was significantly higher than the prevalence of IBR in animals with the absence of ocular/nasal discharges (60.67%). Animals with ocular/nasal discharges were 2.41 (95% CI: 1.03-5.66; p = 0.044) times at higher risk for IBR seropositivity as compared to animals without the clinical signs. The seroprevalence of IBR was 60.94% (39/64) in animals with respiratory problems like coughing and 61.97% (277/447) in animals without respiratory problems. There was no significant association between IBR seropositivity and the presence of respiratory problems (p = 0.874). The insignificant association between IBR and respiratory problems suggests latent infections or the presence of other respiratory diseases (Table 7).

4.1.3. Seroprevalence of infectious bovine rhinotracheitis at the herd level

The overall seroprevalence of IBR at the herd level was 85.21% (95% CI: 78.28-90.21%). The study revealed that the seroprevalence of IBR at the herd level was 88.24% (60/68) in Angolela Tera district and 82.43% (61/74) in Kimbibit district. Even though, the herd prevalence of IBR was higher in Angolela Tera district than in Kimbibit district, there was no significant difference in the herd prevalence between the districts (p = 0.333). The herd prevalence of IBR was significantly associated with the location of herds/farms; the herd prevalence was higher in peri-urban (94.87%; 37/39) and urban (93.75%; 15/16) areas as compared to herds in rural areas (79.31%; 69/87). The herds located in rural areas had lower odds of the disease (OR = 0.21; 0.05-0.94; p = 0.042) as compared to herds located in peri-urban areas (Table 8).

There was a significant association between IBR herd prevalence and herd size; the prevalence was higher in herds with medium and large herd sizes as compared to herds with smaller herd sizes. The herd prevalence of IBR was 66.67% in herds with less than or equal to 5 herd sizes and 87.67% in herds with 6–10 herd sizes, while the prevalence was 90.91% in herds with 11–20 herd sizes and 100% in herds with greater than 20 herd sizes. The result showed that herds with 6–10 herd sizes had higher odds of IBR seropositivity (OR = 3.56; 95% CI: 1.23–10.28; p = 0.019) than herds with ≤5 herd sizes. Accordingly, herds with 11–20 herd sizes were 5 (95% CI: 1.20-20.92; p = 0.028) times more at risk of IBR seropositivity, whereas herds with greater than 20 herd sizes were 10 (OR = 9.76; 95% CI: 5.11-86.32; p = 0.013) times at higher risk of IBR seropositivity as compared to herds with less than or equal to 5 cattle (Table 8).

Table 8: Univariable logistic regression analysis of IBR seroprevalence at the herd level and associated risk factors

Predictive variables	Categories	Total no. of herds	Positive (%)	OR (95% CI)	P-value
District	Angolela Tera	68	60 (88.24)	1	
	Kimbibit	74	61 (82.43)	0.63 (0.242-1.618)	0.333
	Total	142	121 (85.21)		
Location	Peri-urban	39	37 (94.87)	1	
	Rural	87	69 (79.31)	0.21 (0.046-0.942)	0.042
	Urban	16	15 (93.75)	0.82 (0.068-9.627)	0.868
Herd size	≤5	27	18 (66.67)	1	
	6-10	73	64 (87.67)	3.56 (1.230-10.279)	0.019
	11-20	33	30 (90.91)	5.00 (1.195-20.922)	0.028
	>20	9	9 (100)	9.76 (5.109-86.321)	0.013
Production system	Extensive	122	101 (82.79)	1	
	Intensive	8	8 (100)	3.60 (0.201-64.789)	0.385
	Semi-intensive	12	12 (100)	5.30 (0.302-92.913)	0.254
Mating system	AI	24	22 (91.67)	1	
	Both	27	26 (96.30)	2.36 (0.201-27.852)	0.494
	Bull	91	73 (80.22)	0.37 (0.079-0.714)	0.032
Sharing of bulls	Absent	62	52 (83.87)	1	
	Present	80	69 (86.25)	1.21 (0.476-3.054)	0.692
Replacement strategy	Own	27	16 (59.26)	1	
	Purchased	6	5 (83.33)	3.44 (0.352-33.612)	0.289
	Both	109	100 (91.74)	7.64 (2.736-21.331)	0.000

The herd prevalence of IBR was 82.79% (101/122) in herds managed in extensive system, and 100% in herds under semi-intensive and intensive management systems. However, there was no statistically significant difference in the herd prevalence of the disease among the different production systems ($p > 0.05$). There was a significant association between herd prevalence of IBR and breeding (mating) systems. The herd prevalence was 91.67% (22/24) in herds using

only artificial insemination (AI), 96.30% (26/27) in herds using both AI and bull mating systems, and 80.22% (73/91) in herds using only natural (bull) mating systems. Herds using only natural mating systems had lower odds of IBR seropositivity (OR = 0.37; 95% CI: 0.08-0.71; p = 0.032) than herds using only AI systems (Table 8).

The presence of group mating or sharing of bulls was not significantly associated with IBR herd prevalence (p = 0.692); the prevalence was 83.87% in herds that did not share bulls and 86.25% in herds that shared bulls for mating. The herd prevalence of IBR was lower in herds that raise their own replacement (59.26%; 16/27) than in herds that depend on purchasing (83.33%; 5/6) and in herds employing both strategies of herd replacement (91.74%; 100/109). The current finding demonstrated a significant association between IBR herd prevalence and herd replacement strategies: herds that used both replacement strategies were over seven times (OR = 7.64; 95% CI: 2.74-21.33; p = 0.000) more likely to be IBR seropositive than herds that raised their own replacement herds (Table 8).

4.1.4. Multivariable logistic regression analysis of risk factors associated with IBR seroprevalence

The variables with a p-value of less than 0.25 on univariable logistic regression analyses were selected and further included in the final multivariable logistic regression analysis model. Multicollinearity was existed between the variables age and number of parities (Goodman and Kruskal's gamma statistics; gamma = 0.89); thus, the variable number of parities was dropped. Further selection of variables in the final model was based on a backward stepwise selection procedure. Finally, age groups, cattle breeds, abortion history, history of retained fetal membranes, and the presence of ocular or nasal discharge were subjected to the multivariable logistic regression model.

The results of the final multivariable logistic regression model indicated the association of predictor variables with IBR seroprevalence. Animals with the age of 3-6 years old were 3.2 (95% CI: 1.96-7.89; p = 0.005) times at higher risk of IBR seropositivity, while animals above

6 years old were over 9 (OR = 9.16; 95% CI: 3.09-27.16; p = 0.000) times more likely to be IBR seropositive as compared to animals in the reference group (animals below 3 years old, crossbred cattle, and animals without a history of abortion, RFM and ocular/nasal discharges). Local breeds of cattle had lower odds of the disease (OR = 0.48; 95% CI: 0.23-0.99; p = 0.004) as compared to the crossbred cattle (Table 9).

The multivariable logistic regression model depicted that the cows with a history of abortion and retained fetal membrane were 4.51 (95% CI: 1.23-16.53; p = 0.019) and 2.75 (95% CI: 1.72-4.22; p = 0.029) times more likely to be IBR seropositive than cows without a history of abortion and retained fetal membrane, respectively. Animals with a clinical sign of ocular and/or nasal discharges were nearly three times (OR = 2.83; 1.86-9.31; p = 0.030) at higher risk of IBR seropositivity as compared to animals with absence of the clinical signs (Table 9).

Table 9: Multivariable logistic regression analysis of the potential risk factors associated with IBR seropositivity in cattle

Predictive variables	**Categories**	**OR**	**95% CI**	**P-value**
Age	<3	1		
	3-6	3.20	1.961-7.887	0.005
	>6	9.16	3.089-27.158	0.000
Breed	Cross	1		
	Local	0.48	0.231-0.992	0.004
Abortion history	Absent	1		
	Present	4.51	1.230-16.527	0.019
RFM	Absent	1		
	Present	2.75	1.723-4.217	0.029
Ocular/Nasal	Absent	1		
discharges	Present	2.83	1.857-9.310	0.030

4.2. Seroprevalence of Bovine Brucellosis

4.2.1. Seroprevalence of bovine brucellosis at the animal level

Among 511 serum samples screened by RBPT, 7 (1.37%) were found positive for bovine brucellosis. Samples positive on RBPT were further subjected to CFT. Accordingly, of the 7 positive samples tested by RBPT, 5 samples were confirmed to be seropositive for brucellosis. Therefore, the overall seroprevalence of bovine brucellosis was 0.98% (95% CI: 0.41-2.34%) in the present study areas on combined RBPT and CFT tests. The study revealed that the prevalence of bovine brucellosis was 1.45% (4/276) in Angolela Tera district, and 0.43% (1/235) in Kimbibit district. There was a significant difference in the seroprevalence of bovine brucellosis between the districts. The univariable firth's bias reduced logistic regression analysis indicated that the odds of bovine brucellosis in animals in Kimbibit district were lower (OR = 0.39; 95% CI: 0.06-0.48; p = 0.046) than animals in Angolela Tera district (Table 10).

The seroprevalence of bovine brucellosis was higher in smallholder farms (1.15%; 3/261) as compared to commercial farms (0.80%; 2/250). However, there was no significant association between the seroprevalence of bovine brucellosis and the farm types (p = 0.721). In other ways, the seroprevalence of bovine brucellosis was 0.98% in both female (4/409) and male (1/102) animals. Therefore, there was no significant difference in the seroprevalence of bovine brucellosis (p = 0.763) between female and male animals (Table 10).

The prevalence of bovine brucellosis was 0% in animals below 3 years old, 0.81% in animals 3-6 years old, and 1.70% in animals above 6 years old. The result showed that there was a significant association in the seroprevalence of bovine brucellosis among age groups. Animals above 6 years old were 3.5 (OR = 3.53; 95% CI: 1.18-9.11; p = 0.045) times at higher risk of brucellosis seropositivity as compared to animals below 3 years old. The prevalence of the disease was 0.26% (1/385) in crossbred cattle and 3.17% (4/126) in local cattle breeds. The result indicated that there was a significant difference in the seroprevalence of brucellosis between the cattle breeds, where the local breeds of cattle were over nine (OR = 9.42; 95% CI:

1.47-60.43; p = 0.018) times more likely to be seropositive for bovine brucellosis as compared to the crossbred cattle (Table 10).

Table 10: Univariable firth's bias-reduced logistic regression analysis of bovine brucellosis seroprevalence at animal level and associated risk factors

Predictive variables	Categories	Total no. of animals	Positive (%)	OR (95% CI)	P-value
District	Angolela Tera	276	4 (1.45)	1	
	Kimbibit	235	1 (0.43)	0.39 (0.061-0.479)	0.046
	Total	511	5 (0.98)		
Farm types	Commercial	250	2 (0.80)	1	
	Smallholder	261	3 (1.15)	1.35 (0.263-6.885)	0.721
Sex	Female	409	4 (0.98)	1	
	Male	102	1 (0.98)	1.33 (0.207-8.560)	0.763
Age	<3	87	0 (0.00)	1	
	3-6	248	2 (0.81)	1.77 (0.084-37.332)	0.712
	>6	176	3 (1.70)	3.53 (1.180-9.109)	0.045
Breed	Cross	385	1 (0.26)	1	
	Local	126	4 (3.17)	9.42 (1.467-60.429)	0.018
Status of the cow	Dry	33	0 (0.00)	1	
	Heifer	90	0 (0.00)	0.37 (0.007-19.032)	0.621
	Lactating	177	3 (1.69)	1.34 (0.068-26.621)	0.846
	Pregnant	109	1 (0.92)	0.93 (0.037-23.275)	0.963
Parity	0	108	0 (0.00)	1	
	1	164	1 (0.61)	1.99 (0.080-49.320)	0.674
	≥2	137	3 (2.19)	5.65 (3.466-110.504)	0.038

The seroprevalence of brucellosis was 0% in heifers and dry cows, while the prevalence was 1.69% (3/177) in lactating cows and 0.92% (1/109) in pregnant cows. The odds of brucellosis seropositivity were not significantly different with the lactation (p = 0.846) and pregnancy (p = 0.963) status of the cows. The number of parities showed a significant association with the

seroprevalence of bovine brucellosis; the seroprevalence of the disease was 0% in non-parous cows, 0.61% in monoparous cows, and 2.19% in multiparous cows. Therefore, multiparous cows were nearly six times (OR = 5.65; 95% CI: 3.47-110.50; p = 0.038) at higher risk of bovine brucellosis than non-parous cows (Table 10).

4.2.2. Association of bovine brucellosis seroprevalence with reproductive disorders

The results of the present study indicated that the history of reproductive problems were significantly associated with bovine brucellosis seropositivity. The prevalence of brucellosis was 11.11% (3/27) in cows with abortion history, and 0.36% (1/274) in cows without abortion. Accordingly, the cows with a history of abortion were 26 (95% CI: 3.68-184.34; p = 0.001) times more likely to be brucellosis seropositive as compared to those cows without abortion. The cows with a history of RFM had 7.69% (3/39) seroprevalence of brucellosis, while the prevalence was 0.38% (1/262) in cows without retained fetal membrane. The odds of bovine brucellosis in cows with retained fetal membrane were nearly 17 (OR = 16.72; 95% CI: 2.39-116.78; p = 0.005) times higher as compared to the cows without RFM (Table 11).

The prevalence of brucellosis was higher in cows with a history of dystocia (9.52%; 2/21) than in cows with the absence of dystocia (0.71%; 2/280). The cows with a history of dystocia were more than 14 (OR = 14.28; 95% CI: 2.33-87.46; p = 0.004) times at higher risk of bovine brucellosis in comparison with the cows that had not experienced dystocia. The study depicted that the prevalence of bovine brucellosis was 25% (1/4) in bulls with a history of orchitis, and no seropositivity was found in bulls without orchitis. The results indicated that animals with reproductive disorders had higher odds of bovine brucellosis seropositivity as compared to those animals free from reproductive problems (Table 11).

Table 11: Univariable firth's bias-reduced logistic regression analysis of bovine brucellosis seroprevalence in association with reproductive clinical signs

Predictive variables	Groups	Total no. of animals	Positive (%)	OR (95% CI)	P-value
Abortion history	Absent	274	1 (0.36)	1	
	Present	27	3 (11.11)	26.05 (3.680-184.344)	0.001
RFM	Absent	262	1 (0.38)	1	
	Present	39	3 (7.69)	16.72 (2.393-116.775)	0.005
Dystocia	Absent	280	2 (0.71)	1	
	Present	21	2 (9.52)	14.28 (2.332-87.459)	0.004
Orchitis	Absent	98	0 (0.00)	1	
	Present	4	1 (25.00)	84.43 (2.893-463.647)	0.010

4.2.3. Seroprevalence of bovine brucellosis at the herd level

The present study revealed that the overall herd prevalence of bovine brucellosis was 3.52% (95% CI: 1.46-8.26%) in the study areas. The herd prevalence of bovine brucellosis was 5.88% in Angolela Tera district and 1.35% in Kimbibit district. The result indicated that there was a significant difference in the seroprevalence of bovine brucellosis at the herd level between the districts, where the odds of brucellosis were lower (OR = 0.29; 95% CI: 0.05-0.91; p = 0.043) in herds in Kimbibit district as compared to the herds in Angolela Tera district. The herd prevalence of brucellosis was 0% (0/16) in herds in urban areas, 5.13% (2/39) in peri-urban areas, and 3.45% (3/87) in rural areas. Even though, there was found no brucellosis seropositivity in herds located in urban areas, there was no statistically significant difference (p = 0.617) in herd prevalence of brucellosis with herd location (Table 12).

The herd prevalence of bovine brucellosis was 0% (0/27) in herds with less than or equal to 5 herd sizes, 4.11% (3/73) in herds with 6-10 herd sizes, 3.03% (1/33) in herds with 11-20 herd sizes, and 11.11% (1/9) in herds with greater than 20 herd sizes. However, there were no significant differences between the odds of herd prevalence of brucellosis among the different

herd sizes ($p > 0.05$). The prevalence of bovine brucellosis at the herd level was (2.46%; 3/122) in herds under extensive management systems, (8.33%; 1/12) in herds with semi-intensive management systems, and (12.50%; 1/8) in herds under intensive management systems. Even though, there was no significant difference in the odds of the disease, 3 of the total 5 positive herds were herds under extensive management systems (Table 12).

The current result indicated that there was a significant association in the herd prevalence of bovine brucellosis with the presence of group mating or sharing of bulls. Bovine brucellosis herd prevalence was 5.00% in herds that shared bulls for mating and 1.61% in herds that did not share bulls. The cattle herds that shared bulls for mating were over 3 (OR = 3.12; 95% CI: 1.37-15.78; $p = 0.035$) times more likely to be seropositive for bovine brucellosis as compared to herds that did not share bulls. However, the result depicted that there was no significant association between herd prevalence of brucellosis and mating systems. The herd prevalence of brucellosis was 3.30% (3/91) in herds using only natural mating, 3.70% (1/27) in herds using both AI and bull mating, and 4.17% (1/24) in herds using only AI systems (Table 12).

The herd prevalence of brucellosis was higher in herds that used purchased replacements (33.33%; 2/6), as compared to herds that raised their own replacements (3.70%; 1/27) and herds using both replacement strategies (1.83%; 2/109). There was a significant difference; the odds of bovine brucellosis were 9.81 (95% CI: 1.03-93.82; $p = 0.047$) times higher in herds that were based on purchasing for herd replacement as compared to the herds that raised their own replacement herds (Table 12).

Table 12: Univariable firth's bias-reduced logistic regression analysis of bovine brucellosis seroprevalence at the herd level and associated risk factors

Predictive variables	Categories	Total no. of herds	Positive (%)	OR (95% CI)	P-value
District	Angolela Tera	68	4 (5.88)	1	
	Kimbibit	74	1 (1.35)	0.29 (0.045-0.913)	0.043
	Total	142	5 (3.52)		
Location	Peri-urban	39	2 (5.13)	1	
	Rural	87	3 (3.45)	0.62 (0.117-3.294)	0.576
	Urban	16	0 (0.00)	0.45 (0.021-10.000)	0.617
Herd size	1-5	27	0 (0.00)	1	
	6-10	73	3 (4.11)	2.73 (0.137-54.612)	0.511
	11-20	33	1 (3.03)	2.54 (0.099-64.858)	0.573
	>20	9	1 (11.11)	9.71 (0.361-60.987)	0.176
Production system	Extensive	122	3 (2.46)	1	
	Intensive	8	1 (12.50)	6.83 (0.879-53.048)	0.066
	Semi-intensive	12	1 (8.33)	4.45 (0.599-33.110)	0.144
Mating system	AI	24	1 (4.17)	1	
	Both	27	1 (3.70)	0.89 (0.086-9.117)	0.920
	Bull	91	3 (3.30)	0.62 (0.087-4.425)	0.633
Sharing of bulls	Absent	62	1 (1.61)	1	
	Present	80	4 (5.00)	3.12 (1.369-15.776)	0.035
Replacement strategy	Own	27	1 (3.70)	1	
	Purchased	6	2 (33.33)	9.81 (1.027-93.819)	0.047
	Both	109	2 (1.83)	0.41 (0.052-3.251)	0.399

4.2.4. Multivariable firth's bias-reduced logistic regression analysis of risk factors associated with bovine brucellosis seroprevalence

Multivariable firth's bias-reduced logistic regression analysis model was built to identify the important risk factors and predictors of bovine brucellosis with the variables with a p-value of

less than 0.25 on univariable firth's bias-reduced logistic regression analyses. The variables with collinearity effects (parity with age: gamma = 0.89; and dystocia with RFM: p = 0.004) were dropped from the model. The final model was based on the backward elimination of less significant variables. The variables, which included age, cattle breed, history of abortion and RFM were finally fitted to the multivariable regression model.

The result indicated that the odds of bovine brucellosis increased by 3.82 (95% CI: 1.71-7.97; p = 0.004) times in animals above 6 years old as compared to the animals in the reference group (animals below 3 years old, crossbred cattle, cows without a history of abortion and retained fetal membrane). The local cattle breeds had higher odds (OR = 6.20; 95% CI: 2.08-8.21; p = 0.016) of brucellosis seropositivity. The multivariable firth's bias-reduced logistic regression model indicated that a history of abortion increased the odds of brucellosis by 22.35 (95% CI: 3.90-107.91; p = 0.002) times as compared to cows without abortion history. The cows with a history of RFM were 18.74 (95% CI: 3.48-63.86; p = 0.020) times more at risk of brucellosis seropositivity as compared to cows without retained placenta (Table 13).

Table 13: Multivariable firth's bias-reduced logistic regression analysis of potential risk factors associated with brucellosis seropositivity in cattle

Predictive variables	Categories	OR	95% CI	P-value
Age	<3	1		
	3-6	2.31	0.573-9.976	0.148
	>6	3.82	1.712-7.965	0.004
Breed	Cross	1		
	Local	6.20	2.075-8.206	0.016
Abortion history	Absent	1		
	Present	22.35	3.896-107.913	0.002
RFM	Absent	1		
	Present	18.74	3.483-63.863	0.020

4.3. Questionnaire Survey

4.3.1. Socio-demographic characteristics

The questionnaire survey was conducted on 120 respondents (dairy farm owners, attendants, and local farmers), of whom 60 (50%) were from Angolela Tera district and the remaining 60 (50%) were from Kimbibit district. The survey result indicated that the respondents have different age levels, with a minimum age of 21 years and a maximum age of 70 years, with an average age of 42.97 $\pm$ 1.07 years old. The majority of respondents (50.83%) were categorized between the ages of 30-50 years, followed by those above 50 years old (30%). In the present study, out of 120 respondents, 90 (75%) were male and 30 (25%) were female respondents. The educational status of the respondents ranged from illiterate to attending higher education, with the majority of respondents (31.67%) were attended primary school and 27.50% were uneducated. Most of the respondents (48.34%; 58/120) were from rural areas, and (35.83%; 43/120) were from peri-urban areas (Table 14).

4.3.2. Cattle production system

The present study revealed that cattle were the most important livestock species (94.17%) in the study areas. The result indicated that the primary purpose of cattle production was for draught power (65.83%; 79/120), for milk production (25.84%; 31/120), and for market or cash needs (8.33%; 10/120). The majority of households (55.84%) owned small herd sizes (6–10 cattle), while 27.50% households owned medium herd sizes (11–20 cattle), followed by very small ($\leq$5 cattle) and large (>20 cattle) herd sizes (8.33%). Regarding the cattle population and production systems, the study showed that the majority of cattle herds (55%) comprised both local and crossbred cattle breeds, and that 74.17% (89/120) of the cattle production is under extensive management systems. Nearly half of the respondents (49.17%; 59/120) indicated that they used natural (bull) mating systems, while 41.66% (50/120) of households used both bull mating and artificial insemination (AI) systems. The use of group mating or the sharing of bulls was present in 62.50% (75/120) of cattle herds (Table 15).

Table 14: Socio-demographic characteristics of the respondents in the study areas

Demographic variables	Groups	Proportions	Percentage (%)
District	Angolela Tera	60	50
	Kimbibit	60	50
	Total	120	100
Age	<30	23	19.17
	30-50	61	50.83
	>50	36	30.00
Sex	Female	30	25
	Male	90	75
Educational level	College/University	7	5.83
	Illiterate	33	27.50
	Informal	18	15.00
	Primary School	38	31.67
	Secondary School	24	20.00
Location	Peri-urban	43	35.83
	Rural	58	48.34
	Urban	19	15.83

4.3.3. Major reproductive problems

The present study indicated the occurrence of various reproductive problems in dairy cattle on commercial and smallholder dairy farms in the present study areas. The findings revealed that reproductive disorders and infertility problems were very important constraints for the cattle production and dairy industries. The most common reproductive disorders in dairy farms and herds were retained fetal membrane (RFM) and abortion, with 40% (48/120) and 32.50% (39/120) herd prevalence, respectively (Figure 5). Whereas, the results revealed that dystocia had occurred in 22.50% of cattle herds. Accordingly, the prevalence of cervical and/or vaginal prolapses was 14.17% (17/120). The mortality of calves was the other important problem in

the central highlands of Ethiopia. The current finding revealed that neonatal calves' mortality had occurred in 35% of herds (Table 16).

Table 15: Cattle production, management systems, and purposes of cattle keeping in central highlands of Ethiopia

Production variables	Groups	Proportions	Percentage (%)
Most important livestock species	Cattle	113	94.17
	Others	7	5.83
	Total	120	100
Primary purpose of cattle production	Draft	79	65.83
	Market/ Cash	10	8.33
	Milk	31	25.84
Herd size	≤5	10	8.33
	6-10	67	55.84
	11-20	33	27.50
	>20	10	8.33
Cattle breed	Both	66	55.00
	Cross	26	21.67
	Local	28	23.33
Production system	Extensive	89	74.17
	Intensive	5	4.16
	Semi-intensive	26	21.67
Mating system	AI	11	9.17
	Both	50	41.66
	Bull	59	49.17
Group mating	Absent	45	37.50
	Present	75	62.50

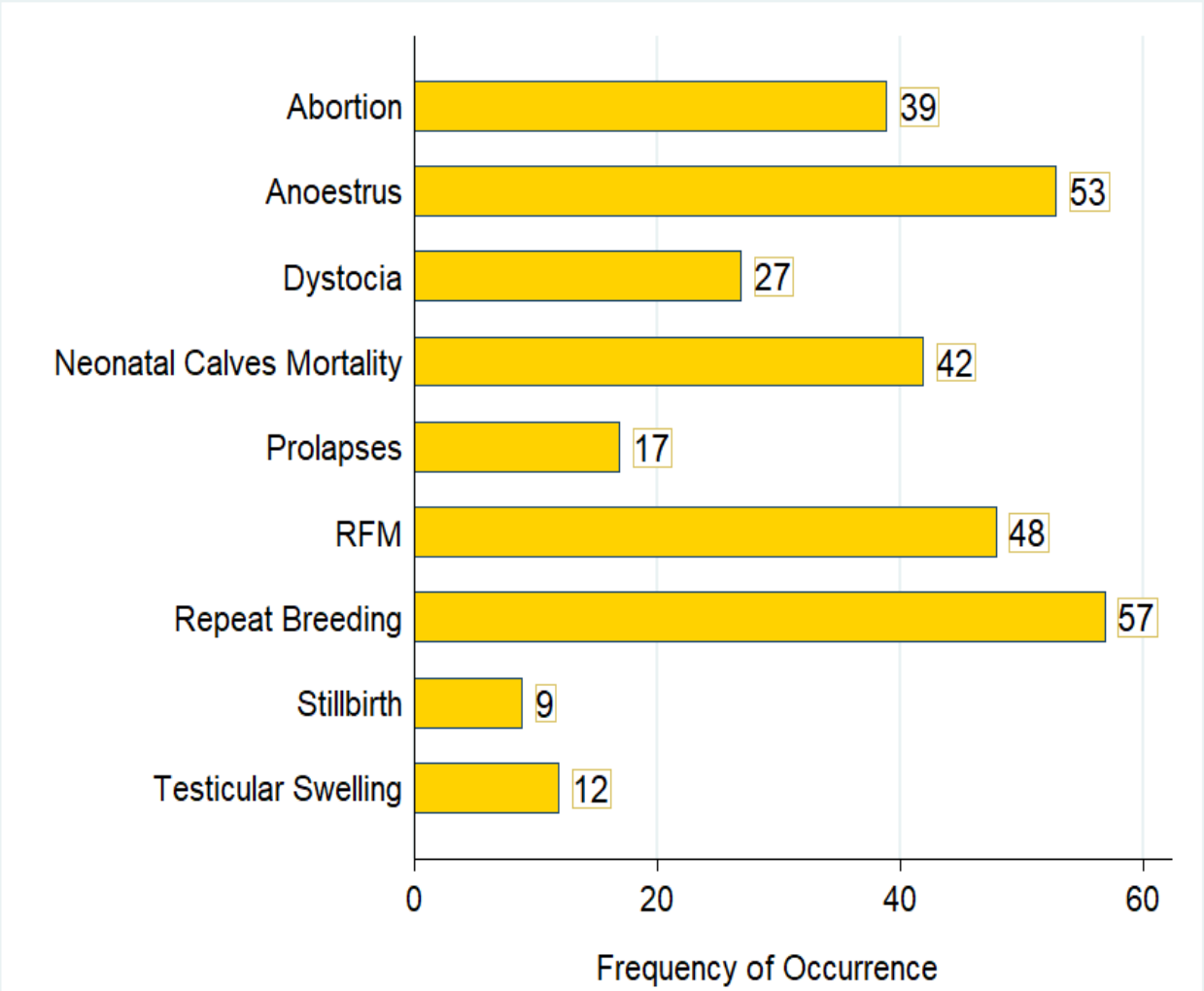

Figure 5: Major reproductive problems in dairy cattle herds/farms in the study areas

Stillbirth became the least common reproductive problem in the study areas, with only 7.50% (9/120) herd prevalence. The present study revealed a higher prevalence of infertility problems on dairy farms and herds. Repeat breeding was found to be the most commonly occurring reproductive problem with 47.50% herd prevalence, followed by anoestrus, which occurred in 44.17% of herds (Figure 5). About 10% (12/120) of respondents indicated the occurrence of testicular swelling in bulls in their herds, while 23.33% (28/120) respondents had experienced lameness/joint swelling in their herds (Table 16).

Table 16: The types of major reproductive problems and their prevalence in the cattle herds

Reproductive problems	Categories	Proportions	Percentage (%)
Abortion	Absent	81	67.50
	Present	39	32.50
	Total	120	100
RFM	Absent	72	60.00
	Present	48	40.00
Dystocia	Absent	93	77.50
	Present	27	22.50
Uterine/cervical prolapses	Absent	103	85.83
	Present	17	14.17
Stillbirth	Absent	111	92.50
	Present	9	7.50
Neonatal calves' mortality	Absent	78	65.00
	Present	42	35.00
Repeat breeding	Absent	63	52.50
	Present	57	47.50
Anoestrus	Absent	67	55.83
	Present	53	44.17
Testicular swelling	Absent	108	90.00
	Present	12	10.00
Lameness/joint swelling	Absent	92	76.67
	Present	28	23.33

4.3.4. Association of major reproductive problems with cattle production and management factors

The prevalence of major reproductive problems was analyzed with respect to different cattle production factors using the Chi-square measure of association. The result indicated that the occurrence of abortion was significantly associated with cattle breeds, production systems, and the presence of group mating. The cattle breeds in the herd were significantly associated with

the occurrence of abortion (p = 0.039), where abortion had occurred in 25.00% (7/28) of cattle herds constituting only local breeds, 28.79% (19/66) of cattle herds constituting both local and crossbred cattle, and 50% (13/26) of cattle herds constituting only crossbred cattle (Table 17).

The prevalence of abortion had a significant variation (p = 0.042) in different production systems and was higher in herds under semi-intensive management system (46.15%; 12/26) followed by the intensive (40%; 2/5) and extensive management systems (28.09%; 25/89). Accordingly, abortion was also significantly associated with the presence of group mating or sharing of bulls in the herds (p = 0.024), and was higher in herds with the presence of group mating (40%). The prevalence of retained fetal membrane (RFM) was significantly associated with cattle production systems (p = 0.041), where RFM had occurred in 29/89 (32.58%), 5/5 (100%), and 14/26 (53.85%) cattle herds under extensive, intensive, and semi-intensive management systems, respectively. There was no significant variation in the prevalence of RFM among cattle herds with different herd sizes, cattle breeds, mating systems, or the presence of group mating (Table 17).

The present study indicated that the prevalence of repeated breeding was significantly associated with mating systems (p = 0.035). Repeat breeding had occurred in 8/11 (72.73%), 26/50 (52.00%), and 23/59 (38.98%) cattle herds that had used AI systems, both AI and bull mating, and only bull mating, respectively. In other ways, the prevalence of anoestrus was significantly associated with cattle breeds (p = 0.044) and mating systems (p = 0.011). The study revealed that anoestrus had occurred in 60.71% of herds with local cattle breeds, 43.94% of herds with both local and crossbred cattle, and 26.92% of crossbred cattle herds. The prevalence of anoestrus was higher (55.93%) in cattle herds that utilized a natural (bull) mating system (Table 17).

Table 17: The prevalence of major reproductive problems in association with cattle production and management factors

Variables	Abortion (%)	X^2	P-value	RFM (%)	X^2	P-value	Repeat breed. (%)	X^2	P-value	Anoestrus (%)	X^2	P-value
Herd size												
≤5	30.00	2.85	0.416	40.00	3.38	0.337	50.00	2.58	0.462	40.00	1.35	0.718
6-10	28.36			40.30			50.75			41.79		
11-20	39.39			33.33			36.36			45.45		
>20	40.00			60.00			50.00			60.00		
Cattle breed												
Both	28.79	7.42	0.039	34.85	2.81	0.245	48.48	0.33	0.847	43.94	6.25	0.044
Cross	50.00			53.85			50.00			26.92		
Local	25.00			39.29			42.86			60.71		
Production system												
Extensive	28.09	6.45	0.042	32.58	6.64	0.041	47.19	1.37	0.503	48.31	4.58	0.101
Intensive	40.00			100			80.00			40.00		
Semi-intensive	46.15			53.85			42.31			30.77		
Mating system												
AI	27.27	0.54	0.765	45.45	0.16	0.922	72.73	7.49	0.035	54.55	9.02	0.011
Both	30.00			40.00			52.00			28.00		
Bull	35.59			38.98			38.98			55.93		
Group mating												
Absent	20.00	5.13	0.024	42.22	0.15	0.700	53.33	0.98	0.322	44.44	0.27	0.962
Present	40.00			38.67			44.00			44.00		

4.3.5. Farmers' knowledge, attitudes and practices (KAP) assessment

The present study revealed that most of the respondents (55.83%; 67/120) had been involved in assisting the cows during parturition in the study areas. However, nearly 75% (50/67) of the respondents had not used protective materials such as gloves, plastic materials, or masks. The majority of respondents disposed of fetal membranes, retained placenta, and aborted fetuses by throwing them into water canals or dips (52.50%) and placing them in open dumps or fields (30.83%). The result indicated that the practice of raw milk consumption was 26.67% (32/120), while 64.17% (77/120) of the respondents had the practice of consuming raw meat in present study areas. However, most of the respondents (76.67%; 92/120) had no awareness of the disease brucellosis, where only 23.33% of the respondents were aware of brucellosis as the disease causing various reproductive problems in animals (Table 18).

In the current study areas, the risk of zoonotic transmission of brucellosis was unknown by most of the respondents (85.83%; 103/120). Accordingly, the respondents who understood the zoonotic infection of brucellosis were only 14.17% (17/120). Hence, the study indicated that 17 respondents (14.17%) understood the zoonotic transmission of brucellosis by contact with aborted fetuses, placenta, and fetal membranes. However, only 9.17% of the respondents were aware of the zoonotic transmission of brucellosis through drinking contaminated raw milk. The current findings indicate lower rates of awareness of zoonotic brucellosis (Table 18).

4.3.6. Association of KAP level with socio-demographic characteristics

The Chi-square measure of association on the KAP level with regard to socio-demographic factors revealed that raw milk consumption was significantly associated with the age of the respondents. The majority of raw milk consumers were age of above 50 years old (44.44%; $p = 0.015$). The result indicated that the practices of raw milk consumption was also significantly varied between districts ($p = 0.039$), where a higher raw milk consumption was found in Kimbibit district (21/60) than in Angolela Tera district (11/60). The awareness of disease transmission through raw milk and meat consumption was significantly associated with age

groups (p = 0.008), where respondents below 30 years old were the most aware (69.56%), while the least aware (38.88%) age groups were those above 50 years old (Table 19).

The educational status of the respondents was significantly associated with the awareness of disease transmission through raw milk and meat consumption (p = 0.006), where respondents who attended higher education (100%), secondary school (79.17%), and primary school (63.16%) were understood the risk of diseases through raw milk and meat consumption. Accordingly, the location or residency of respondents was significantly associated (p = 0.029) with awareness of disease transmission through raw milk and meat consumption that the respondents in urban (63.16%) and peri-urban (74.42%) areas being more aware of the risk than those in rural (48.28%) areas (Table 19).

The use of protective materials was significantly associated with age groups (p = 0.005), educational levels (p = 0.001), and location/residency (p = 0.025). The use of protective materials such as gloves and masks when assisting the cows' during parturition was higher in respondents below 30 years old (34.78%) as compared to the other age groups of respondents. Similarly, the respondents who attended higher education (71.43%) and those who were urban residents (31.58%) had relatively higher practices of using protective materials while assisting parturition (Table 19).

The current study indicated that awareness of the zoonotic transmission of brucellosis was significantly associated with the educational level (p = 0.000) and location (p = 0.020) of the respondents. The results showed that awareness of the zoonotic transmission of brucellosis was higher among educated respondents, with 43% of those attending higher education and 25.00% in secondary school. Regarding the location or residency of the respondents, 8/43 (18.60%), 4/58 (6.90%), and 5/19 (26.32%) were aware of the zoonotic risk of brucellosis in peri-urban, rural, and urban areas, respectively. The sex category did not show any significant associations in the present findings (Table 19).

Table 18: Knowledge, attitudes and practices (KAP) of the respondents regarding brucellosis and its zoonotic transmission

KAP variables	Categories	Proportions	Percentage (%)
Assisting cows during parturition	Absent	53	44.17
	Present	67	55.83
	Total	120	100
Use of protective gloves	Absent	50	74.63
	Present	17	25.37
Fetal membranes and aborted fetuses' disposal	Burying/burning	11	9.17
	Thrown on field or Open dumps	37	30.83
	Water canal/dips	63	52.50
	To carnivores	9	7.50
Raw milk consumption	Absent	88	73.33
	Present	32	26.67
Raw meat consumption	Absent	43	35.83
	Present	77	64.17
Awareness on diseases transmission through raw milk and meat consumption	Absent	48	40.00
	Present	72	60.00
Awareness on brucellosis in animals	Absent	92	76.67
	Present	28	23.33
Awareness on zoonotic transmission of brucellosis	Absent	103	85.83
	Present	17	14.17
Zoonotic transmission by contact with fetal membranes	Absent	103	85.83
	Present	17	14.17
Zoonotic transmission through raw milk consumption	Absent	109	90.83
	Present	11	9.17

Table 19: The association of knowledge, attitudes and practices of the respondents with socio-demographic characteristics

Variables	Raw milk consumption (%)	X^2	P-value	Diseases trans. with raw milk and meat (%)	X^2	P-value	Zoonotic Brucellosis (%)	X^2	P-value	Use of protective materials	X^2	P-value
Age												
<30	21.74	8.43	0.015	69.57	9.55	0.008	21.74	2.98	0.225	34.78	10.59	0.005
30-50	18.03			68.85			14.75			11.48		
>50	44.44			38.89			8.33			5.56		
Sex												
Female	26.67	0.00	1.000	56.67	0.27	0.893	16.67	0.64	0.424	16.67	0.21	0.650
Male	26.67			61.11			13.33			13.33		
Educational level												
Higher Edu.	28.57	2.28	0.684	100.00	14.56	0.006	42.86	23.2	0.000	71.43	23.05	0.001
Illiterate	30.30			42.42			6.06			6.06		
Informal	22.22			44.44			5.56			5.56		
Primary S.	21.05			63.16			13.16			10.53		
Secondary S.	28.57			79.17			25.00			20.83		
Location												
Per-urban	23.26	0.52	0.773	74.42	7.13	0.029	18.60	7.79	0.020	16.28	7.42	0.025
Rural	27.59			48.28			6.90			6.90		
Urban	31.58			63.16			26.32			31.58		
District												
Angolela Tera	18.33	4.26	0.039	63.33	0.56	0.456	15.00	0.35	0.553	11.67	0.62	0.432
Kimbibit	35.00			56.67			13.33			16.67		

5. DISCUSSION

The present study indicated the significance and distribution of infectious bovine rhinotracheitis (IBR), bovine brucellosis, and major reproductive problems in North Shewa, the central highlands of Ethiopia. The result of the current study showed a higher seroprevalence of IBR that the overall animal-level seroprevalence was 61.84% (95% CI: 57.53-65.97%) in the study areas. Accordingly, studies conducted on the prevalence of IBR in Ethiopia were also reported such a higher seroprevalence of the disease. The result in this study was lower than the studies that reported IBR prevalence of 67% in Gobe and Ghibe (Bekele *et al*., 1989), 77.6% in North Western Ethiopia (Zewde *et al*., 2021), and 79.1% in central Ethiopia (Messele *et al*., 2021).

However, the seroprevalence of IBR in the present study was relatively higher than the findings that reported 41.80% on the first IBR investigation in the country (Lefevre, 1975), 41% in major milkshed areas of Ethiopia (Sibhat *et al*., 2018), and 25.60% in South Wollo (Tadeg *et al*., 2021). These variations in the seroprevalence of IBR in the country might be seen due to the difference in study animal population, management systems and study areas. The presence of animal movements, poor farm biosecurity measures, high stocking densities and mismanagement practices increase the risk of introduction and transmission of the disease.

The finding of the current study was in close agreement with the seroprevalence studies of IBR that reported 61.54% in Southern India (Krishnamoorthy *et al*., 2015), 62.50% in Iraq (Rhaymah *et al*., 2012), and 64.50% in Mexico (Romero-Salas *et al*., 2013). However, the present finding was very lower than the findings that reported 85.7% in Egypt (Mahmoud *et al*., 2009), 93.75% in Sudan (Hekal *et al*., 2019), and 96.2% in Korea (Choi *et al*., 2014). In contrast, the current result was very higher than the findings that reported IBR prevalence of 16.1% in Cameroon (Daniel *et al*., 2018), 17.1% in Turkey (Yeşilbag and Gungor, 2008), and 17.4% in Meru County, Kenya (Kipyego *et al*., 2020). These differences in the prevalence of IBR could be due to the variations in disease introduction, status and distribution, and control and prevention strategies in different countries. Some countries that implement robust disease surveillance and control systems significantly reduce the incidence and impact of the disease.

In the present study, the univariable logistic regression analysis revealed that there was no significant difference in the seroprevalence of IBR between the districts. However, the result indicated that the prevalence was higher in Kimbibit (66.38%) than in Angolela Tera district (57.97%). Similarly, Tadeg *et al*. (2021) also revealed insignificant association in the disease prevalence between the districts. In contrast to this finding, significant associations of IBR prevalence among districts were reported by the studies conducted in major milkshed areas of Ethiopia (Sibhat *et al*., 2018), and Northwestern, Ethiopia (Zewde *et al*., 2021). The difference in the study animal populations, production systems, or status of the disease and potential factors in the districts might be the reasons responsible for the variations in the findings.

The insignificant association in seroprevalence of IBR between commercial and smallholder farms suggested the cosmopolitan distribution of the disease and poor biosecurity measures in the study areas. Culled animals, breeding bulls and heifers from the commercial dairy farms were sold to the local farmers. In agreement with the current finding, studies also reported that there was no significant difference in the prevalence of the disease between commercial and smallholder farms (Sibhat *et al*., 2018; Kipyego *et al*., 2020). There was also no significant association in the prevalence of IBR with sex groups. The current finding was in agreement with several studies (Derrar *et al*., 2019; Zewde *et al*., 2021; Selim *et al*., 2022; Patel *et al*., 2023) that showed no significant variation in the prevalence of IBR between male and female animals. However, Saravanajayam *et al*. (2015) reported that there was a significant association in the prevalence of IBR with sex group that the prevalence was higher in females. This variation in the association of IBR prevalence between sex groups was seen due to the error in sampling proportions, as males were only 2% of the total sample size in their study.

The finding of the present study indicated that there was a very significant association in the prevalence of IBR with age of the animals. Animals above six years old were nearly 6 times (OR = 5.58; 95% CI: 3.18-9.78, p = 0.000) more likely to be IBR seropositive than animals below 3 years old. The result was in agreement with the findings of several studies (Carbonero *et al*., 2011; Saravanajayam *et al*., 2015; Sibhat *et al*., 2018; Patel *et al*., 2023). In contrast to these findings, (Paudel *et al*., 2022; Selim *et al*., 2022) reported insignificant associations in

IBR prevalence with the age of the animal. The differences might be seen due to variations in age classifications and study animal populations. The higher prevalence of IBR in adults as compared to young animals indicated an increasing trend in exposure to the virus and predisposing factors as age of the animal advances, rather than age-dependent susceptibility to the virus.

The current study showed a significant association between the prevalence of IBR and cattle breeds (OR = 0.62; 95% CI: 0.41-0.93; p = 0.022), the prevalence was higher in crossbred cattle than local breeds. Similar findings were reported in Spain (Gonzalez-Garcia *et al*., 2009), in India (Saravanajayam *et al*., 2015), and in Colombia (Ortiz-González *et al*., 2022). Whereas, (Sibhat *et al*., 2018; Derrar *et al*., 2019) reported insignificant associations of IBR prevalence with cattle breed types. The types of studied cattle breeds and management systems could be the factors that resulted in such variations among the findings. The movement of crossbred cattle from farms to farms as a replacement, and the distribution of improved crossbred dairy cattle into the smallholder farmers increase the risk of IBR infection in crossbred cattle breeds.

The prevalence of IBR was higher (75.23%) in pregnant cows, and lower in heifers and dry cows. Pregnant cows were 2.24 (OR = 2.24; 95% CI: 1.99-5.06; p = 0.048) times at higher risk for IBR seropositivity. The result of the current study agreed with the findings reported in dairy cattle herds in Poland (Rypuła *et al*., 2012), and in dairy animals in India (Kathiriya *et al*., 2018). Accordingly, these studies also reported the prevalence of the disease was higher in pregnant and lactating animals as compared to dry cows and heifers. The implication of the results is the effect of pregnancy stress on reactivated infection with latent BoHV-1 infection.

The finding of this study depicted that there was a very significant association between the prevalence of IBR and the number of parities in dairy cows. Multiparous cows were nearly 4 (OR = 3.94; 95% CI: 2.28-6.80; p = 0.000) times at higher risk of IBR infection as compared to non-porous cows. Accordingly, studies conducted in southern Mexico (Romero-Salas *et al*., 2013), and South Wollo, Ethiopia (Tadeg *et al*., 2021) reported a significant association of IBR prevalence with the number of cows' parity. In contrast, a study conducted in North-western Ethiopia reported insignificant difference in the odds of IBR infection among number

of parities (Zewde *et al*., 2021). The higher odds of IBR seropositivity in multiparous cows could be attributed to the subsequent viral exposure arousal along with age of the animal.

In the present study, the cows with a history of abortion were more than 5 (OR = 5.28; 95% CI: 1.55-17.98; p = 0.008) times more likely to be IBR seropositive than cows without abortion history. The result of the study was in agreement with the findings of different studies (Krishnamoorthy *et al*., 2015; Sibhat *et al*., 2018; Messele *et al*., 2021; Ortiz-González *et al*., 2022) that reported the prevalence of IBR was higher in cows with abortion history. However, insignificant associations of abortion with IBR seropositivity were reported in some studies (Zewde *et al*., 2021; Paudel *et al*., 2022). The disagreement in the findings might be seen due to the difference in sampling proportion, or the prevalence of other diseases causing abortion. The findings suggested that IBR is one of the potential causes of abortion in dairy cattle.

The significant association in the seropositivity of IBR with the history of RFM in the current study was in agreement with findings of the studies conducted in India (Krishnamoorthy *et al*., 2015), in major dairy areas of Ethiopia (Sibhat *et al*., 2018), and in South Wollo, Ethiopia (Tadeg *et al*., 2021). The odds of IBR seropositivity in animals with history of RFM was 2.55 (95% CI: 1.13-5.77; p = 0.024) times higher than animals without RFM. Hence, IBR needs to be highly considered behind the reproductive disorders in dairy cattle. In contrast, the study conducted in Nepal reported no significant difference in IBR seroprevalence in cows with RFM and those without RFM history (Paudel *et al*., 2022). The selection of smaller sample size in this study might be the reason contributing for the variation in the findings.

IBR is the viral disease affecting the respiratory and reproductive systems of cattle. Though, in the current study there was no significant association in IBR seropositivity with the presence of respiratory problems. However, studies conducted in Estonia (Raaperi *et al*., 2012) and northwestern Ethiopia (Zewde *et al*., 2021) reported significant association in the prevalence of IBR and presence of respiratory problems. Whereas, animals with the presence of ocular/nasal discharge were 2.41 (95% CI: 1.03-5.66; p = 0.044) times at higher risk of IBR seropositivity as compared to animals free of the discharges. In line with this result, Raaperi *et al*. (2012) also

reported ocular/nasal discharge were significantly associated with the prevalence of respiratory diseases (BRSV and BoHV-1). The presence of ocular/nasal discharge is one of the major clinical signs of IBR infection in animals. Therefore, the presence of significant association between IBR seropositivity and ocular/nasal discharge in the present study areas could suggest the presence of active BoHV-1 infections that needs further confirmation with virus isolation.

The current study indicated that the overall seroprevalence of infectious bovine rhinotracheitis at the herd level was 85.21% (95% CI: 78.28-90.21%) in the study areas. The result revealed a higher herd prevalence, indicating the extent of the disease distribution in North Shewa, the central highlands of Ethiopia. Studies conducted in Ethiopia and other parts of the world also reported nearly similar findings. Sibhat *et al*. (2018) reported 81.8% herd-level prevalence of IBR in major milkshed areas of Ethiopia, while the study conducted in dairy and dual-purpose cattle herds in Ecuador reported 82.1% herd prevalence (Carbonero *et al*., 2011). But the finding of the present study was lower than IBR herd prevalence of 94% in Costa Rica (Raizman *et al*., 2011), and 94.7% in western Ethiopia (Sibhat *et al*., 2018).

The herd-level prevalence of IBR in the current study areas was relatively higher than the studies that reported a herd prevalence of 30.9% in Kenya (Kipyego *et al*., 2020), and 53% in dairy cattle herds in Poland (Rypuła *et al*., 2012). The variations in the findings of IBR herd prevalence might be seen due to differences in the status of disease in different countries, herd management practices, and study herd types. The farms with loose biosecurity measures, uneven herd screening and management systems that aggravate stress and immunosuppression are tremendously at higher risk of the disease.

The herd prevalence of IBR was higher in Angolela Tera district (88.24%) than in Kimbibit district (82.43%), though there was no statistically significant difference. In line with the current finding, studies conducted in Cameroon (Daniel *et al*., 2018), in South Wollo, Ethiopia (Tadeg *et al*., 2021), and in Gujarat, India (Patel *et al*., 2023) reported insignificant difference in IBR herd prevalence between the districts/provinces. However, studies conducted in China (Yan *et al*., 2008), Lebanon (Abboud *et al*., 2016), and Nepal (Paudel *et al*., 2022) reported significant

differences in the occurrence of IBR between different districts or villages. The variations in the results of these studies could be attributed to the differences in cattle movement, livestock markets, cattle production systems and management practices in the districts/provinces.

The significant association in the prevalence of IBR with the farm/herd locations in the current study was in agreement with (Maresca *et al*., 2018; Waldeck *et al*., 2021), who stated that the disease prevalence was significantly varied with herd location areas and distance from cities. The current finding revealed that herds located in rural areas were less likely (OR = 0.21; 95% CI: 0.05-0.94; p = 0.042) to be IBR seropositive than herds located in peri-urban areas. The higher herd prevalence in urban and peri-urban areas could be related with the expansions of large and small-scale dairy farms, and the introduction and distribution of improved dairy animals into the mixed crop-livestock productions without adequate quarantine and testing. Thakur *et al*. (2020) and Tadeg *et al*. (2021) reported insignificant difference in herd prevalence of IBR in different management systems which was in accordance with the current finding. Though, the prevalence was higher in herds managed intensively than in extensive system.

The findings of the current study revealed a significant association in the herd prevalence of IBR with herd size, where large herds were found to be 100% IBR seropositive, while herds with medium size (11-20 cattle) were 5 (95% CI: 1.20-20.92; p = 0.028) times at higher risk as compared to herds with ≤5 cattle. Hence, in agreement with this finding several studies (Brock *et al*., 2020; Waldeck *et al*., 2021; Paudel *et al*., 2022) indicated that herd size was the significant factor in occurrence of IBR in cattle herds. Contradictory findings were also reported in Irish beef herd (Grady *et al*., 2008), Estonia (Raaperi *et al*., 2012), and Algeria (Derrar *et al*., 2019). The major factors contributing to these variations in the findings could be the difference in herd size and classifications. The presence of large herds and intensifications in the farms increase the chance of overcrowding and stress that aggravate the spread of disease.

The prevalence of IBR was higher in herds that used only AI system (91.67%) as compared to herds that used natural mating system (80.22%). The significant difference in the prevalence of IBR with breeding systems suggested the risk of AI in the transmission of IBR on

commercial dairy farms. In accordance with the current finding, a significant association of IBR herd prevalence with breeding systems was reported in England (Williams and Winden, 2014), Brazil (Almeida *et al*., 2021), and the Netherlands (Waldeck *et al*., 2021). However, some of these studies indicated that the use of bull mating increased the risk of IBR transmission. In other ways, (Kipyego *et al*., 2020; Tadeg *et al*., 2021) reported the absence of significant association in the prevalence of the disease with different breeding methods. BoHV is among the major diseases that able transmit through fresh and frozen semen, and with a higher risk of transmission through AI. Dairy farms should consider the disease status of the source bull and farms, while importing semen besides other routes of IBR transmission.

There was a very significant association between the prevalence of IBR and herd replacement strategies in the current finding. The prevalence of the disease was significantly higher in herds using both strategies (OR = 7.64; 95% CI: 2.74-21.33; p = 0.000) than herds raising their own replacements. The finding strongly agreed with the results (Sibhat *et al*., 2018; Derrar *et al*., 2019; Zewde *et al*., 2021) that reported origin of animals was a significant factor in the occurrence of IBR. Even if, few studies revealed that the prevalence was higher in homegrown animals, the introduction of newly purchased animals into herds was a major risk for IBR introduction and transmission. The current finding indicated that the introduction of replacement herds without the necessary prerequisites exposed the farms/herds to the disease.

Generally, the current study indicated that age and breed of the animal, history of abortion, retained placenta, and presence of ocular/nasal discharges were the most important risk factors and predictors of IBR infection in cattle. Irrespective of the clinical disease being most severe in young animals, the seroprevalence of IBR was higher in adults that the prevalence increased as age of the animal become advanced (Sibhat *et al*., 2018; Selim *et al*., 2022). The possible explanation is that build-up of infection due to longer exposure, rather than age-dependent susceptibility. The significant association in the prevalence of IBR with the reproductive and respiratory clinical signs was the obvious indicator that IBR could be the important cause of abortion, RFM, and ocular/nasal discharge (Trangadia *et al*., 2010; Graham, 2013). Therefore, commercial and smallholder dairy farms need to give higher attention to IBR control measures.

The current study revealed that the overall seroprevalence of bovine brucellosis was 0.98% (95% CI: 0.41-2.34%) by the combined RBPT and CFT tests. The prevalence of bovine brucellosis was very low (below 1%) in the study areas. Similar findings were reported in various parts of the country, where bovine brucellosis seroprevalence of 0.78% in Goha Tsion (Pal *et al*., 2016), 0.8% in Kembata zone (Mitiku *et al*., 2022), and 0.6% in central Ethiopia (Getahun *et al*., 2023) were reported. The current finding was also comparable with the studies that reported prevalence of 1.0% in western Ethiopia (Adugna *et al*., 2013), 1.3% in eastern Ethiopia (Terefe *et al*., 2017), and 1.21% in Debre Birhan (Lakew *et al*., 2022). However, the current finding was higher than the results that reported 0.1% in Arsi zone (Degefa *et al*., 2011), 0.2% in Ambo town (Bashitu *et al*., 2015), and 0.06% in Addis Ababa (Edao *et al*., 2018).

The seroprevalence of bovine brucellosis in the present study was lower than the findings that reported 2.4 % in Alage district (Asgedom *et al*., 2016), 2.08% in and around Kombolcha town (Tesfaye *et al*., 2017), 2.4% in Borena zone (Edao *et al*., 2020), 5.7% in Afar region (Negash and Dubie, 2021), and 6.4% in Southwestern Oromia (Etefa *et al*., 2022). The findings indicated that the prevalence of bovine brucellosis was higher in lowlands as compared to highland areas. The possible explanations for these differences are the presence of herd mixing in communal grazing and watering points, keeping of multiple livestock species, holding of large herds and sharing of bulls in lowlands, particularly in pastoral and agro-pastoral areas.

Bovine brucellosis was reported in Africa and various parts of the world. The finding of the present study was nearly similar to the studies that reported 0.9% in Tanzania (Asakura *et al*., 2018), 1.1% in India (Behera *et al*., 2020), and 0.95% in Sudan (Abubaker, 2022). However, several studies reported a higher prevalence of bovine brucellosis of 12.44% in Kenya (Enström *et al*., 2017), 8.3% in India (Shome *et al*., 2019), 3.18% in South Africa (McCrindle *et al*., 2020), 7.71% in Pakistan (Khan *et al*., 2020), and 20.7% in Egypt (Elhaig and Wahdan, 2023). The variations in the findings could be due to the differences in disease status in different countries, livestock production systems, or diagnostic tests employed in the studies. Besides, loose disease control and traditional livestock husbandry practices in sub-Saharan and some Asiatic countries are supposed to increase the transmission and impact of brucellosis.

The result of the present study dictated that the prevalence of bovine brucellosis was higher in Angolela Tera (1.45%) than in Kimbibit (0.43%) district, and that animals in Kimbibit district were less likely (OR = 0.39; 95% CI: 0.06-0.48; p = 0.046) to be seropositive for brucellosis. In line with this, different studies (Abera, 2018; Madut *et al*., 2018; Edao *et al*., 2020; Mfune *et al*., 2021) indicated a significant difference in the prevalence of bovine brucellosis in different districts. In contrast, studies conducted in northern Nigeria (Mai *et al*., 2012), in Afar region (Negash and Dubie, 2021), in western Ethiopia (Sima *et al*., 2021), and in Somalia (Hassan *et al*., 2021) reported no significant associations in the prevalence of brucellosis with districts. The difference in the prevalence of bovine brucellosis between the districts could be attributed to the sharing of bulls for breeding, geographic proximity of Angolela Tera district to lowland areas that share livestock markets, and communal grazing in the winter and spring seasons.

Even if the prevalence of bovine brucellosis was higher in smallholder farms (1.15%) than in commercial farms (0.80%), there was no significant difference in the prevalence of the disease. Accordingly, (Matope *et al*., 2009; Asgedom *et al*., 2016; McCrindle *et al*., 2020) also reported that there was no significant difference in the prevalence of brucellosis between commercial and smallholder farms. However, Mai *et al*. (2012) and Borba *et al*. (2013) reported significant associations of brucellosis prevalence with farm and herd types. The present finding suggested that the purchase and introduction of replacements without screening for brucellosis in commercial farms. Moreover, small-scale farms were also purchase dairy animals from the local markets that supposed to increase the introduction of the disease into the farms.

The present study dictated that the prevalence of bovine brucellosis was significantly associated with the age of the animal. The prevalence of the disease was 0% in animals below 3 years old, while animals above 6 years old were nearly 4 (OR = 3.53; 95% CI: 1.18-9.11; p = 0.045) times more likely to be brucellosis seropositive than animals below 3 years old. The finding was in line with the results reported in South Sudan (Madut *et al*., 2018), Borena zone (Edao *et al*., 2020), Zambia (Mfune *et al*., 2021), and western Ethiopia (Etefa *et al*., 2022). The major justifications behind the findings are that brucellosis is more common in sexually mature animal due to the preference of the bacteria to sexual organs and fetal tissues. The

exposure to the disease also increases with the age of animal. Though, some studies reported insignificant associations (Pandeya *et al*., 2013; Sima *et al*., 2021; Getahun *et al*., 2023). The main reason for these differences were the variations in age classifications in the studies.

The significant association in the seropositivity of bovine brucellosis with the cattle breeds in the present study was in agreement with other findings (Borba *et al*., 2013; Terefe *et al*., 2017; Sima *et al*., 2021). The prevalence of bovine brucellosis was higher in the local cattle breeds (OR = 9.42; 95% CI: 1.47-60.43; p = 0.018) than in the crossbred cattle. In other ways, some studies reported that there was no significant difference in the prevalence of bovine brucellosis in different breeds of cattle (Hailemichael *et al*., 2020; Mitiku *et al*., 2022; Getahun *et al*., 2023). The variation in the study populations, production systems, and sampled proportion might be the underlining factors for differences in the findings of the studies. The possible explanations for the higher prevalence of the disease in local breeds include the fact that in the present study areas, local cattle breeds are mostly kept under traditional husbandry practices, where herd mixing in free-grazing and watering sources is common. Local breeds are also brought from lowland areas, including the neighbouring Afar region.

The current study revealed a higher seroprevalence of brucellosis in lactating (1.69%) and pregnant cows (0.92%), and no seropositivity was found in heifers. Accordingly, the prevalence of bovine brucellosis was 0% in non-parous cows, while multiparous cows were nearly six (OR = 5.65; 95% CI: 3.47-110.50; p = 0.038) times at higher risk of brucellosis seropositivity as compared to non-parous cows. In agreement with the present finding, significant association of bovine brucellosis with number of parities were reported in Alage district (Asgedom *et al*., 2016), central Ethiopia (Abera, 2018), Borena zone (Edao *et al*., 2020), and western Oromia (Etefa *et al*., 2022). The presumed factors for a higher prevalence of brucellosis in multiparous, lactating and pregnant cows include *Brucella* organism tropism to the reproductive tracts of mature productive animals and fetal tissues. The higher concentration of erythritol, a sugar that serves as a growth stimulant for *Brucella* species, during pregnancy in placental tissues also aggravates the risk of the disease in multiparous and pregnant animals. The increased exposure time with the age of the animal is also supposed to be a contributing factor.

The strong association between the seropositivity of bovine brucellosis and the history of abortion in dairy cows (OR = 26.05; 95% CI: 3.68-184.34; p = 0.001) was in line with (Tasiame *et al*., 2016; Madut *et al*., 2018; Hailemichael *et al*., 2020; Asmare *et al*., 2018; Sima *et al*., 2021). The findings indicated that the cows with a history of abortion had higher odds of brucellosis seropositivity as compared to the cows without a history of abortion. The results strengthen the importance of brucellosis as a cause of reproductive disorders in dairy cows and suggest that the disease should receive higher consideration in the diagnosis of bovine abortion, particularly in late-stage of pregnancies. Insignificant associations of brucellosis with a history of abortion were also reported by some researchers (Muflihanah *et al*., 2013; Bashahun *et al*., 2015; Bifo *et al*., 2020). The deviations in the findings might be seen due to the presence of other reproductive diseases or management factors contributing to abortions in dairy cattle.

The present study indicated that the history of dystocia and retained placenta in dairy cows were significantly associated with bovine brucellosis in the study areas. The cows with a history RFM were 16.72 (95% CI: 2.39-116.78; p = 0.005) times at higher risk, while the presence of dystocia in cows increased the chance of brucellosis seropositivity over 14 times (OR = 14.28; 95% CI: 2.33-87.46; p = 0.004). In agreement with the present findings, several studies (Mee, 2008; Alemu *et al*., 2014; Lakew *et al*., 2022; Getahun *et al*., 2023) showed that the history of RFM and dystocia was significantly associated with brucellosis. In contrast, studies conducted around Bahir Dar (Asmare *et al*., 2018), and Hawassa town (Abera *et al*., 2019) reported insignificant associations in the prevalence of brucellosis with the presence of RFM history. The difference in management practices or the presence of other disease factors could be the underlining causes of the disagreements in the findings. The current findings and many studies signify the fact that brucellosis infections of the uterus (metritis) and placental membrane (placentitis) are the major contributors to dystocia and RFM in cows.

The overall herd-level prevalence of bovine brucellosis was 3.52% (95% CI: 1.46-8.26%) in the current study. The finding of the current study was in close agreement with the findings that reported brucellosis herd prevalence of 4.76% in Jimma zone (Bashahun *et al*., 2015), 5.4% in selected towns of Oromia region (Abera, 2018), and 4.8% in southern Oman (Marzooqi

et al., 2022). Though, there are several studies in Ethiopia and across the world that reported a higher herd prevalence of bovine brucellosis, such as 11.4% in Brazil (Borba *et al*., 2013), 45.9% in Alage district, Ethiopia (Asgedom *et al*., 2016), 33.33% in South Africa (McCrindle *et al*., 2020), and 21.14% in Zambia (Mfune *et al*., 2021). The differences in the status of brucellosis in the countries, study herds and management systems or diagnostic techniques used in the studies might be linked to the variations in the findings. The lower herd prevalence of bovine brucellosis in the current study areas is supposed to be due to the holding of small herd sizes in mixed crop-livestock production in most central highlands, lesser communal grazing and herd mixing, and relatively restricted movement of animals.

The herd prevalence of bovine brucellosis was 5.88% in Angolela Tera district and 1.35% in Kimbibit district, where herds in Kimbibit district were less likely (OR = 0.29; 95% CI: 0.05-0.91; p = 0.043) to have brucellosis seropositivity. This finding was in line with the studies (Bashahun *et al*., 2015; Abera, 2018; Edao *et al*., 2020) that reported a significant difference in the prevalence of bovine brucellosis in different districts. However, Hailemichael *et al*. (2020) and Sima *et al*. (2021) reported that there was no significant association in prevalence of the disease with the districts. Besides, the prevalence of brucellosis was higher in herds located in rural (3/87) and peri-urban (2/39) areas as compared to herds in urban area (0/16). The relative higher herd prevalence of brucellosis in Angolela Tera and, accordingly, in rural and peri-urban areas in current study suggested that herd mixing during free-grazing seasons and introduction of draught bulls from lowland areas predisposed to an increased risk of bovine brucellosis.

There was no significant association in herd prevalence of bovine brucellosis with herd size in the present study. In accordance, the findings of (Asgedom *et al*., 2016; Khan *et al*., 2020; Hailemichael *et al*., 2020) also indicated the absence of significant associations with herd size. However, herd size was reported as a significant factor for bovine brucellosis in different studies (Tasiame *et al*., 2016; Terefe *et al*., 2017; Madut *et al*., 2018). The differences in herd size classifications and herd management systems could be the reasons for variations in the results of the studies. Moreover, in central highlands of Ethiopia, particularly smallholder farmers hold small herd sizes, and large herds are mostly found in commercial dairy farms.

The prevalence of bovine brucellosis was significantly higher in herds that shared bulls for mating (OR= 3.12; 95% CI: 1.37-15.78; p = 0.035) than in herds that used their own bulls. Accordingly, even if there was no significant difference in the prevalence of the disease in herds using different breeding systems, 3 of the total 5 positive herds were herds that used natural mating. Similarly, studies conducted in selected towns of Oromia (Abera, 2018), in Sendafa (Bifo *et al*., 2020), and in Wolaita zone (Hailemichael *et al*., 2020) reported the absence of significant difference in herd prevalence of brucellosis with the breeding strategies. The probable justifications for a higher prevalence of brucellosis in herds that shared bulls and more positives in herds that used natural mating include the effect of herd mixing, exchange of bulls between herds, and the possible venereal transmissions of brucellosis.

The significant association in the herd prevalence of bovine brucellosis with herd replacement strategies in the present study was in agreement with the findings reported in the Colombian herds (Cardenas *et al*., 2019), and central Ethiopia (Getahun *et al*., 2023). The current finding stated that herds that purchased replacements were 9.81 (95% CI: 1.03-93.82; p = 0.047) times more at risk of having brucellosis seropositivity. However, Bifo *et al*. (2020) and Etefa *et al*. (2022) reported that there was no significant association in herd prevalence of brucellosis with replacement strategies. The variations in the findings might be seen due to the difference in biosecurity measures, source of herds, and biosafety and management practices in the farms.

Finally, the findings of the present study revealed that the most important risk factors and indicators of bovine brucellosis were the age of the animal, breeds of cattle, the presence of abortion history and retained fetal membrane, sharing of bulls and herd replacement strategies. The present finding and several studies signifies the fact that besides a longer period of exposure with the age of the animal, brucellosis is primarily a disease of sexually mature animals (Khan *et al*., 2021; Lakew *et al*., 2022; Getahun *et al*., 2023). Moreover, the findings indicated the presence of strong associations (higher odds) between brucellosis seropositivity and the history of reproductive disorders such as abortion and RFM. Generally, the findings suggested that bovine brucellosis could be the major cause of reproductive problems in dairy cattle, and due attention should be given during the diagnosis of reproductive problems.

The findings of a semi-structured questionnaire survey indicated the occurrence of major reproductive problems, the awareness and practices of the farmers on handling reproductive disorders in dairy cows, and the zoonotic transmission of bovine brucellosis. The present finding revealed that cattle were the most important livestock species (94.17%) in the mixed crop-livestock production system of the central highlands of Ethiopia, and that the primary purpose of cattle production was draught power for cultivation (65.83%), followed by milk production (25.84%). Similarly, (Amsalu and Addisu, 2014) reported that cattle were the most important livestock species for cultivation in the mixed farming system of Ethiopia. In the central highlands of Ethiopia, the presence of higher cultivation of crops and the potential for dairy production are the most probable factors for the higher necessity of cattle production.

In the present study areas, most farmers (55.84%) were holding small herds (6–10 cattle), while 27.50% of the farmers were holding medium herds (11–20 cattle). The result agreed with the findings that reported average 8 herd size in Bench-Maji zone (Mengistu *et al*., 2017), and Gamo-Gofa zone (Kebede *et al*., 2017). The holding of relatively small herds in the central highlands is supposed to be due to an intensive land-use system and the gradual decline of grazing lands. The cattle population was composed of both local and crossbred cattle in the majority of herds (55%), and extensive system was the most common cattle production system (75.83%) in the study areas. Accordingly, Belay (2016) reported that most cattle herds in urban and peri-urban areas of Sidama region constituted both local and crossbred cattle breeds, while extensive system (Mekuria, 2016; Tesfa *et al*., 2022) was the major means of cattle production in most smallholder livestock production systems of Ethiopia.

The result indicated that most farmers used only natural mating (49.17%), and sharing of bulls (group mating using selected bulls) was the common practice (62.50%). The study conducted in Gamo-Gofa zone also reported similar findings that natural controlled breeding and herd mating using selected bulls were the most common breeding system (Kebede *et al*., 2017). Goraga *et al*. (2019) also reported that sharing of bulls were common in smallholder dairy farming systems. The present finding supposes that the desire of most farmers to improve local cattle breeds to maximize dairy production intensifies their tendency to share selected bulls.

The current study revealed the occurrence of various reproductive disorders and infertility problems in dairy cattle. The findings indicated that abortion had occurred in 32.50% of herds. This finding was in close agreement with Lakew *et al*. (2022) and Weldegebriel (2015) that reported herd-level abortion of 27% and 28.30%, respectively. However, there were studies that reported a low herd prevalence of abortion of 2.9% (Tulu and Negera, 2022), 4.17% (Tolosa *et al*., 2021), and 5% (Debebe and Fesseha, 2020). The present study revealed 40% RFM in dairy herds. In accordance, 35.8% RFM in dairy cattle in central zone of Tigray was reported (Weldegebriel, 2015), while 13.2% RFM was reported in dairy cattle in and around Hawassa town (Debebe and Fesseha, 2020). The differences in herd management practices, reproductive diseases conditions, or retrospective data collection are supposed to be the underlining reasons for the variations in the findings. The collection of 5-year retrospective data and higher dairy production in the current study areas increased the sensitivity of the study.

The present findings indicated the significant associations in the occurrence of abortion with production system ($p = 0.042$), cattle breeds in the herd ($p = 0.039$), and the presence of group mating ($p = 0.024$), and RFM with management systems ($p = 0.041$). In accordance with the present study, (Deresa *et al*., 2020; Bitew and Prasad, 2011; Elemo *et al*., 2022) reported significant differences in the occurrence of the reproductive disorders with herd characteristics and management systems. The findings signify the impacts of management system on reproductive disorders, and that the use of group mating in fact increases the transmission of reproductive diseases that cause several reproductive problems, such as RFM and abortions in dairy cows.

The findings of this study showed a higher infertility problem of 47.50% repeat breeding (RB) and 44.17% anoestrus in dairy cattle herds. Accordingly, Eshete *et al*. (2023) reported 38.4% RB problems in northern highlands of Ethiopia, while Dinka, (2013) reported 26.8% RB at animal-level in Assella town. Anoestrus was reported to be 38.6% in dairy cattle in Wukro (Kidusan, 2009), and 20% in central Tigray (Weldegebriel, 2015). The significant associations between RB and mating systems ($p = 0.035$), and anoestrus with cattle breeds ($p = 0.044$) and breeding methods ($p = 0.011$) were in line with (Gebremeskel *et al*., 2019; Eshete *et al*., 2023). The higher RB (72.73%) in herds that used AI suggested the lower efficiency of AI, insemination timing and technical limitations. Higher herd prevalence of anoestrus in local breeds (60.71%), and in herds used bull mating (55.93%) are supposed to be due to diseases and mismanagement practices.

The KAP assessment indicated that most people (55.83%) were involved in assisting the cows during parturition. Accordingly, Lakew *et al*. (2022) reported 59.3% and Lemu *et al*. (2020) reported 51.82%. In contrast, Edao *et al*. (2018) reported a higher (94.62%) in Addis Ababa dairy cattle, and a lower (41.94%) in Borena zone (Edao *et al*., 2020). However, nearly 75% respondents had not used protective materials. Similarly, 84% was reported in central Tigray, while a higher status (93%) was reported in Debrebirhan (Weldegebriel, 2015; Lakew *et al*., 2022). The higher involvements in assisted parturition and lower rate of using protective gloves in the current study areas could be due to minimal awareness of the public on brucellosis and its zoonotic transmission.

The practices of raw milk and raw meat consumption were found to be 26.67% and 64.17%, respectively. Studies indicated higher percentages of raw milk consumption, where 65.53% in central Ethiopia (Getahun *et al*., 2023) and 67.69% around Addis Ababa (Edao *et al*., 2018). The differences in the findings could be due to the difference in practices, culture and level of awareness of the study population. The result showed significant difference in raw milk consumption among age groups of the respondents ($p = 0.015$). Accordingly, awareness on disease transmission through raw milk and meat consumption was significantly influenced by age ($p = 0.008$), educational status ($p = 0.006$) and resident ($p = 0.029$). In agreement, (Amenu *et al*., 2019; Deneke *et al*., 2022; Mamo *et al*., 2022) showed significant differences that indicate awareness raising could significantly decrease public exposure to zoonotic diseases.

The current finding revealed that only 14.17% of the respondents were aware of zoonotic risk of brucellosis. Close findings were reported that 18.97% in Oromia region (Lemu *et al*., 2020), and 18.4% pooled awareness in Ethiopia (Sibhat *et al*., 2022). The awareness on the zoonotic risk of brucellosis was significantly associated with educational level ($p = 0.000$) and residents ($p = 0.020$) of the respondents. Similarly, (Alqahtani *et al*., 2021; Zhang *et al*., 2019) reported that awareness on zoonotic transmission of brucellosis was significantly different by occupation, educational status and residency. The findings suggested the fact that education and trainings have inevitable effects on raising public awareness, particularly to the neglected zoonotic brucellosis. Generally, this study showed high rates of practices that could expose the public to zoonotic brucellosis, while lower rates of awareness on biosafety and preventive measures that suggested the need for extensive intervention measures in central highlands of Ethiopia.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study revealed the widespread distribution of infectious bovine rhinotracheitis in commercial and smallholder dairy farms in North Shewa, the central highlands of Ethiopia. The study showed a higher prevalence of the disease, with a seroprevalence of 61.84% at the animal level and 85.21% at the herd level. The current findings indicated a relatively lower prevalence of bovine brucellosis in the study areas, which revealed an overall seroprevalence of 0.98% at the animal level and 3.52% in the herds. The study identified that age, breed, parity, history of abortion, retained placenta, and ocular or nasal discharges were significant risk factors of IBR. Whereas, bovine brucellosis was significantly associated with districts, age, breed, parity, history of abortion, retained placenta and dystocia. The current study determined the occurrence of various reproductive problems in dairy cattle. Repeat breeding, anoestrus, abortion, retained placenta and dystocia were identified as the most important reproductive problems in both commercial and smallholder dairy farms. The knowledge, attitudes and practices assessment of farmers indicated higher practices of assisted parturition, improper disposal of fetal membrane and aborted fetuses, and raw milk and meat consumption, while the use of protective materials and awareness of zoonotic brucellosis were lower. Thus, the higher prevalence of IBR and the presence of bovine brucellosis in North Shewa, the central highlands of Ethiopia, signifies the extent of the diseases distribution, and their impacts on the growing dairy industry, and the risk of brucellosis to public health.

Based on the present findings, the following recommendations are forwarded to mitigate further spreading of the diseases and their impacts on dairy production, animal and public health:

- The higher prevalence of infectious bovine rhinotracheitis warrants the immediate implementation of integrated control and prevention measures and the initiation of vaccination for *Bovine Herpes Virus-1* with IBR-marker vaccines in Ethiopia.
- Strict farm biosecurity measures to prevent the introduction of infected animals with the diseases and latent carriers of BoHV-1 in to the herds.
- Regular herd screening, isolation of animals with reproductive and respiratory clinical signs, testing for the diseases, and separation of positive reactors.

- Replacement animals should be purchased form diseases free herds and re-tested in quarantine before introduction.
- Awareness raising for the farmers on the management of reproductive problems, handling of aborted fetus and fetal membranes, and zoonotic transmission of brucellosis through meetings, short-term trainings, and public education channels.

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8. ANNEXES

Annex 1: Competitive IBR gE ELISA Procedures

The serological investigation of infectious bovine rhinotracheitis was conducted using competitive IBR gE ELISA. The competitive ELISA kit is designed for the detection of antibodies against the gE glycoprotein of the BoHV-1 virus in the serum samples. The test has the capability of differentiation of naturally-infected animals in a vaccinated population provided that the animals are vaccinated with BoHV-1 gE-deleted vaccines.

Principle

The microwells are coated with purified gE recombinant protein. The samples to be tested and controls are added to the wells. Anti-gE antibodies, if present form antigen-antibody complex which mask the gE epitopes. Anti-gE horseradish peroxidase (HRP) conjugate is added to the wells that fixes to the remaining free gE epitopes, forming antigen-conjugate-HRP complex. After elimination of the excess conjugates by washing, the substrate solution (TMB) is added. The resulting coloration depends on quantity of specific antibodies present in the specimens.

- Blue solution appears in the absence of antibodies which become yellow following the addition of the stop solution
- No coloration appears in the presence of antibodies

Reagents and Materials

The necessary reagents and materials required for the ELISA procedure include microplates coated with purified gE recombinant protein, positive and negative controls, dilution buffer 2, dilution buffer 100, concentrated conjugate, wash concentrate, substrate solution, stop solution, mono or multi-channel pipettes, disposable tips, 96-well microplate reader, distilled or deionized water and manual or automatic wash system.

Procedures

Sample preparation: in order to avoid the difference in incubation time between specimens, 96-well plates containing test and control specimens were prepared before transferring them into ELISA microplate using multichannel pipette.

Wash solution preparation: the concentrated Wash solution (20X) was allowed to room temperature and mixed thoroughly to completely solubilized. The Wash solution (1X) was prepared by diluting the concentrated Wash solution in 1/20 in distilled water.

All the reagents were allowed to room temperature 21 ºC (±5ºC), and homogenized before use.

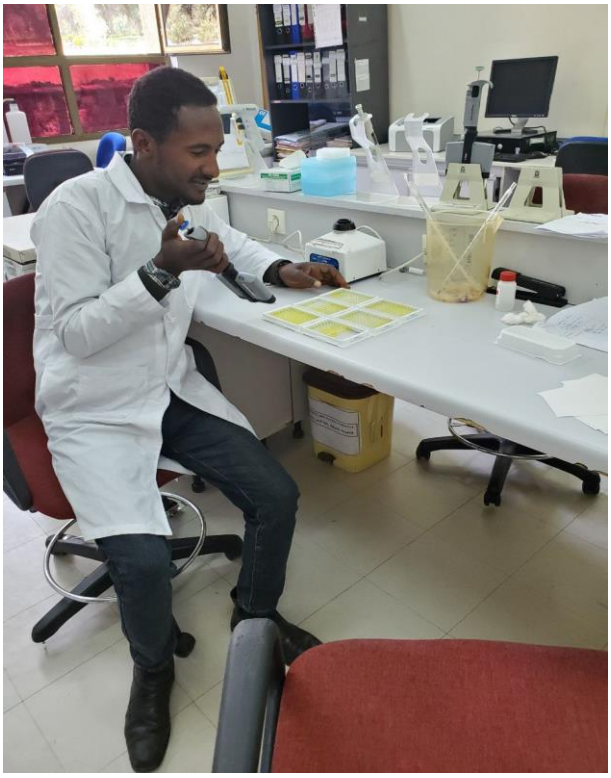
1. 50 µl of the Dilution buffer 2 was transferred to each well. 50 µl of the Positive control to wells A1 and B1, and the Negative control to wells C1 and D1 were added. Then 50 µl each sample to be tested was added in to the remaining wells.
2. The plate was covered and incubated overnight (16-20 hours) at 21 ºC (±5ºC).
3. The wells were emptied, and washed each well 3 times with approximately 300 µl of the Wash solution by avoiding drying of the wells between washes.
4. The Conjugate was prepared by diluting the concentrated conjugate to 1/10 in the Dilution buffer 100, and 100 µl of the Conjugate was added to each well.
5. The plate was covered and incubated again for 30 ± 3 minutes at 21 ºC (±5ºC).
6. The wells were emptied again and washed each well 3 times with approximately300 µl of the Wash solution by avoiding drying between consecutive washes.
7. 100 µl of the Substrate solution was added to each well.
8. The plate was covered and incubated for 15±2 minutes at 21 ºC (±5ºC) in the dark
9. 100 µl of the Stop solution was added to each well in order to stop the reaction.
10. The microplate was read and recorded the optical density (OD) at 450 nm.

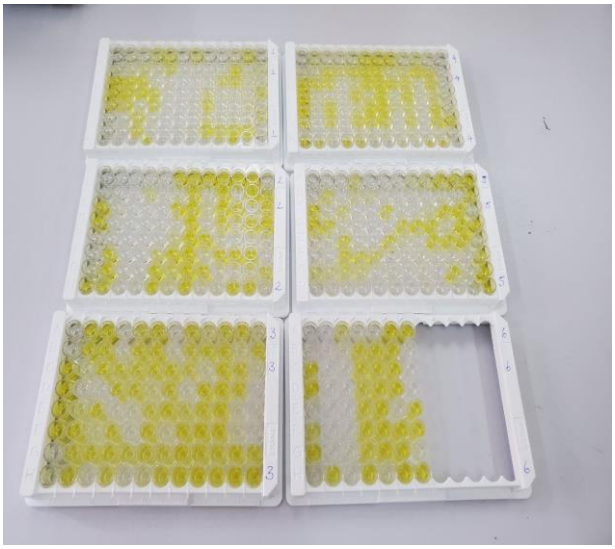
Interpretation

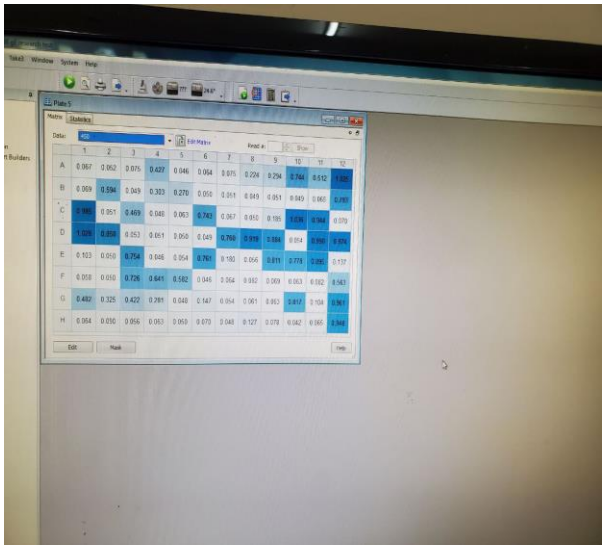
For each sample, the competition percentage (S/N%) was calculated:

$$S/N\% = \frac{\text{OD of the sample}}{\text{OD of the Negative controls}} \times 100$$

- Samples with S/N less than or equal to 60% were considered positive.
- Samples with S/N greater than 60% were considered as negative.







Annex 2: Rose Bengal Plate Test (RBPT) for Bovine Brucellosis

The investigation of bovine brucellosis was started by serological assay of all the serum samples using RBPT screening test. The plate agglutination test used antigen prepared from *Brucella abortus* (strain 99) stained with Rose Bengal dye and suspended in acid buffer (pH 3.65) for the detection of *Brucella* antibodies in the serum. RBPT detects antibodies against *B.abortus*, *B.melitensis* and *B.suis* in the serum samples.

Reagents and Materials

Rose Bengal plate test procedures require RBT *Brucella* antigen, positive and negative control sera, plate, magnifying glass, micro pipette of 30 µl, disposable pipette tips and mixing sticks.

Procedures

1. The serum samples and reagents were allowed to room temperature for 30 minutes, and the antigen was vigorously mixed before using; since active materials straight from the refrigerator react poorly and reduce sensitivity of the test.
2. 30µl of each serum sample was placed onto the white tile or enamel plate.
3. 30 µl of the Rose Bengal antigen was placed near the test samples.
4. The antigen and serum were mixed thoroughly using a clean mixing stick to spread the mixture over the entire surface of the circle.
5. The plate was rocked using automatic rocker for about 4 minutes.
6. The tests were examined for agglutination in a good light.
7. Magnifying glass was used when micro-agglutinations were suspected.

Interpretation

0 = no agglutination, + = barely perceptible, ++ = fine agglutination and +++ = coarse clumping. The sera samples that showed presence of agglutination (even slight) are considered positive for RBPT.


HITACHI
GUEST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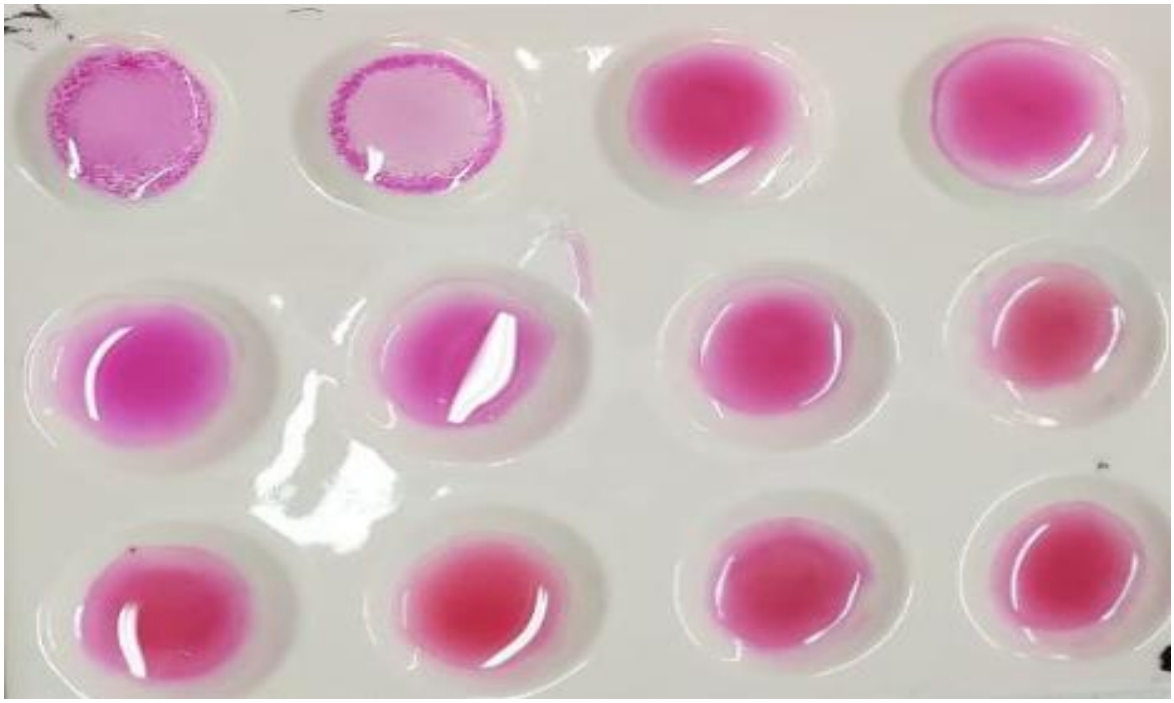

Annex 3: Complement Fixation Test (CFT) for Bovine Brucellosis

Complement fixation test was used to confirm the sera samples which were positive on RBPT. CFT is based on the detection of antibody in the serum. Therefore, samples positive on RBPT were further subjected to CFT test.

Principle

The complement which is a heat labile globular protein present in normal serum, can only bind antigen-antibody complexes. The binding of complements on Ag-Ab complexes on red blood cells or other cells resulted in the lysis of the cell. The Ag-Ab complex fixes the complements, but the fixation of complement with Ag-Ab complex do not produce any visible agglutination. The sheep RBC coated with anti-sheep RBC antibody is used as indicator system.

Reagents and Materials

The reagents necessary for CFT procedures include antigen, complement, positive and negative controls, Ambocepter (indicator system), and buffered diluent. The main materials required are micro-titer plate, single and multi-channel pipettes, disposable pipette tips, centrifuge, incubator, refrigerator, shaker and water bath for incubation.

Preparation of sheep red blood cells (SRBC) and Amboceptor titration

About 10 ml of SRBC was centrifuged at 2500 rpm for 5 minutes. The buffered diluent was then added after discarding the supernatant. The SRBC was washed with the diluent by centrifuging and removing the supernatant for about three times. The packed cell volume (PCV) was measured during the last wash. A 2% suspension of sheep red blood cells was prepared for Amboceptor titration.

Amboceptor titration

The Amboceptor is titrated for determination of hemolysin activity

- ❖ In the first row of the rack five test tubes were prepared, similarly 5 test tubes were arranged in the second row.
- ➢ 1:500 dilution was prepared by mixing 10 µl the amboceptor with 4990 µl buffered diluent (Veronal buffered diluent) in the first tube of the first row.
- ➢ 1:750 dilution was prepared by mixing 10 µl the amboceptor with 7490 µl VBD in the first tube of the second row.
- ➢ - 1ml of the buffered diluent (VBD) was added to the test tubes 2-5 in both rows
- ➢ 1 ml of the 1:500 and 1:750 dilution amboceptor were transferred from the first test tube to the second to fifth tubes, in accordingly rows.
- ➢ Amboceptor was then diluted successively from tube 1 to tube 5 in 0.5 ml amount in both rows. The dilution rans from 1:500 to 1:8000 in the first row, and from 1:750 to 1:12000 in the second row.
- ➢ 1ml of VBD was added to each of the test tubes.
- ➢ 0.5 ml of SRBC were added to the test tubes, and were shacked well.
- ➢ The mixtures were allowed stay for 10-15 minutes.
- ➢ 1 ml of the complement at working dilution was added and incubated in water bath at 37 ºC for 30 minutes.
- ➢ The minimum hemolytic dose (MHD) that cause complete lysis in the last tube was recorded, and the working dilution of amboceptor is four times the MHD.

Evaluation of Complement

The freeze-dried complement was reconstituted according to its instruction.

- ➢ The complement was prepared in 1:100 dilution
- ➢ Complement was added into the 9 tubes increasing by 5 µl every time, starting with 10µl.
- ➢ Diluent was added into the 9 tubes in decreasing amount by 5µl, starting with 40µl.

- 25 µl of a diluent was added into the tubes with syringe
- The test tubes were placed in water bath at 37 ºC for 1 hour
- 25 µl of 2% SRBC was added into the tubes
- Then 25 µl of amboceptor at working dilution 1:1,000 was added into the tubes.
- The tubes were properly mixed and placed in the water bath at 37 ºC for 30 minutes

The test was read by recording the minimum hemolytic dose of the complement (MHD), which was represented by the first tube showing complete hemolysis. The next tube contains full hemolytic dose (FHD). The complement dilution = 2FHD/initial dilution of complement.

Procedure

1. The sera were prediluted to 1: 2.5 and incubated at 58 ºC in a water bath for 30 minutes in order to inactivate the native complement.
2. 25 µl of diluted test sera were placed in wells of first and second rows of U- bottom plates.
3. 25µl of VBD was added to all except those of the first row.
4. Then, serial doubling dilution were made by transferring 25µl volumes of serum from second row on wards continuing for at least four dilutions.
5. 25 µl of antigen diluted to working dilution excluding those of the anti-complementary controls, which received 25µl VBD was added to all wells.
6. 25 µl of complement at working dilution was added to all wells except the control wells.
7. Control wells containing serum + complement + diluent, antigen control has antigen + Complement + diluent, complement control has complement + diluent, and hemolytic system has diluent set up to 75µl total volume in each case before hemolytic system was added.
8. The plates were incubated for 30 minutes at 37 ºC with warm fixation (agitations)
9. 25µl of SRBC suspension (2%) and amboceptor mixture was added to each well. Then the plates were sealed, and placed on shaker and incubated at 37 ºC for 30 minutes.

10. Before reading the result on the plates were left in the refrigerator at +4 ºC for 1 hour in order to allow the none-lysed cells to settle.
11. The plates were taken out from refrigerator and left at room temperature for 10 minutes
12. Finally, the results were read. Positive reactions were indicated by sedimentation of SRBC and absence of hemolysis, while negative reactions by hemolysis of SRBC.

Interpretation

- The sera with the absence of hemolysis were considered as positive on CFT. Ag-Ab reactions fix complements, so no free complement is available to lyse the RBC. The sera with strong reaction, more than 75% fixation of complement at a dilution of 1:10, and at least 50% fixation of complement at a dilution of 1:5 was classified as positive.
- The sera with hemolysis of RBC were taken as negative on CFT. There is no complement fixation occurred, so the complement remains free and hemolyze RBC.

Annex 4: Data recording format for blood sample collection and retrospective data collection

No	Farm name/ Owner name	Location/ District	Animal ID	Herd size	Sex	Age (years)	Breed	Status of cow	Parity	Farm type	Production ssystem	Replacement strategy	Abortion or Orchitis history	RFM	Dystocia/Prolapses	Ocular/Nasal disc.	Resp. problems	Mating system	Sharing of bulls
1																			
2																			
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Annex 5: Consent Form for Research

Title of Study: Seroepidemiology of Infectious Bovine Rhinotracheitis and Bovine Brucellosis, and Major Reproductive Problems in Commercial and Smallholder Dairy Farms in North Shewa, Central Highlands of Ethiopia

Principal Investigator: Dr. Aweke Engdawork (MSc student at AAU College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia

Some general rules you should know about this research: Participation in a research project is requested of you. It is entirely optional for you to take part in this study. You are free to decide whether to take part in this study, and you are not subject to any consequences if you decide not to. Gaining more knowledge about a certain topic or issue is the goal of research investigations. Being a part of a study does not assure you of any personal benefits. Detailed information on the study in which you are being requested to participate is included in this consent form. This consent form includes a description of the study in which you are being asked to take part. You have the right to ask the researcher for clarification or further information if you do not understand something in this form. We'll provide you with a copy of this consent form. Please feel free to get in touch with the above-mentioned researcher if you ever have any questions concerning your involvement.

Purpose of this study: The health and productivity of animals can be constrained by a variety of infectious diseases. Dairy productions are vulnerable to several reproductive diseases. Ethiopia suffers from many of these infectious reproductive diseases. Moreover, some infectious diseases possess zoonotic risks. Therefore, understanding the major constraints of dairy production and thereby the distribution of reproductive diseases has very significant importance in the control of the problems to maximize production. This research project focuses on the investigation of Infectious Bovine Rhinotracheitis and Bovine Brucellosis, Major Reproductive Problems in Commercial and Smallholder Dairy Farms, and what the community is practicing to assess the KAPs of farmers related to zoonotic brucellosis. If you accept to take part in this study, you will be required to respond orally to questions from a survey I will be giving out. You'll need to finish the questionnaire in about 15 minutes.

Risks: This study has very little risk. You will not be able to identify yourself from this study because no personal information about you will be collected.

Benefits: Identifying the major reproductive problems and infectious reproductive diseases in dairy cattle helps to implement the appropriate control and prevention measures, thereby increasing dairy production and improving the health of humans and animals.

Confidentiality: To the fullest extent permitted by law, study-related data will be kept private. Neither verbal nor written reporting will include any allusions to the study that might identify you. On any study materials, you won't be required to write your name, ensuring that no one can connect your answers to you personally.

Compensation: There is no compensation for taking part in this study. You can reach me by phone at +251915570173 or by email at awekeengdawork@gmail.com at any time if you have any questions concerning the study.

What if you have questions about your rights as a research participant? Through my principal advisor, Dr. Haileleul Negussie, or the Regulatory Compliance Administrator of Ethiopia, you may reach out to the AAU College of Veterinary Medicine and Agriculture with your concerns if you feel that you have not been treated as described in this form or that your rights as a research participant have been violated during this project.

Consent To Participate: I was given the opportunity to hear or read the material above, and I understood it. This form was delivered to me. I hereby consent to participate in this study with the understanding that I may withdraw at any time without penalty or loss of any advantages to which I am otherwise entitled.

Name of participant _____ **Date_____**

Signature_____ **Phone №_____**

Annex 6: Questionnaire survey for major reproductive problems and KAP assessment

I. GENERAL INFORMATION

1. Personal Address

Name of respondent: _____

Age: _____ Sex: M ☐ F ☐

Position in house: Household head ☐ Spouse of head ☐ Boy ☐ Girl ☐

Other: _____

2. Location

Region: _____ Zone: _____

District: _____ Peasant association: _____

3. Educational Level

Illiterate ☐ Elementary ☐ College/University ☐

Informal ☐ High school ☐

4. Family Size

Males: _____ Females: _____ Children < 15 yrs.: _____

5. Residence: Urban ☐ Peri-urban ☐ Rural ☐

6. Occupation

Farmer ☐

Government employer ☐

Private organization employer ☐

Self-employer ☐

Other: _____

7. Agroecology

Highland ☐

Midland ☐

Lowland ☐

II. LIVESTOCK PRODUCTION

1. Types of livestock and their importance

Livestock type	Breed type		Total	Rank most important species (1-3)
	Local	Cross		
Cattle				
Sheep				
Goat				
Poultry				
Equines				
Others				

2. Does livestock production is the major farming activity? Yes ☐ No ☐

3. Major farming activities (tick in the first column and rank in the secondcolumn)

Livestock production	☐	☐
Crop production	☐	☐
Mixed type production	☐	☐

4. System of production

Extensive	☐
Semi-intensive	☐
Intensive	☐

5. Number of cattle with age and sex

No.	Sex	Below 1 year	1 to 3 years	Above 3 years
1	Male cattle			
2	Female cattle			

6. Herd mixing

Communal grazing	☐	Housing	☐
Free grazing	☐	Breeding	☐
Watering points	☐	Agricultural activities	☐

7. **Purpose of keeping cattle (tick in the first column and rank (1-3) in the second column)**

Purpose	**Tick**	**Rank**	**Purpose**	**Tick**	**Rank**
Milk			Hide		
Meat			Dowry/Cultural		
Draft (work)			Ceremonies		
Market (cash)			Tangible asset		
Breeding			Investment		
Manure			Other (specify)		

8. **Members of household responsible for cattle activities**

No.	**Main Activities**	**Adults**			**Children (<15 years)**		
		Males	**Females**	**Laborers**	**Boys**	**Girls**	**Laborers**
1	Purchase cattle						
2	Selling cattle						
3	Herding						
4	Breeding						
5	Caring sick animals						
6	Feeding cattle						
7	Milking						
8	Making dairy products						
9	Selling dairy products						
10	Others						

9. **Grazing (feeding)**

Season	Free grazing	Herded	Paddock	Tethered (outdoor)	Yard	Stall	Others
Winter							
Summer							

10. Feed supplementations

Feeds	Roughage (crop residue)	Minerals (salts) and vitamins	Bought in feed (concentrates)	None	Others
Winter					
Summer					

11. Watering **Dry season** **Wet season**

Animals go to water	☐	☐
Water is fetched (provided)	☐	☐
Both	☐	☐

12. Source of water

Source	Borehole	Dam/pond	River	Water well	Municipal/piped	Spring	Other
Winter							
Summer							

13. Frequency of watering

Frequency	Freely available	Once a day	Twice a day	Every other day	Once in 3 days	Others
Winter						
Summer						

14. Housing: Does cattle have a house? Yes ☐ No ☐

No.	**Housing**	**Winter**	**Summer**
1	In the house together with family		
2	Stall/sheds		
3	Kraal		
4	Yard		
5	Others		

15. Are calves housed together with adults? Yes ☐ No ☐

16. Materials used for housing

Roof: Iron sheets ☐ Grass/bushes ☐ Open ☐ Others: _____

Floor: Earth/mud ☐ Concrete ☐ Others: _____

Wall: Wooden ☐ Stone/bricks ☐ Mud ☐ Iron sheets ☐ Others: _____

17. Trend within the herd

Increasing ☐ Decreasing ☐ Stable ☐ Unknown ☐

18. Mating system used

Mating	Uncontrolled	Hand mating	AI	Both	Group mating
Tick					
Rank (1-3)					

19. Animal health: Did you get veterinary services? Yes ☐ No ☐

Access to veterinary service: Government ☐ Private ☐ Traditional ☐

III. REPRODUCTIVE PROBLEMS ASSESSMENT

1. **Have you ever seen reproductive problems in your herd in the last five years?**

   Yes ☐ No ☐

2. **If you answer yes to the question above;** list the types of reproductive problems and rank the most common problems

No	**Reproductive problems**	**Presence**	**Rank (1-3)**
1	Abortion		
2	Retained placenta		
3	Dystocia		
4	Cervical/vaginal prolapse		
5	Stillbirth		
6	Neonatal calves' mortality		
7	Repeat breeding		
8	Anoestrus		
9	Others (specify)		

3. **Animals with reproductive problems**

   Are they housed together with others? Yes ☐ No ☐

   Do you have a separate calving pen? Yes ☐ No ☐

4. **Did you see any abortions in your herd of cattle?** Yes ☐ No ☐
5. **How many animals did abort within the last 5 years in your herd?** _____
6. **At what stage of pregnancy did the abortion occur?** _____
7. **At which stage of parity did the abortion observed?** _____

IV. FARMERS' KNOWLEDGE, ATTITUDES AND PRACTICES ASSESSMENT

1. **What do you think are the causes of abortion?**
   a. _____
   b. _____
   c. _____
2. **What is the local name for diseases that causes abortion? If any**
   a. _____
   b. _____
3. **Is there any treatment/vaccination given?** Yes ☐ No ☐
4. **Do you assist the dairy cows during parturition?** Yes ☐ No ☐
5. **If yes, do you use protective gloves and masks when assisting with the laboring,** and during handling of placentas and aborted fetuses? Yes ☐ No ☐
6. **Where do you dispense placentas, dead and aborted fetuses?**

   Burying/burning ☐

   Thrown on field or Open dumps ☐

   Water canal/dips ☐

   To carnivores ☐

   Other (Specify)_____

7. Where do you dispose of animal carcasses?

1. _____
2. _____
3. _____

8. What do you do when your animal is infected with reproductive disorders?

Activities	**Mark/tick (√)**	**Activities**	**Mark/tick (√)**
Separate the infected animal		Call the local veterinarian	
Sell to market		Give treatments	
Sell to neighbor		Nothing	
Sell to butcher			

9. Do you milk your cattle? Yes ☐ No ☐

10. What do you do with the milk produced from your herd?

Sell raw milk ☐
Processed cheese ☐
Processed butter ☐
Processed yogurt and cream ☐
Other (specify) _____

11. Who is responsible for milking?

Woman ☐
Man ☐
Children ☐
Labor ☐

12. Do you consume milk? **Yes** ☐ **No** ☐

13. How do consume milk?

Raw ☐
Boiled ☐
Other: _____

14. Do you know any disease transmitted to humans by drinking raw milk?

Yes ☐ No ☐

15. Have you seen any lameness or swelling around the knee in your cattle?

Yes ☐ No ☐

16. Did you see any testicular swelling in your bulls? Yes ☐ No ☐

17. Do you consume raw meat from cattle? Yes ☐ No ☐

18. Do you heard of the disease brucellosis? Yes ☐ No ☐

19. If yes to the above question, to which animals does this affects?

Cattle ☐ Donkey ☐

Sheep ☐ Horse ☐

Goat ☐ Others: _____

20. Do you believe that brucellosis can be transmitted to humans?

Yes ☐ No ☐

21. If yes, what do you believe is the means of transmission?

Physical contact with animals ☐

Through contact with fetuses or fetal membranes ☐

Through drinking contaminated raw milk ☐

Through contact with untested humans ☐

Other (specify): _____

22. What are the symptoms of this disease in humans? (mention)

No symptoms ☐

Abortion ☐

Fever ☐

Joint pain ☐

Loss of appetite ☐

Loss of body weight ☐

Other (specify): _____

Annex 7: Ethical Clearance

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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/14/03/15/2023

Name and affiliation of applicant: **Aweke Engdawork (DVM, MSc student)**
Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Epidemiology of infectious reproductive diseases and major reproductive problems in commercial and smallholder dairy farms in North Showa zone, Central Ethiopia*

Date of application:	**January, 2023**
Nature of the project:	**Field investigation**
Target animal species:	**Cattle**
Number of animals involved:	**511**
Study area:	**North Showa, Ethiopia**

Minutes No. and date of review: **VM/ERC/03/15/022, 25/01/2023**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of Aweke Engdawork.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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

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Fax 251-11-4339933	Tel. +251 114338450	P.o.x. Box}34	Bishoftu, Ethiopia

SEROEPIDEMIOLOGY OF INFECTIOUS BOVINE RHINOTRACHEITIS AND BOVINE BRUCELLOSIS, AND MAJOR REPRODUCTIVE PROBLEMS IN COMMERCIAL AND SMALLHOLDER DAIRY FARMS IN NORTH SHEWA, BY AWEKE

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