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SEROEPIDEMIOLOGY AND ALSO DETECTION OF BOVINE VIRAL DIARRHEA VIRAL ANTIGEN FROM DAIRY FARMS IN HOLETA TOWN, ETHIOPIA

MSc THESIS

By

WORKU BERHANU SEFER

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF CLINICAL STUDIES

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SEROEPIDEMIOLOGY AND ALSO DETECTION OF BOVINE VIRAL DIARRHEA VIRAL ANTIGEN FROM DAIRY FARMS IN HOLETA TOWN, ETHIOPIA

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Epidemiology

By

Worku Berhanu Sefer

June, 2021

Bishoftu, Ethiopia

Signature Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As MVSc research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance by Worku Birhanu, entitled: "**SEROEPIDEMIOLOGY AND AlSO DETECTION OF BOVINE VIRAL DIARRHEA VIRAL ANTIGEN FROM DAIRY FARMS IN HOLETA TOWN"**; I recommend that it can be accepted as fulfilling the thesis requirement for the Degree of Master of Veterinary Science in Veterinary Epidemiology.

Submitted by: Worku Berhanu _____ _____

Name of student Signiture Date

Approved for submittal to MSc research Thesis assessment committee:

Dr. Haileleul Negussie _____ _____

Major Advisor Signature Date

Dr.Haileleul Negussie _____ _____

Departement chairperson Signature Date

Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As a member of the Examining Board of the final MVSc open defense, we certify that we haveread and evaluated the thesis prepared by: Worku Birhanu,entitled: "**SEROEPIDEMIOLOGY AND AlSO DETECTION OF BOVINE VIRAL DIARRHEA VIRAL ANTIGEN FROM DAIRY FARMS IN HOLETA TOWN**; we recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Veterinary Science in Veterinary Epidemiology.

Dr. Bulto Giro	_____	_____
Modulator	Signature	Date
Dr. Tesfaye Rufael	_____	_____
External Examiner	Signature	Date
Dr. Yasmin Jibril	_____	_____
Internal Examiner	Signature	Date
Dr. Haileleul Negussie	_____	_____
Major Advisor	Signature	Date
Dr. Asamenew Tesfaye	_____	_____
Co-Advisor	Signature	Date
Dr.Haileleul Negussie	_____	_____
Departement chairperson	Signature	Date

STATEMENT OF THE AUTHOR

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Name: Worku Birhunu Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

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LIST OF ABBREVIATIONS

AB	Antibody
BVDV	Bovine Viral Diarrhea Virus
BRD	Bovine Respiratory Disease
CPE	Cytopathic Effect
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
IHC	Immunohistochemistry
IPMA	Immune Peroxidase Monolayer Assay
MAb	Monoclonal Antibody
MD	Mucosal Disease
Ncp	Noncytopathic
PCR	Polymerase-chain reaction
PI	Persistent infection
RNA	Ribonucleic acid
TI	Transient infection
USD	US dollars
VI	Viraemic infect

ABSTRACT

A cross sectional study was conducted from November 2020 to April 2021 to detect the antigen, investigate seroepidemiology, and associated risk factors bovine viral diarrhea virus (BVDV) infection in Holeta town at Walmera district of Ethiopia. A total of 337 sera samples were collected from unvaccinated cattle. The sera samples were subjected to BVDV antibody and antigen detection using indirect enzyme linked immunosorbent assay and antigen-capture ELISA test, respectively. In this study, 15.43% (n = 52) and 64.71% (n = 11) overall seroprevalence of bovine viral diarrhea virus antibody was observed at individual and herds level, respectively. All antibody-negative serum samples (n =285) were tested for antigen using antigen-capture ELISA, of which 0.35% (n = 1) was found antigen-positive for BVDV.This is the first report of BVDV antigen prevalence (0.35%) in the study areas and Ethiopia. A statistically higher seroprevalence (P = 0.000) of BVDV was observed in cows with a history of abortion 52% (OR: 7.2; 2.98-16.56), cows with repeated breeder 50% (OR: 6.2, 2.85-13.12), the introduction of new animal to the herd 50% (OR: 6.97;3.01-16.13), animals reared in intensive farms 26.43% (OR: 4.36;0.28-8.32) and parity ≥ 2 24.17% (OR:4.42;1.92-10.14). Additionally, Congenital defects 41.67% (OR: 4.22;1.28-13.86, P=0.017), large herd size 24.22% (OR: 3.36; 1.358.35,P=0.009), adults 23.12% (OR: 3.78;1.63 8.77, P=0.002) and farms used both AI and bull breeding 17.44% (OR: 2.17;0.93-5.03,P=0.070) were identified as potential risk factors and significantly associated with bovine viral diarrhea seropositivity. Seroprevalence of BVDV was not associated with body condition scores and sex at (P>0.05). This study revealed that cattle residing in Holeta town have greater exposure to BVDV infection and varied with reproductive problem, management, and farming system of the animals. The finding has also detected the antigens that helpful to further isolate the dominant genotypes BVDV circulating in the study area

Keywords: Antigen capture-ELISA, BVDV, Dairy cattle, Holeta, Ethiopia, Seroepidemiology

1. INTRODUCTION

The bovine viral diarrhea virus (BVDV) is a major pathogen that affects cattle worldwide and causes significant economic losses. It is a pathogen in the genus *Pestivirus of the Flaviviridae* family that is economically significant (Brodersen, 2014). The noncytopathic (ncp) and cytopathic (cp) biotypes of BVDV have been identified based on their effects on cell cultures. Only ncp BVDV can cause long-term infections, while the emergence of cp BVDV from a spontaneous mutation in animals that have been infected with ncp BVDV for a long time is crucial in the pathogenesis of Mucosal disease (MD) (Moennig and Becher, 2018).

BVDV is a high morbidity and mortality rates associated with an increased premature culling and a decreased reproductive performance, which is caused by early embryonic death, premature birth, congenital defects, weak calves, stillbirths, and the birth of persistently infected (PI) offspring (Lindberg, 2016). Due to the disease's broad nature, the transmission and absence of intervention, it has become a globally endemic and one of the most important cattle diseases (Khodakaram-tafti *et al*., 2017).

The virus survive in the cattle population depends on the characteristic of persistently infected host. Persistent infection arises by the unique ability of the BVDV to survive by inducing immune tolerance in the bovine fetus through evasion of both innate and acquired immunity in utero(Brodersen, 2014). BVDV transmission can occur either directly or indirectly through inhalation or ingestion of virus-contaminated materials. The main transmission route in infected herds is direct contact with a PI animal (Lindberg, 2003).

Horizontal transmission occurs primarily through contact with PI and TI animals excrete the virus. The transmission of the virus across the placenta to the developing foetus is a result of viremia during early pregnancy. This does not occur in those animals with antibody and provides good evidence for protection against viraemic spread of BVDV in seropositive animals(Brownlie, 2014).

The presence of genetic variation and BVDV's ability to persist in carriers limit its control in the cattle population. BVDV control efforts have primarily focused on detecting PI cattle. This

process may overlook the detection and management of transient BVDV infections, which are frequently associated with cattle morbidity and mortality due to BVDV's immunosuppressive effects and its potentiating effect on secondary infections (Peddireddi *et al*., 2018).

Bovine viral diarrhea viruses are capable of infecting cattle of all ages. Farmers incur significant costs as aresult of BVDV due to increased production losses and mitigation expenses.Global BVDV production losses have been estimated to be up to 687.80 USD per animal (Pinior *et al*., 2019).

In Ethiopia, BVDV infection may have a negative impact on animal production. Milk production and reproduction quality, for example, decline, and young stock develop at a slower pace. The use of competitive ELISA for BVDV antibody testing shows a higher prevalence of the virus. In Ethiopia there is no the national sero prevalence data of BVDV. It is not possible to confirm PI status or determine the predominant BVDV genotype, whether BVDV- 1 or BVDV-2. knowing the BVDV genotype and subtype is critical for controlling the infection by vaccination strategies. The global distribution of BVDV has shown heterogeneity in genotype and sub-type (Tadesse *et al*., 2019a). In Ethiopia, the epidemiology, genotype of the virus, clinical importance, and economic loss associated with BVDV infection in cattle are all critically lacking. Therefore, the specific objectives of this study are:

- To determin e the seroepidemiology and assess associated risk factors with BVDV
- To detect the antigen of the virus from dairy farms in Holeta, Ethiopia

2. LITERATURE REVIEW

Cornell University researchers discovered bovine viral diarrhea (BVD) in 1946 (Moennig and Becher, 2018). Olafson and colleagues were the first to identify the clinical manifestations of infection with bovine viral diarrhea virus, reporting on a new disease in cattle characterized by acute gastroenteritis and gastrointestinal erosions. The virus was recognized and its epidemiology was gradually better understood during the 55 years followed (Lindberg, 2016).

Mucosal disease was successfully reproduced experimentally in 1984-1985, resulting in a basic understanding of the disease. The study of the molecular structure of the BVD virus was also increased at this time, and the virus's genome was cloned and sequenced as a result (Deregt and Loewen, 1995). It is found in almost every cattle-producing country on the planet, with at least 88 countries having it and 107 countries reporting mitigation activities between 1960 and 2017(Rocha, 2020).

In immunotolerant animals, the causal relationship between infection in the first trimester of pregnancy, establishment of persistent infection, and subsequent death from mucosal disease is now apparent (Lindberg, 2016). The virus's first isolates were non-cytopathic, but cytopathic strains were later discovered, and it is now known that this distinction can differentiate the virus in cell culture (Brownlie, 2014).

2.1. Etiology

BVDV is an enveloped, spherical virus that is relatively small (40-60 nm). The genome is a 12,500 base pair single-stranded positive sense ribonucleic acid (RNA) molecule (Lindberg, 2016). Pestivirus is divided into four species: BVDV-1 and BVDV-2 (previously known as genotypes 1 and 2), classical swine fever virus, and border disease virus (Khodakaram-tafti *et al.*, 2017). The virus has a lot of genetic diversity, and two distinct species (BVDV-1 and BVDV-2) have been identified, each with several sub genotypes (Moennig and Becher, 2018). Each of these genotypes is further subdivided into several subgenotypes (BVDV-1a to 1q and BVDV-2a to 2c) that are distributed worldwide(Peddireddi *et al*., 2018).

There are hundreds of different strains of the virus, characterized by viral nucleotide sequence comparison or by monoclonal antibody (MAb) serotyping, which can also be categorized under two biotypes based on their growth characteristics in cell cultures(Tadesse *et al*., 2019).

Two genotypes, BVDV type 1 and BVDV type 2, are identified as distinct species within this genus, with further classification as cytopathic (cp) and noncytopathic (ncp) based on in vitro cell culture characteristics and genetic differences(Brodersen, 2014). Despite their denomination, the name of the biotypes does not correspond to the pathogenicity of the virus in the field, but rather to the effect the virus has when grown in cell culture. Noncytopathic strains are adapted to persist by avoiding the induction of a type I interferon response in the fetus, they can establish persistent infections, whereas cp strains cannot. Among the pestiviruses, two other important animal pathogens can be found: classical swine fever virus and border disease virus in sheep (Lindberg, 2016). Molecular characterization of BVDV strains in the 1980s and early 1990s resulted in the establishment of the first viral genomic sequences providing the basis for the segregation of BVDV strains into two separate species, BVDV-1 and BVDV-2, which can be further subdivided into subgenotypes(Moennig and Becher, 2018).

2.2. Epidemiology

BVDV is a major cattle pathogen with a global distribution that causes significant economic losses (Kadir Yesilbag, 2017). Between 1960 and 2017, it was found in most cattle-producing countries, with at least 88 countries reporting reported infections and 107 countries reporting prevention activities (Rocha, 2020). Actually endemic in the majority of countries around the world, with control programs underway in Germany, Scotland, Belgium, Northern Ireland, and Ireland, as well as regional programs in a number of European countries (Yarnall and Thrusfield, 2015).

The prevalence of PI in animals worldwide ranged from low (0.8 percent in Europe, North America, and Australia), medium (>0.8 percent to 1.6 percent in East Asia), and heavy (>1.6 percent in West Asia). In Europe, the prevalence of PI has decreased over time, while the prevalence of BVDV has risen in North America (Donoso *et al*., 2018).

In Africa countries the detected virus in cattle includes: South Africa it ranging from 37% to 100%. In Namibian in the late study (1980s), 49% of cattle had neutralizing antibodies to BVDV. On the Kafue flats in Zambia in 1987, an antibody prevalence of 76.2% was found. In Tanzania, a 34% seroprevalence have been observed. In Botswana, however, knowledge of BVDV prevalence is limited and dated. In the 1970s, two cattle herds with clinical signs indicative of BVDV infection were investigated, and an antibody prevalence of 42% and 70% found. Also, testing was conducted in a nearby village without clinical signs of BVDV, which detected 19% seropositivity(Lysholm *et al*., 2020).

In Ethiopia the level seroprevalence (51.7%) was the highest result yet reported in cattle. seroprevalence was previously reported 9.59% by Nigussie *et al.,* (2010) in Jimma zone by using indirect ELISA, 11.7% prevalence reported by Asmare *et al.,* (2013) in central and southern parts of Ethiopia, 32.9% prevalence recently reported by Asmare *et al.,* (2018) in Ethiopian dairy cattle with history of reproductive disorders by using competitive ELISA and 32.6% prevalence by Aragaw *et al.* (2018) in three milk sheds sample using competitive ELISA. This result was also higher than previous reports from other East African countries; 19.8% in Kenya and 10.7%in Sudan(Tadesse *et al*., 2019).

Table 1: The mean prevalence of countries

	Before 2008	After 2008
Antibody positive herds		
Asia	83%	73%
Europe	*57%*	46%
Oceania	87%	78%
Africa	83%	74%
Central America	54%	-
North America	61%	-
South America	93%	67%
Persistently infected and or viremic infected animal (PI/VI or VI/PI)		
Asia	6.9%(PI or VI): 4%(PI)	4.5%(PI or VI): 0.2%(PI)

	9.1%(VI)	5.8%(VI)
Europe	5% (PI or VI): 3.6% (VI)	0.5%(PI or VI): 0.2%(PI)
	6.5%(VI)	1.5%(VI)
Oceania	12.7%(PI)	0.3%(PI)
Africa	11.7%(VI)	19.1%(VI)
Central America	-	-
North America	8.6%(PI or VI):0.9%(PI)	3.6%(PI or VI): 0.5%(PI)
	13.7%(VI)	6.2%(VI)
South America	1.2%(VI)	2,3%(VI)

Source (Richter *et al*., 2019).

From several and different genotypes of BVDV have been detected in Africa;BVDV-1a is the most frequent genotype (Kadir Yesilbag, 2017).

The various BVDV subgenotypes predominate in different countries. The most frequently-reported BVDV-1 subgenotypes are 1b, followed by 1a and 1c. The highest number of various BVDV subgenotypes has been documented in European countries, indicating greater genetic diversity of the virus on this continent. Current segregation of BVDV field isolates and the designation of subgenotypes are not harmonized(Kadir Yesilbag, 2017). BVDV in Chilean cattle is composed of the subgenotypes BVDV-1a, BVDV-1b, and BVDV-1j, with BVDV-1b and BVDV-1j as the predominant subgenotypes(Donoso *et al*., 2018).

A wide range of ruminant hosts, including cattle, sheep, goat, pigs, yak, buffalo, llama, alpaca, camel, deer, and bongo can be infected with BVDV (Kadir Yesilbag, 2017). These animals act as an important source of infection to cattle. Seropositive rate of the virus more in older cattle than in younger animals; because of the increment of exposure to respiratory viruses during life(Khezri, 2015). Although the virus is named for its primary host, its prevalence in non-bovine species has become increasingly recognized. The virus has been isolated in over 40 species and serological evidence indicates that most wild ruminants are susceptible to BVDV infection(Khodakaram-tafti *et al.*, 2017). Common use of pastures by PI and healthy animals in the summer is the important for transmission within a population(Khezri, 2015). BVDV and border disease virus can be transmitted between cattle and sheep(Lindberg, 2016).

Contamination of fetal bovine serum (FBS) by the ncp biotype has long been known and remains a recognized risk factor for the worldwide distribution of BVDV. Because FBS is used in the production of vaccines and other biological products, the global trade of infected FBS products is a potential source of trans boundary spread of BVDV(Luzzago *et al.*, 2014). Open breeding herd is the primary risk factor for disease introductions there are likely specific purchased cattle are at increased risk of generating outbreaks in the destination herd(Gates et al., 2014).

Herd size and density are significant risk for the prevalence of infection in populations where BVDV is endemic. To date, BVDV control efforts have been focused primarily on detecting PI cattle. This process can ignore detection and management of transient BVDV infections, which are frequently associated with morbidity and mortality in cattle because of the immunosuppressive effects of BVDV and its potentiating effect on secondary infections. Of particular importance is the documented association of transient BVDV infections and bovine respiratory disease (BRD) in feedlot cattle(Peddireddi *et al*., 2018).

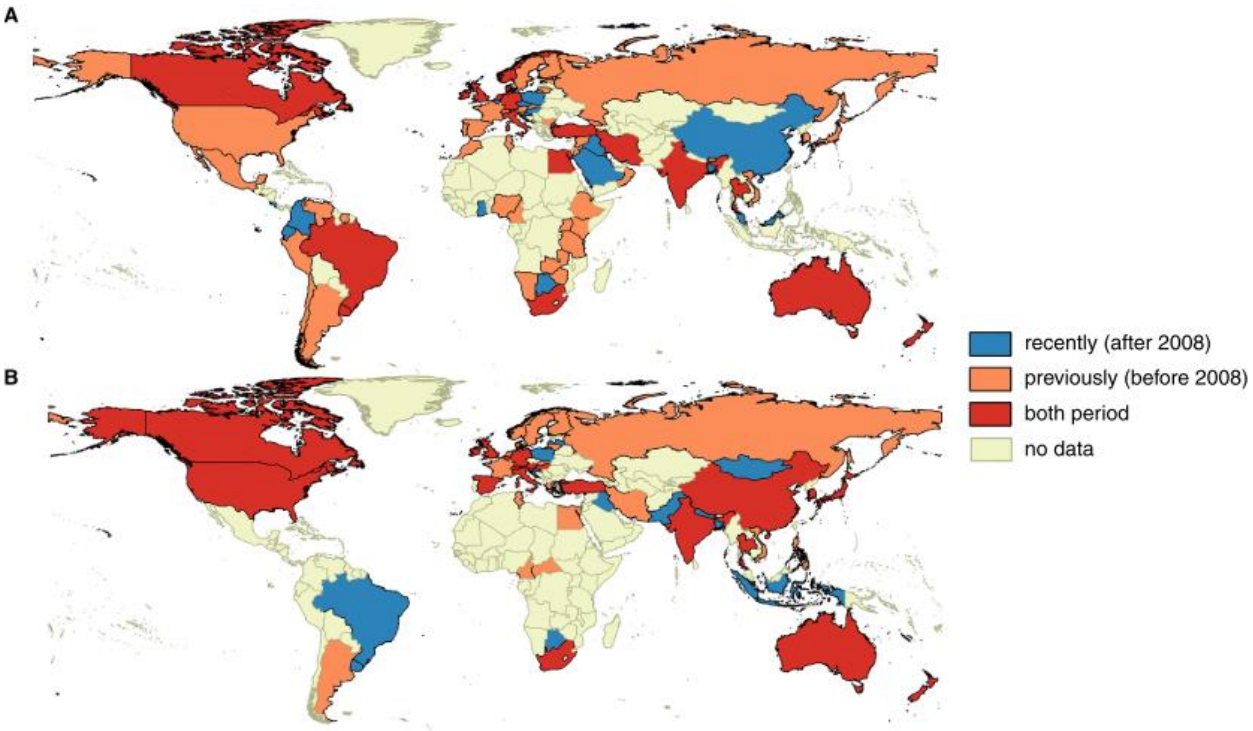

Figure 1: Worldwide BVDV infections map

Source;(Richter *et al*., 2019)

(a) Antibodies positive and (b) persistently infected (PI) and viremic infected (VI) infections

2.2.1. Transmission

BVDV can be transmitted both by direct contact between an infected and a susceptible animal, and indirectly through different types of vehicles(Lindberg, 2011). The principal part of transmission route in infected farms is direct contact with a PI animal. The horizontal transmission of BVDV may be direct or indirect via inhalation or ingestion of virus-contaminated materials(Khodakaram-tafti *et al.,* 2017).Two distinct BVDV infections in cattle are transient and persistent infections. Transient or acute infections are associated with respiratory or reproductive diseases(Peddireddi *et al.,* 2018). The importance of the virus is due to the occurrence of persistently infected (PI) animals, resulting from infections of pregnant cows or heifers during gestation prior to the development of the fetus' immune system. In such a case, the virus is recognized as "self", and an animal born alive will spread BVDV lifelong.

Persistently infected cattle may develop fatal mucosal disease(Casaubon *et al.*, 2012). The most frequent route of BVDV infections is by oro-nasal uptake of the virus. Acute natural BVDV infections of BVDV seronegative cattle result in a transient viraemia, starting 3 days post-infection(Sarrazin, 2015).

Direct contact with PI animals is the most efficient route of transmission. In contrast, animals subjected to a primary BVDV-infection have been are poor transmitters of the virus even in the presence of a concurrent infection(Lindberg, 2016). Exposing susceptible cattle to the virus PI cattle are a means of introducing infections into a herd (Peddireddi *et al.*, 2018).

Other mechanisms of vertical transmission include: contaminated semen, embryo transfer, and contaminated modified live vaccines. Infected bulls can shed BVDV in semen for prolonged periods, and cattle have been infected following insemination with frozen semen from these animal(Khodakaram-tafti *et al.*, 2017). The majority of transient infection is caused by NCP viruses. Infected animals shed virus in nasal and oral secretions, less so in feces and urine. This form of infection is important in pregnant cattle because of the ability of the virus to cross the placenta and cause intrauterine infections of the fetus(Khodakaram-tafti *et al.*, 2017).

BVDV can be transmitted to the fetus in pregnant cattle during the 40 to 120 days of gestation, thereby inducing fetus immune tolerance to the virus and resulting in the delivery of PI calves(Isoda *et al.*, 2017). Indirect transmission of BVDV by rectal palpation, nose tongues, ambient air, fetal fluids and contaminated pens and injectable have been shown experimentally, but only where the initial contact was PI(Lindberg, 2011).

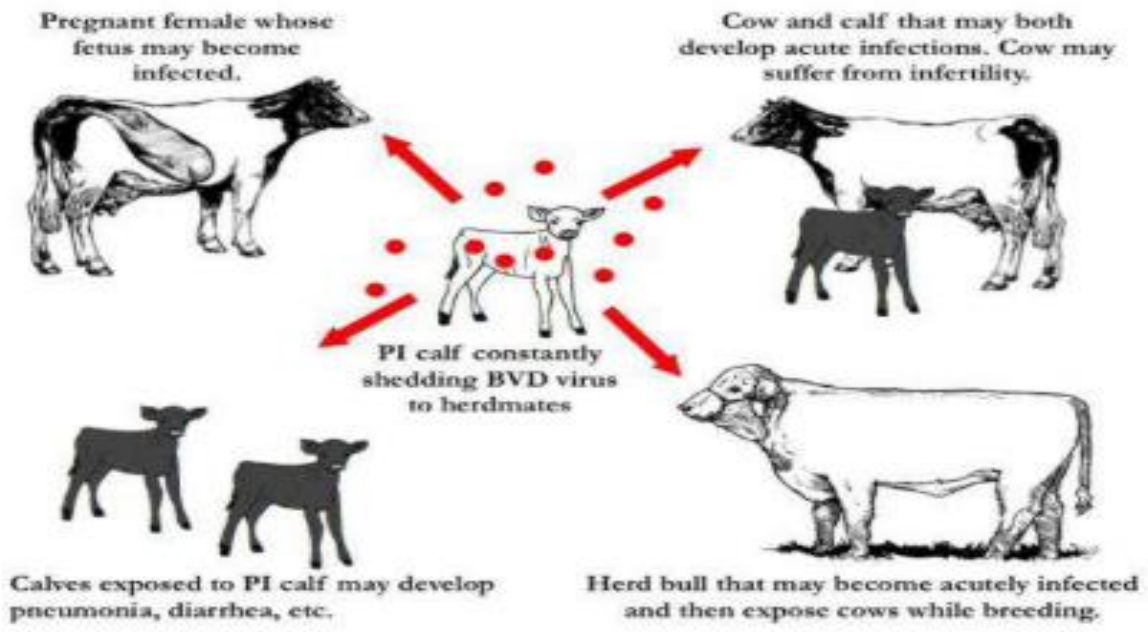

Figure 2. Transmission pattern of PI cattle excrete the virus
Source (Khodakaram-tafti *et al.*, 2017).

2.3. Pathogenesis of BVDV

The pathogenesis of BVDV infection is complex, with infection pre- and post-gestation leading to different outcomes. Infection of the dam during gestation results in fetal infection, which may lead to embryonic death, teratogenic effects or the birth of persistently, infected (PI) calves. Bovine virus diarrhea virus (BVDV) disease in cattle ranges from the transient acute infections, which may be inapparent or mild, to mucosal disease, which is inevitably fatal. Venereal infection is important in the transfer of virus to the fetus and congenital infections can cause abortions, malformations and the development of persistently viraemic calves (Brownlie, 2014).

2.4. Clinical sign

Clinical signs of acute transient postnatal infection temperatures as high as 42^{o}C, diarrhea, ulceration of the muzzle and oral cavity, and leucopenia. Few or no clinical signs were detected in other infected animals(Brodersen, 2014). Persistently infected cattle may develop the fatal

mucosal disease(Casaubon *et al.*, 2012). During acute outbreaks, cattle infected with BVDV may exhibit non- specific clinical signs of depression, in appetence, fever, and diarrhea leading to transient declines in milk production, growth performance, and animal fertility(Gates *et al.*, 2014).

BVDV cause fetal infections with a noncytopathic BVDV biotype, at 2 to 4 months of gestation. neonate will be immunotolerant to the homologous BVDV strain, remain PI with that strain of BVDV for its life, and serve as an important reservoir for the transmission of virus in a susceptible population(Peddireddi *et al*., 2018). More serious complications arise when virus crosses the placental barrier in pregnant cattle. Fetal infections have been associated with early embryonic death, abortions, stillbirths, congenital abnormalities, and the development of persistent infections in calves that gain immunotolerance to BVDV through vertical transmission of the virus during early gestation(Gates *et al*., 2014).

2.5. Diagnosis

There are different reliable methods for the detection of BVDV infected animals and, more importantly, differentiate acutely infected from PI animals because the identification and removal of PI animals that serve as the natural reservoirs are essential in preventing the spread of BVDV(Khodakaram-tafti *et al*., 2017). PI cattle with BVDV can be identified by virus isolation from whole blood (buffy coat) or other tissues, micro titer virus isolation (Immune Peroxidase Monolayer Assay; IPMA) from serum, immunohistochemistry (IHC) staining of viral antigen in skin biopsies, antigen- capture enzyme-linked immunosorbent assay (ELISA) and polymerase-chain reaction (PCR) methods(Khezri, 2015).The direct test includes virus isolation, antigen capture ELISA and polymerase- chain reaction (PCR)(Tadesse *et al*., 2019).

Detection of virus specific antibodies by using different serological tests such as virus neutralization test and enzyme linked immunosorbent assay are an important ways for the indirect detection of the virus. The presence of BVDV in a sample can be demonstrated by isolation and detection in cell culture, by detection of viral antigens, or by detection of viral nucleic acid(Lindberg, 2016).

Peripheral blood leukocytes, serums, and nasal swabs were collected for viral isolation and serology(Fulton *et al*., 2005). Culture and identification of BVDV from clinical specimens remains the "gold standard" diagnostic technique. Unfortunately, viral isolation methods are labor intensive and take several days to be completed, and may not differentiate between TI and PI animals, unless positive cattle are re-tested and remain positive at a later date of 3 weeks. Antigen-capture enzyme-linked immunosorbent assay has good sensitivity, specificity and repeatability for detecting antigen from BVDV; it is a robust, economical method of identifying PI cattle, easy to transfer and to perform(Khodakaram-tafti *et al*., 2017).

Table 2. The diagnostic test for detection of PI

Test	**Cost**	**Advantages**	**Disadvantage**	**Specimens/shipping**
Virus isolation 1-3week turnaround	Moderate to high cost	Gold standard for BVDV -High specificity Virus is available for study at a later date	-Slow procedure Labor-intensive Potential false negative due to interference by maternal Ab - Retest positive animals in 3-4 weeks to distinguish between PI and TI	-Whole blood (10 ml) or serum (2-3 ml) and tissue samples -Send in container with cold packs -Do not freeze the samples

Immunohistochemistry (IHC) 2-5day turnaround	Low cost	-High sensitivity -Usually identifiesony PI -TI animals usually test negative	-Labor-intensive Formalin usage -Will not generally identify TI animals	-Skin samples-ear notch and tissue samples -Send fresh on wet ice or stored in 1:10 volume of 10% neutral bufferedformalin –Sample can be held in formalin forseveralweeks
Antigen-Capture ELISA of serum 1-2day turnaround	Low cost	-High sensitivity -Easy to carry out	-Potential false negative due to the interference bymaternal antibodies –Variation of viremia -To distinguish between PI and TIanimals,retest 3weeks later	-Serum (2 ml) -Send in insulated container with cold packs
Antigen-Capture skin ELISA of skin 1-2 day turnaround	Low cost	-High sensitivity -Usually identifies only PI animals –TI animals usually test negative	-Will generally not identify TI animal	-Skinsamples -ear notches –Sendin insulated container with cold packs -Do not allow to dry out -Whole
Antigen-Capture ELISA of	Low cost	High sensitivity	-Labor-intensive to prepare buffy coat -Not used in	-Whole blood (10 ml) using EDTA or heparin -Tissues –Send in

tissue/leukocytes 1-3daysturnaround			a large screening	insulated container with cold packs -Whole
PCR 1-3 days turnaround	Moderate to high cost (can be reduced pooling samples	-High sensitivity -Can detect 1 ng/ml BVDV RNA	-Potential of false positive due to laboratory contamination -Retest samples in 3 weeks to distinguish between PI and TI animals	-Whole blood (10 ml) or serum (2-3 ml) -Ear notches in red top tubes -Milk, semen and tissues -Send in insulated container with cold pack

Source; (Khodakaram-tafti *et al*., 2017)

2.6. Economic importance of BVD

BVD causes substantial costs for farmers through increased production losses and mitigation expenditures. Worldwide BVDV production losses have been estimated to be up to 687.80 US dollars (USD) per animal. Depending on the time and duration of infection, the virus can cause a considerable number of direct losses, suchas morbidity and mortality due to immunosuppression, reduced reproductive performance (e.g., first service conception, extended calving intervals), stillbirth and abortion, congenital deformities and malformations, growth retardation, reduced milk production and average daily weight gain(Pinior *et al*., 2019). The mean financial loss per cow per year in beef herds where the disease is not diagnosed quickly and without intervention or re-infection was estimated to be €46 (£37)(Sarrazin, 2015).

2.7. Treatments

There is no treatment for subclinical BVD or acute mucosal disease. Support therapy in the form of fluids and anti-inflammatory agents can be used for acutely infected animals. PI's whether

diseased or apparently healthy should be removed from the herd as soon as possible (Pinior *et al*., 2019).

2.8. Control and prevention

Currently, the only approaches that have been successful in reducing the impact of BVDV infections at a larger scale are those that put emphasis on biosecurity in general, and control of direct animal contacts in particular with or without the complementary use of vaccines(Lindberg, 2011). Any successful BVD control program requires the removal of PI animals in order to protect susceptible or incompletely protected animals. Immediately after detection, viremic cattle should to be removed from the herd(Moennig and Becher, 2018).

Due to the nature of the infection, there is no treatment to fully cure an infected animal and the key lies in the prevention of disease. Persistently infected animals in the domestic and wild populations are important reservoirs of the virus and shed large amounts of virus throughout their lives and it spreads among herds. All control programs which are in use in many countries, largely depend upon the detection and removal of PI animals, and preventing the introduction of PI animals in the herds. Detection of PI animals at early stage, particularly soon after birth is of significant benefit to implement BVDV control programs(Khodakaram-tafti *et al.*, 2017).

Vaccination can represent an accompanying tool to prevent BVDV, but without removing PI animals it does not enable the elimination of the virus in a susceptible population(Kadir Yesilbag, 2017). There are diverse BVDV strains and thus, current vaccines contain representative genotype 1 and 2 viruses (BVDV-1 & 2) to broaden coverage. BVDV modified live virus (MLV) vaccines are superior to killed virus vaccines, but they are susceptible to neutralization and complement-mediated destruction triggered by passively acquired antibodies, thus limiting their efficacy(Lokhandwala *et al*., 2017).

Table 3. Types of BVD vaccines

Name of veterinary medicinal product	Type (live/dead) and strain(s)	Way of administration	Duration of immunity/booster interval	Manufacturer
Bovela lyophilisate and solvent for suspension for injection for cattle	Modified live bovine viral diarrhoea virus type 1, non-cytopathic parent strain KE-9 and modified live bovine viral diarrhoea virus type 2, non- cytopathic parent strain NY-93	Intramuscular injection	1year	Boehringer Ingelheim
Bovidec	Bovine viral diarrhoea (BVD) virus strain KY1203nc (inactivated)	Subcutaneous infection	A single annual booster dose is recommended	Novartis Animal Vaccines Ltd
Bovilis BVD Suspension for injection for cattle	Inactivated antigen of cytopathogenic BVDV strain C-86	Intramuscular injection	One vaccination every 6 months	MSD Animal Health

Source; (More *et al*., 2017)

BVDV control consists of three essential measures: preventive measures (biosecurity), removal of PI animals (virus elimination) and follow-up of the BVDV status (monitoring). Immunization through vaccination is as an optional fourth element(Sarrazin, 2015).

2.9. The status of BVDV in Ethiopia

In Ethiopia, few studies were conducted on the disease indicated that 9.6%, 16.6% and 6.11% seroprevalence of BVDV was reported in dairy cattle herds in Jimma, south western Shoa, and West Shoa, respectively (Nigussie *et al.,* 2010). Seroprevalence of 11.7% of BVDV was also reported in breeding and dairy farms of southern and central Ethiopia (Asmare *et al.,* 2012). There is no study conducted to determine the rate of persistent of infection caused by BVDV in Ethiopia (Tulu *et al.,* 2018).

The individual level seroprevalence of bovine viral diarrhea virus in the 420 cattle tested was 51.7% in which 217 animals were found seropositive. The herd level prevalence of the virus was 95.6% of 43 farms have at least one seropositive for BVDV antibody out of 45 sampled dairy farms(Tadesse et al., 2019a). Out of a total of 1379 dairy cattle examined serologically for exposure to BVDV, 449 (32.6%) were positive. The true animal-level prevalence estimate was 28.5%. BVDV seroreactors were detected in all the 15 conurbations involved in the study with prevalence ranging between 10.0 and 71.9% in Bishoftu and Holeta, respectively. Of the total of 149 herds sampled, at least one reactor was found in 104 (69.8%). All 19 herds tested in western Ethiopia were positive while 51 of the 77 (66.2%) and 34 of the 53 (64.2%) from southern and central Ethiopia, respectively, were positive(Aragaw *et al*., 2018).

The higher seroprevalence was estimated in adult age categories, cows with history of repeat breeding compared to cows with history of abortion and farms introduced new animals to their herds. Among other suspected risk factors for BVDV infection, age, introducing of new animals to herd and animals with history of reproduction problems were potential risk factors for BVD in Jimma town dairy farms(Tadesse *et al.,* 2019).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Holeta town at Walmera District of West Shoa Zone of Oromia regional state, which is located 30km to the west along the main road to Ambo. Geographically, the district is found 9^{o} 0' 0''-9^{o} 10' 0' N latitude and 38^{o} 25' 0''- 38^{o} 30' 0'' E longitudes. The study area has an altitude of 2400m.a.s.l and receives an average annual rainfall of about 1000mm.The mean minimum and maximum temperatures are 6 and 22^{o}C, respectively (WDLDFO, 2017). The mean relative humidity is 59%. The study area obtains a short rainy season (March to May), long rainy season (June to September) and dry season (October to February) (HARC, 2008). The total human population of the district is 104,932 and cattle are the dominant livestock of the smallholder farmer in the area, although limited number of small ruminants and equines are kept (WDLDFO, 2017). This area is purposively selected based on the relative abundance of dairy farms and the long tradition of keeping improved dairy cattle.

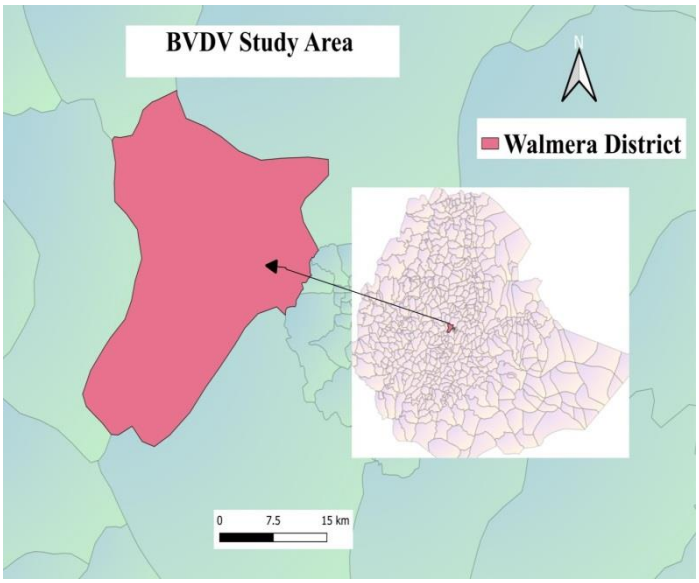

Figure 3: Study Area map

3.2. Study animals

The study animals were Holstein-Friesians crosses breeds reared in small-holder; medium and large commercial dairy farms located in and arround Holeta town. Small-holder dairy farms are those holdings with up to 1-15 dairy cows, medium with 15-25 and large commercial dairy farms more than 25 dairy cows and produce milk basically for sale. unvaccinated animals were selected for the study but in Ethiopia, cattle are generally not vaccinated against BVDV.

All of the sampled animals were Holstein-Friesian cross breed. Relevant individual animal data and herd- level information were collected using farmers interview through a semi- structured questionnaire. Information of each sampled animal including herd size, sex, age, parity, reproductive disorder, introduction of new cows to the herd were recorded using a structured questionnaire to identify the potential risk factors associated with the virus. Large, medium, and small-holder dairy farms were selected for sample collection.

3.3. Study design, sampling method, and sample size Determination

A cross-sectional study design was conducted to address the objectives of the study from November 2020 to April 2021 on randomly selected 17 farms/herds out of 133 registered dairy farms at Walemera District Livestock development and Fishey Office (WDLDFO) in Holeta town. All cattle above six months of age were included in this study. Depending of the herd sizes, herds were classified into three categories(I-herds with 1-15 animals, II- herds with 15-25 and III-herds with above 25 animals).

Animals were also grouped into three age categories (calves with 6m-1 year, heifer with 1-2 years and adults with above 2 years were selected. Out of 133 registered dairy farms of the town, 17 farms were selected by simple random sampling techqnique. Thereafter, all animals within the randomly selected farms were included into the sample. The sample size was determined based on the previous BVDV expected prevalence of 32.6% in intensive dairy farms at centeral part of Ethiopia reported by Aragaw *et al*. (2018) was used to calculate the sample size. Accordingly,a total of 337 blood sample was collected.

$n=[Z^2 \times Pexp(1-Pexp)] / d^2$

$n=[1.96^2 \times 0.326(1-0.326)]/0.05^2 = 337$

Where, n, sample size; z, confidence statistic; Pexp, expected prevalence (32.6%); d, desired absolute precision

3.4. Sample collection and Serological assay

Two sets of blood samples were collected from 337 randomly selected cattle from jugular vein under sterile conditions. One set was with EDTA and another set without EDTA for separation of the buffy coat and sera, respectively. From each randomly selected animal, 10 ml of blood was drawn and kept overnight in an upright position. The serum samples were decanted into labeled sterile cryovials and transported on ice box with ice pack to the National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratory and preserved at -20°C until diagnosis.

3.4.1. Indirect Enzyme-Linked Immunosorbent Assay (iELISA)

The presence of antibodies to BVDV was tested using an indirect Enzymes-linked Immmunosorbent assay (iELISA) antibodies anainst BVDV p80 antibody (IDEXX, France) according to the manufacturer instruction. Each serum sample was diluted in a 50 µl dilution buffer, and the positive serum was resespended in 1/100 and dispensed at 100µl in microplate wells. The plate was incubated at 18-26^0c for one hour. After incubation, the plate was wash 3 times with a 300µl of washing solution, and 100µl of diluted conjugate solution was added to each well. The plate was incubated for 30 min. at 18-26^0c. Then, the plate was washed 3 times and 100µl of chromogen solution was added to each well. After incubation for 20 minutes at room temperature(21^0C) away from direct light, the reaction was stopped with 100µl of stop solution. Next, BioTek EL800 Microplate Reader used to measure the optical dencity (OD) of each samples and controls at 450nm. A serum with absorbance value (S/N) with ≤50% a acut-off level of 0.4 was considered to be BVDV positive. The serum with absorbance value in between 0.4 and 0.5 were considered as doughtful and value greater than 0.5 were considered as negative according to manufacturer of the ELISA kit.

3.4.2. Antigen capture ELISA (ACE) assay

Detection of BVDV antigen in serun sample was performed using the manufacturer's instruction (BVDV Ag/Serum Plus, IDEXX, Switzerland). This kit is desgined to detect BVDV Erns antigen in serum, plasma, whole blood, and ear notch tissue in cattle. The test kit has diagnostic specificity of 100% (95% CI: 95.69-100) and sensitivity 100% (95% CI: 90.50-100). A micro titration format was configured by immobilizing specific monoclonal antibodiesfor BVDV(Erns) on the plates that would then bind BVDV antigen in the sample. After inubation (2 hours 37°C), of the test sample in the plate, captured BVDV antigen was detected by specific antibodies and horseradish peroxidase conjugate. Next, an unbound congugate was washed away and a substrate/chromogen solution was added. In the presence of enzyme, substrate was conveted into a product that react with the chromogen to generate a blue colour. Upon the addition of the stop solution, a yellow colour was generated.

The absorbance was measured using BioTek EL800 Microplate Reader a spectrophotometer dual wave length of at 450 nm or using 450 nm and 650nm. The corrected OD value of the samples were calculated by using the absorbance obtained with the test sample and corrected for the absorbance the absorbance negative control. The test protocol include adding a positive and negative control to each plate for every run of the assay.

COD Sample =S-N =Sample A (450nm) -NC mean

Note: COD Sample- corrected optical density of sample, NC mean –average optical dencity of two test reading sign of the negative control from the plate. S-N =Sample ≤0.300 was recorded as a negative result, S-N =Sample > 0.300 was recorded as a positive result.

3.5. Questionnaire Survey:

The questionnaire survey was conducted for collecting specific information on dairy cattle movement and transmission, the health status, management and bio-security measures of BVDV using the local language and completed at the time of blood sampling took 20 to 30 minutes. Two questionnaire formats, one for the serum sampled individual animal history and the other with a semi structured questionnaire format for the herders, were used in this study. The questionnaire focus on animal husbandry and housing practices, knowledge about BVD, the production and

reproduction status of the herd and handling, and the history of abortion in the farms. Besides, information related to dairy farm structure, the composition of dairy herds, management (closeness of calves with mothers, housing layout). Additionally, age, sex, herd size, farm types, parity, birth of congenitally defective calf, retention of fetal membranes, and repeat breeding were recorded.

3.6. Ethical clearance

Ethical clearance for this study was obtained from animal research ethical review committee of Addis Ababa University College of Veterinary Medicine and Agriculture with the reference number: VM/ERC/19513/2021 for collecting blood samples from the dairy cows.

3.7. Data management and analysis

Data generated from questionaire survey and laboratory investigations were recorded and coded using Microsoft excel for window 2010 and transfered to STATA13.1 (Stata Corp. College Station, Texas) software for further analysis. chi-square test was conducted to determine the association between the BVDV status of the aninals and the risk factors (sex, age, parity, herd size, breeding system, introduction of new animals to the farm, reproductive problems, farming systems). Differences among groups of each factors were considered statistically significant at $P<0.05$ for all parameters were tested. Logistic regression analysis was used to determine the association between seropositive results for BVDV and risk factors. Before regression analysis, the data was cheked for fulfillments of assumptions, such as correlation of each variables (not more than 0.7), correlation of independent variables with dependent variable (minimum of 0.3), and multi-collinearity tests using Variance inflation factors (VIF) greater than 10 and tolerance value less than 0.1 (lan Dohoo, 2003).

The strength of the association between outcome and explanatory variables was assessed by using the odd ratio (OR). Multivariable logistic regression procedures were used to model the effects of potential risk factors on out come variable (BVDV antibodies). The forward elimination procedure was used to eliminate the factors that were not significant at P>0.05 in the overall model. Factors that were significant at P <0.05 were left in the final model and model it was examined by post-eliminatin goodness-of-fit test, namely the Hosmer-Lemeshow test (p = 0.7866) indicates that the model fit to the data and the receiver operating curve (ROC) for reliability (lan Dohoo, 2003).

Variables with a P-value (≤0.25) in the univariable logistic regression analysis with no multicollinearity were entered into the final multivariable logistic regression model. There was no significant interaction between variables. A Hosmer-Lemeshow goodness-of-fit value (P=0.51), indicated that the model was fit to the data. The final multivariable logistic regression model of backward elimination method retained herd size, sex, age, farming system, introduction of new animals, abortion, repeat breeder, parity and breeding system which were independently associated with (P<0.05) BVDV seroprevalence of Holeta town dairy cattle. Finally, those variables with P < 0.05 (OR, 95% CI) were considered as a significant potential risk factors for BVDV antibody seropositive results (Table6).

4. RESULTS

Sero prevalence

From the 337 tested sera samples of cattle, 52 (15.43%) were positive for antibodies against BVDV using indirect antibody ELISA technique. Antibody-negative serum samples were tested for antigen using antigen-capture ELISA,of which 0.35%(n=1) was found antigen positive for BVDV. Out of 17 cattle herds, 11 (64.71%) were seropositive farms for BVDV antibodies. The sero-prevalence of BVDV was significantly different ($P < 0.05$) between age, herd size, parity, abortion, repeat breeding, introduction of new animal to the farm, congenital defect and among farming systems were highly significant impact on BVDV. Although not statistically significant ($P > 0.05$), the prevalence of BVDV was relatively higher in female animals (16.56%; n=50) compared to males (5.71%; n=2), the breeding system used both AIand bull (17.44%; n=45) than only used AI (8.86%; n=7) and body condition score of thin animals (25.81%; n=8) compared with good body condition (13.99%; n=41) and emaciated (23.08%;n= 3). Significantly (P=0.00) higher sero prevalence was observed in adults (23.12%; n=43) copmpred to calves (7.37%; n=7) and heifers (3.57%; n=2), respectively.

Significantly (P=0.000) higher prevalence was recorded in animal with history of abortion cases (52%; n=13), introduction of new animals to herd (50%, n=13), repeat breeder (50%; n=16), congenital defect (41.67%; n=5), parity greater≥2 (24.17%; n=29), intensive farms (26.43%; n=37) and farms with above 25 herd size (24.22%;n=39) as shown in Table4.

Table 4. Prevalence of different risk factors in cattle

Variables	No. of animals	No. of Positive	Prevalence	χ^2- value	P-value
Sex					
Female	302	50	16.56%	2.8254	0.093
Male	35	2	5.71%		
Age					
Adult	186	43	23.12%	19.1913	0.000
Heifer	56	2	3.57%		
Calf	95	7	7.37%		
Herd size					
Small (<15)	69	6	8.7%	18.4159	0.000
Medium(15-25)	107	7	6.5%		
Large (>25)	161	39	24.2%		
Parity					
≥2	120	29	24.2%	17.7517	0.000
<2	63	13	20.63%		
Null	154	10	6.49%		
Abortion					
Yes	25	13	52%	27.6739	0.000
No	312	39	12.5%		
Repeat breeder					
Yes	34	16	50%	28.9892	0.000
No	303	36	11.88%		
new animal Introduced					
Yes	26	13	50%	25.8016	0.000
No	311	39	12.54%		

Congenital defect					
Yes	12	5	41.67%	6.5637	0.010
No	325	47	14.46%		
Breeding system					
AI	79	7	8.86%	3.4128	0.065
AI and bull	258	45	17.44%		
Farming system					
Intensive	140	37	26.43%	22.2001	0.000
Semi-intensive	197	15	7.61%		
Body condition score					
Emaciated	13	3	23.1%	3.6039	0.165
Thin	31	8	25.8%		
Good	293	41	13.99%		
Overall prevalence	337	52	15.43%		

Cows with history of abortion were seven times (OR: 7.02; 95%CI: 2.98- 16.56, P = 0.000) more likely to be infected with BVDV compared to cows without a history of abortion and problems of repeat breeder were six times (OR: 6.11; 95%CI: 2.85-13.12, P = 0.000) more likely to be infected with BVDV than no history of repeat breeder. Introduction of new animal to the farms were seven times (OR: 6.97, 95%CI: 3.01-16.13, P = 0.000) more likely to be infected with BVDV than no introduced new animals to their farms. Cattle rear in the intensive farming system were four times (OR: 4.4, 95%CI: 2.28-8.32, P = 0.000) more likely to be infected with BVDV than cattle reared semi-intensive farming system. Cows of greater or equal to two parity were four times (OR: 4.42, 95% CI: 1.92-10.14, P = 0.000) more likely to be infected with BVDV than less than two parities cows and nulliparous animals. In age category, adults above two years old were 3.78 times(OR:3.78, 95% CI: 1.629- 8.77, P=0.002) more likely to be infected than heifers and calves above six months of age. Animals with history of congenital defect were 4 times (OR: 4.22; 95%CI: 1.28-13.86, P = 0.017) more likely infected with BVDV than calves with out

history of congenital problems. However, sex, breeding system and body condition score were not significantly associated (P>0.05) BVDV seroprevalence (Table5).

Table 5. Results of univirable logistic regression for association of potential risk factors

Variables	Prevalence	Univariable logistic regression analysis	
		OR(95%CI)	P-value
Herd Size			
Large	24.22%	3.356 (1.3487-8.35)	0.009
Medium	6.54%	0.735 (.236-2.286)	0.595
Small	8.70%	*	*
Sex			
Female	16.56%	3.27 (.76-14.08)	0.111
Male	5.71%	*	*
Age			
Adult	23.12%	3.78(1.629- 8.77)	0.002
Heifer	3.57%	0.465(.093- 2.32)	0.351
Calf	7.37%	*	*
Farming system			
Intensive	26.43%	4.358(2.28-8.32)	0.000
Semi-intensive	7.61%	*	*
Introduction of new animal			
Yes	50.00%	6.974 (3.01-16.13)	0.00
No	12.54%	*	*
Party			
≥2	24.17%	4.42 (1.92-10.14)	0.000
<2	20.63%	3.60(1.40-9.25)	0.008
Nulliparous	6.49%	*	*
Abortion			
Yes	52.00%	7.02(2.98- 16.56)	0.000
No	12.50%	*	*
Repeat breeder			
Yes	50.00%	6.11(2.85-13.12)	0.000
No	11.88%	*	*
Breeding system			
AI and Bull	17.44%	2.17 (.93- 5.03)	0.070
AI	8.86%	*	*
Congenital defect			
Yes	41.67%	4.224 (1.28-13.86)	0.017
No	14.46%	*	*
Body condition score		*	

Emaciated	23.08%	1.14(.253- 5.30)	*
Thin	25.81%	0.54(.143- 2.05)	0.84
Good	13.99%		*

Reference category*

Table 6. Results multivariable logistic regression for association of potential risk factors

Variables	**Multivariate logistic regression analysis**	
	OR(95% CI)	**P-value**
Herd size		
Medium	1.14(.22- 5.88)	0.874
Large	3.49(.53- 22.73)	0.191
Sex		
Female	0.99(.18- 5.31)	0.99
Age		
Adult	2.9 (1.03- 1.03)	0.043
Heifer	.32(.058- 1.759)	0.190
Farming system		
Intensive	4.6(1.61- 13.08)	0.004
New animal introduction		
Yes	6.44(2.17-19.06)	0.001
Congenital defect		
Yes	15.22(3.15- 73.55)	0.001

Table 7. Results of multivariate logistic regression for reproductive problems

Variables	Multivariable logistic regression analysis	
	OR(95% CI)	**P-value**
Abortion		
Yes	6.33 (2.40 - 16.71)	0.000
Repeat breeder		
Yes	5.98 (2.49 - 14.30)	0.000
Party		
≥2	3.55(1.46- 8.59)	0.005
<2	1.58(.53- 4.72)	0.408
Breeding system		
AI and Bull	4.565(1.65- 12.59)	0.003

Questionnaire results

Considering that farms with at least one positive animal were classified as positive for BVDV. The overall farm-level prevalence was (64.71%; n=11) from a total of 17 farms . Although not statistically significant at (P >0.05), the herd level prevalence highest in farms was found in large herd size (100%;n=3), herd with Tail to Tail arrangemen in a farms (100%;n=3), farmers were got bull from borrowing for breeding (83%;n=5), farms with medium herd size (15-25) (80%;n=4), herds having congenital defect at birth (78%;n=7), a poor biosecurity measure of farms (77.78%;n=7), semi-intenisive farms (75%;n=4), herds with abortion (75%;n=6), Face to Face herds arrangement in the house (72.7%;n=8), share common pen with calves (71.4%;n=5), service used both AI and bull while cows were get heat (70%;n=7), herd with retain fetal membrane (69%;n=9) were found highest risk for BVDV prevalence in the herds. Additionaly, farms did not take any measure to minimize disease transmition (66.67%;n=8); calves mixed with other and Share same barn but no close contact to others in the herds (66.67%;n=4) compared with farms have separate calves pen (60%;n=3) relatively higher risks of BVDV prevalence were recorded insignificantly at (P>0.05).

Relatively lower farm level BVDV prevalence also recorded in intensive farming system (62 %;n=8), farmers were not used bull (only used AI) service for cows(50%;n=3) compared with used own bull when breeding (60%, n=3), and borrowed bull users, Very good (0%), good (57%;n=4) and compared to poor biosecurity measure farms.

This questionnaire result showed that, significantly at ($P<0.05$) higher herd level BVDV prevalence was recorded in farms were poor ventilation (100%;n=7) compared with very good(0%;n=3) and good ventilation(57%;n=4), farmers introduce new anima1 to their farms (81.8%;n=9) compared to not introduce new animals to their farms (33%;n=2), and herds get health care only when ill (80%;n=5) compared to herds were got health care regurarly (25%;n=4) as shown in table 8. This finding revealed that, overall biosecurity measures, management and movement of animl were the risk for BVDV in the study area with the over all herd prevalence of 67.71%.

Table 8. Prevalence and risk factors in herd

Variables	**No. of respondents**	Fr**equency**	**Prevalence**	χ^2 value	P -value
Herd size					
Small	9	4	44%	3.7663	0.152
Medium	5	4	80%		
Larg	3	3	100%		
Farming system					
Intensive	13	8	62%	0.2427	0.622
Semi intensive	4	3	75%		
Have you introduce new animal to the farm?					
Yes	11	9	82%	3.9963	0.046
No	6	2	33%		
Service used whilecows					
AI	7	4	57%	0.2981	0.58

AI and bull	10	7	70%		
Where you get the bull?					
No used	6	3	50%	1.5283	0.466
Own bull	5	3	60%		
Borrowing	6	5	83%		
Have you had any animal in herd with retain Fetal membrane?					
Yes	13		69	0.4953	0.48
No	4		50%		
Have you had any animal in herd with abortion?					
Yes	8	6	75%	0.7012	0.40
No	9	5	56 %		
Animal in herd with repeat breeding?					
Yes	12	9	75%	1.8932	0.17
No	5	2	40%		
Congenital defectat birth					
Yes	9	7	78	1.431	0.23
No	8	4	50 %		
Is there calf death?					
Yes	8	5	62.5	0.0322	0.86
No	9	6	66.7 %		
Farm management /husbandry/housing					
What is the housing situation calves?					
Separate pen	5	3	60%	0.0687	0.96
Mixed with other	6	4	66.7%		
Share same barn but no close contact	6	4	66.7%		
Do you have sick animals isolation pen?					
Yes	4	3	75%	0.2427	0.62
No	13	8	61.54%		
How is the cow layout in the house?					

Face to face	11	8	72.7%	4.16	0.125
Tail to tail	1	1	100%		
One row	4	1	25%		
Herds share common pen with calves?					
Yes	10	6	60%	0.2355	0.627
No	7	5	71.4%		
How often animal get health care					
Only when ill	13	10	76.9%	3.61	0.05
Regularly	5	4	25%		
The overall bio-security measure of the farm					
What is the Overall biosecurity measure of the farm?					
Good	7	4	57%	02.68	0.26
Very good	1	0	0%		
Poor	9	7	77.78%		
Ventilation					
Poor	7	7	100%	9.49	P = 0.009
Good	7	4	57%		
Very good	3	0	0%		
Do you take any measure to minimize disease transmition?					
Yes	5	3	60%		
No	12	8	66.67 %	.0687	P= 0.79
Over all prevalence	17	11	64.71%		

5. DISCUSSION

In this finding, an individual and herd level BVDV seroprevalence of 15.43% and 64.7% was recorded, respectively. The animal level seroprevalence of the present study was in comparable with previous reports of (Nigussie *et al.,* (2010**)** with overall seroprevalence of 11.46% in three agroecological zones of Ethiopia (Jimma, Shoa and South Shoa zones) of Ethioipia. Asmare *et al.,* (2012) with a seroprevalence of 11.7% in breeding and dairy farms of central and southhern Ethiopia and Asnake, (2020) with a seroprevalence of 8.4% in dairy cattle in Asela town. This study was also in agreement with previous report in Sudan (Saeed *et al*., 2015), Egypt (Soltan *et al*., 2015) and Kenya (Callaby *et al*., 2016) with 10.7%, 10.4% and 19.8% seroprevalence, respectively. On the other hand, the current findings was lower than the finding reported by (Aragaw *et al*., (2018), (Asmare *et al.,* (2012) and (Tadesse *et al.,* (2019) with seroprevalence of 32.6%, 32.9% and 51.9%, respectively in dairy cattle in Ethiopia. and reported seroprevalence of 27% in Ecuador (Roon *et al*., 2020), 36% in Colombia (Ortega1 *et al*., 2020), 40% in Egypt (Selim *et al*., 2018) and 33.2 in Malaysia(Daves *et al.*, 2016)**.**

The difference in seroprevalence between nations and countries could be attributable to differences in management systems, diagnostic test used, sample size, study design, and environmental (agroecological) conditions (Houe, 1999), (Fernandes *et al.,* 2015), (Fernandes *et al*., 2015).and (Saa *et al*., 2012). The present finding was the lowest seroprevalence reported when compared with the different studies, 78.8% in Mexico (Montiel, 2019), 77.9% in Iran (Shirvani *et al*., 2012) and 64.4% in Nigeria (Bello *et al*., 2016), respectively in different parts of the world. The low prevalence of the this study might be due to differences in study season, breeds of animal, cattle management systems, biosecurity measures and the specificity and sensitivity of the kits used. The antibodies detected in thes countries might be due to vaccination as opposed to situation in Ethiopia where there is no vaccination.

A number of studies conducted in different countries reported that a herd is more likely to have persistently infected cattle are simultaneously mixing with small ruminants or contact with wild animals (Rêgo *et al*., 2016), (Handel *et al*., 2011) and (Bedeković *et al*., 2013)**.** In area residing with high cattle density is likely to lead to increased prevalence of antibody. Many studies

indicated that prevalence was higher in large herds than in small herds, (Givens *et al.*, 2012), (Sarrazin, 2015) and (Rajeev *et al*., 2017). These were comparable with the present study with 24.22% above 25 herd size (large) and 6.54 % in 15–25 animals (medium) and 8.70% 1–15 animals (small) herd size, respectively. The higher seropositive cattle with the history congenital defect (41.1%) were found compared without history of congenital defect 14.46% in this study.

A significant (P=0.000) higher seropositive cows with history of abortions were sobserved compared without history of reproductive problems (52%). Cows with history of abortion were seven times (OR: 7.23; P = 0.000) more likely to be infected than cows with no history of reproductive problems. This result was in agreement with other studies that higher prevalence of BVDV antibody in cows with history of abortions than cows without history of reproductive problem (Asmare *et al.,* 2012), (Okumu, 2014), (Tadesse *et al.*, 2019) and (Asnake, 2020)**.** This results also in agreement with findings of (Derdour *et al*., (2017**)**and (Thapa *et al*., (2019) where there was significant association between abortion and seropositivity.

In this study, the higher seroprevalence was observed in cows with history of repeat breeder compared to the cows without history of reproductive problems (50.0%; P = 0.001) and six times (OR: 6.0; p = 0.000) more likely infected than animals with out history of reproductive problems, which is disagree with finding of (Tadesse *et al.*, 2019) and in agreement with previous findings of **(**Nigussie *et al.*, 2010**),** Asmare *et al.*, 2012) and (Asnake, 2020) in Ethiopia**.** BVDV infection of pregnant cows and heifers cause reproductive disorders such as early embryonic death, fetal death and mummification, birth of calves with congenital defects, calves with poor growth rates, increased age at first calving and decreased ovarian function in affected herds (Kaiser *et al.*, 2013**)** and **(**Ang *et al*., 2012**)**. Transient infection of the dam occurs prior to embryo attachment to the endometrium, infection is avoided as BVDV does not penetrate the zona pellucida. However, following attachment embryonic infection can occur and may lead to embryo loss with the dam returning to heat **(**GÜR, 2011**)**. In addition, the seroprevalence was higher in adult cattle than young animals of less than 6m-1years and 1-2 years old observed in this study, which is in agreement with previous findings (Nigussie *et al.*, 2010). This might reflect the lower chance of transmission of the virus from pregnant uterus to the fetus and higher possibility of getting the virus from the environment, which is shed by lifelong carrier animals (Nigussie *et al*., 2010).

In this study, the higher seroprevalence was observed in intensive farming system (26.43%) as compared to semi- intensive farming system (7.61%). This result is in disagreement with previously reported in Mexico by **(**Montiel, (2019**)**. This might be due to the fact that, cows in an intensive system where contact between animals from PI calves is common, close contact between infected calves with animals share the same barns and entrance of new animals from different sources is frequent, the prevalence is high in intensive than semi-intensive farm. This may due to a high rate of contact between animals within intensively managed herds, that facilitating the transmission of infections among the animals. Therefore, conditions in the intensive farming systems for the pathogen are adverse and have more probable transmission than in the semi-intensive system.

In current study, herd seroprevalence of 64.7% were reported. This finding in agreement with a herd prevalence of 69.8% in Ethiopia (Aragaw *et al*. (2018), 65.5% in Brazil (Fernandes1 *et al*., 2015), 66% in Great Britain (Velasova *et al*., 2017**)**, 69% in Colombia (Ortega *et al*., 2020) Incontrast, higher herd seroprevalence (95.6%) reported by Tadesse *et al.* (2019) in Ethiopia, 92% in Cameron (Handel *et al*., 2011) and lower herd seroprevalence (22.2%**)** reported by Asnake, (2020). However, on the current study, the antigen of the virus has been detected. This is the first report of BVDV Ag prevalence (0.35%) in the study areas and in Ethiopia . This also indicates that the virus circulates in the study area and the presence of persistently infected animals that causes the main source of BVDV transmission.

6. CONCLUSION AND RECOMMENDATIONS

The present study demonstrated that BVDV infection was highly spread in dairy herds in and around Holeta town, Ethiopia. It also suggested that the importance of BVDV was growing in Ethiopia as the seroprevalence according to a few earlier reports from the country. On the current study, the antigen of the virus was detected (0.35%). This is the first report of BVDV Ag prevalence in the study areas and in Ethiopia. This also indicates that the virus circulates in the study area and the presence of persistently infected (PI) animals. The higher seroprevalence was obtained in dairy cows with a history of abortion, repeat breeding, farms introduced new animals to their herds, animals with congenital defect and intensive farming. Therefore, based on this study the following recommendations are forwarded:

- Dairy farm owners have to isolate and know the BVDV status of new animals before introducing to their herds,
- Animals with signs of repeat breeder, abortion, and congenital defects were the alarms of BVDV infection in the farm so farmers should be know the status of their herds
- Detection focus on early detection of calves for PI to eliminate from the herds due to the main source of infection through out its life.
- Farmers and owner of dairy farms need to be aware of the severity and economic importance of the disease.
- Need to isolate the dominant genotypes whether BVDV1 or BVDV2 knowing the genotype and sub-type of BVDV is very important in term of control of the infection through vaccination approaches.
- Improve overall bio-security measures of the farms to prevent the virus.

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8. APPENDICES

Appendix 1. Test procedure for BVDV p80 Protein antibody test

All reagents must be allowed to come to 18-26^{0}c before use. Mix reagents by gentile inverting or swirling.

1. Obtain coated plates and record the sample position
2. Dispense Dilution Buffer N.9, Controls and samples

a. Bovine individual serum and plasma samples: Short incubation (1 hour (±5min.) at 18-26^{0}c)

- Dispense 50µl of Dilution Buffer in each well.
- Dispense 50µl of Negative control (NC) into two appropriate wells.
- Dispense 50µl of Positive control (PC) into one appropriate wells
- Dispense 50µl of sample into remaining wells (1 well per sample).
- Homogenize contents of the well using a microplate shaker.
- Cover the microplate and incupate for 1 hour (±5min.) at 18-26^{0}c

3. Remove the solution and wash each well with approximately 300µl of wash solution 3-5 times. Avoid plate drying between plate washing and prior to the aadition of the next reagent.Tap each plate onto absorbent material after the final wash to remove any residuals wash fluid.
4. Dispense 100µl of Diluted conjugate into each well.
5. Cover the microplate and incubate for 30minutes ((±3min.) at 18-26^{0}c
6. Remove the solution and wash each well with approximately 300µl of wash solution 3 times. Avoid plate drying between plate washing and prior the aadition of the next reagent.Tap each plate onto absorbent material after the final wash to remove any residuals wash fluid.
7. Dispense 100µl of TMB substrate N.9 into each well.
8. Incubate for 20minutes (±3min.)at 18-26℃ away from direct light
9. Dispense 100µl of stop solution N.3 into each well.
10. Measure and record absorbance value of samples and controls at 450nm.
11. Calcultions:

Control

$NC\bar{x} = \underline{NC1A(450)+NC2A(450)/2}$

Validity criteria

$NC\bar{x} \geq 0.0800$ PC/ $NC\bar{x} < 0.20$

Samples $S/N\% = 100 \times [SampleA(450)]/NC\bar{x}$

12. Interpretation

Serum samples

BVD/MD diagnostic for bovine individual serum and plasma samples and BD diagnostic for individual serumand plasma and pooled samples from sheep

Negative	Suspect	Positive
$S/N \geq 50\%$	$40\% < S/N < 50\%$	$S/N \leq 40\%$

BVD/MD diagnositic for pool of Serum samples

Negative	Suspect	Positive
$S/N \geq 60\%$	$50\% < S/N < 60\%$	$S/N \leq 50\%$

Appendix 2. Test procedure for BVDV Antigen Test Kit/serum Plus

All reagents must be allowed to come to 18-26^{0}c before use. Reagents should be mixed by gentile inverting or swirling.

1. Obtain coated plates and record the sample position. If using partial plates, remove only those wells sufficient for samples to be tested. Place the remaining wells, along with the desiccant, in the extra zip lock bag provided and return to 2-8^{0}C
2. Dispense 50µl of Detection antibodies to each well.
3. Dispense 50µl of Negative control (NC) into appropriate wells.
4. Dispense 50µl of Positive control (PC) into appropriate wells
5. Dispense 50µl of sample into remaining wells.
6. Mix the content of the microwells by gently tapping the plate or use a micro shaker.
7. Incubate for 2 hours(±5min.) at 37^{0}c(±3^{0}c) or overnight912-18 hours) at 2-8^{0}c. with eitheroption, the plate should be tightly sealedor incubated in a humid chamber using plate covers to avoid any evaporation.
8. Remove the solution and wash each well with approximately 300µl of washing solution 5 times. Avoid plate drying between plate washing and prior to the aadition of the next reagent.Tap each plate onto absorbent material after the final wash to remove any residuals wash fluid. Important! Control carefully that no trace of blood are left on the walls or edges of the wells. Additionally 2-3 wash can be necessary to remove the blood before proceeding to the next step.
9. Dispense 100µl of conjugate into each well
10. Incubate for 30minutes (±2min.)at 18-26℃.
11. Repeat step eight.
12. Dispense 100µl TMB substrates N.12 solution into each well.
13. Incubate 10 minutes (±1min.)at 18-26℃.
14. Dispense 100µl stop solution N.3 into each well.
15. Measure and record the absorbance of the samples and controls at 450nm or using a dual wavelength of 450nm and 650nm.
16. Calculation

Control

$NC\bar{x}$ =[NC1A(450)+NC2A(450)]/2 $PC\bar{x}$=[PC1A(450)+ PC2(450)+PC2A(450)]/2

Validity criteria

$NC\bar{x} \leq 0.250$ $PC\bar{x} - NC\bar{x} \geq 0.150$

For invalid assay, technique may be suspected and the assayshould be repeated following a though review of the package insert.Note IDEXX has instrument and softwaresystem available that calculate means and S-N and provide data summeries.

S-N=SampleA(450)- $NC\bar{x}$

The presence and absence of BVDV antigen in the sample is determined by the corrected OD value (S-N) for each sample.

17. Interpretation

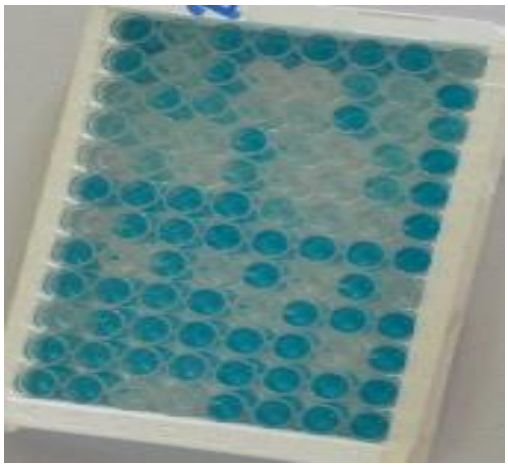
Serum, plasma and whole blood samples

Negative	Positive
S-N≤0.300	S-N>0.300

Positive results from this assay are valid for calves of any age. Circulating high titers of maternal BVDV antibodies might be interfere with the detection of BVDV antigen in serum,plasma and whole blood. Detection of BVDV antigen in serum ,plasma and whole blood samples can be less sensitive after antibody intake through colostrum.”False-negative” results can occur after colostrum intake(“diagnosis gap”). Inorder to exclude influence of colostral antibodies, it is recommended to tes calves before colostrum intakeefer to regulation in your country if different from this description.

Appendix 3. BVDV ANTIBODY POSITIVE RESULT

The white areas with OD values indicates Ab positive for BVDV



ANNEX 4: BVDV ANTIGEN POSITIVE RESULTS

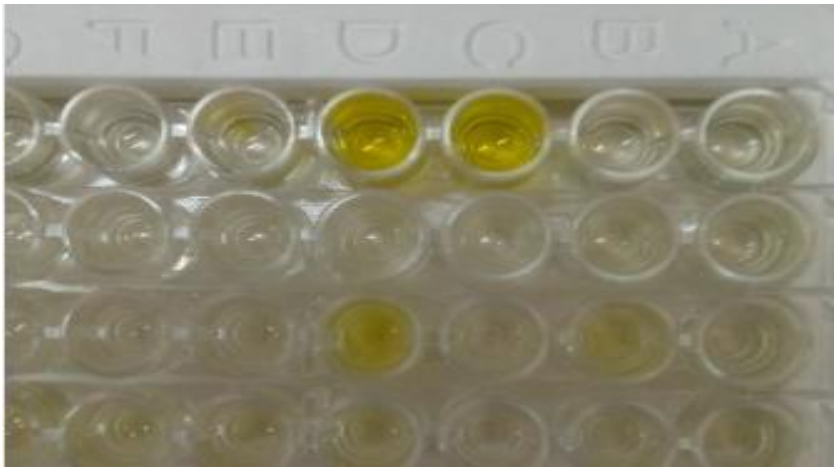
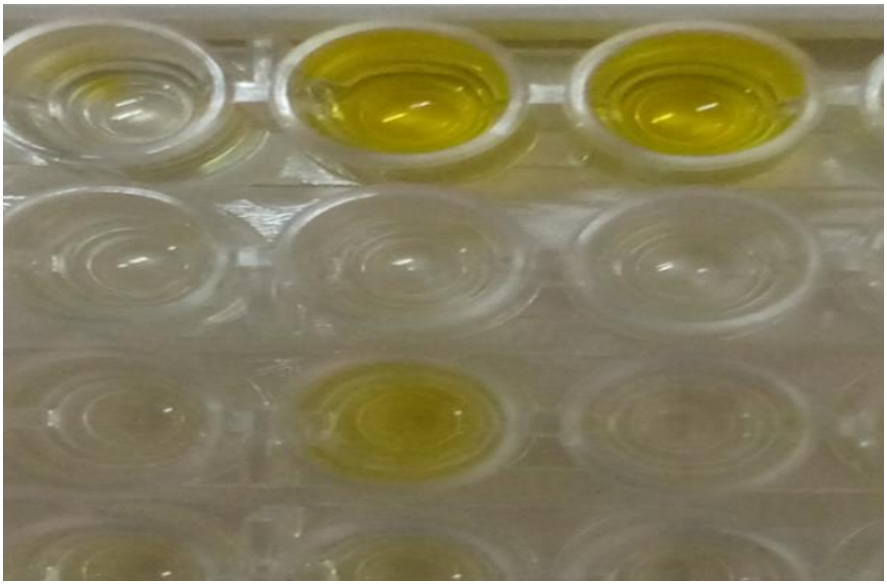
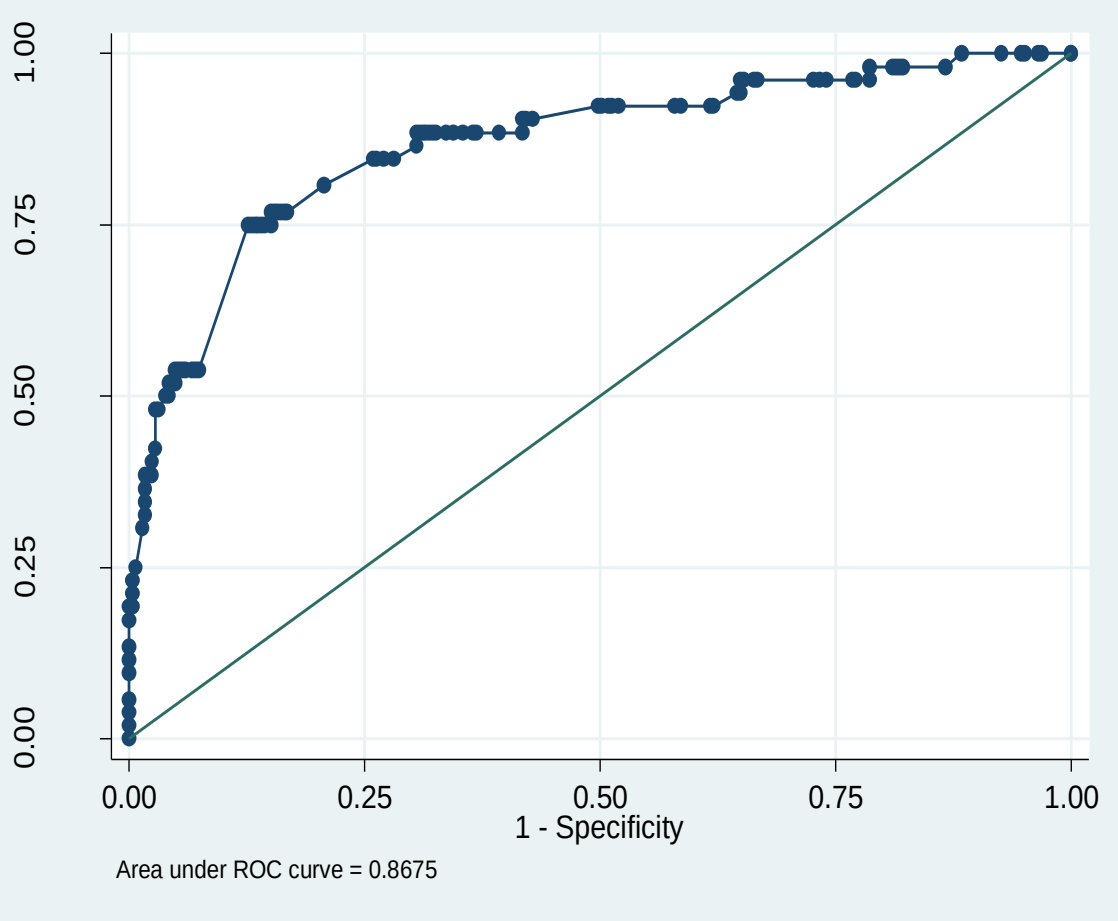


Figure 2: BVDV Ag +Ve shown below the two positive control



Upon the addition of the stop solution, a yellow colour was developed (positive for BVDV)

ANNEX5: THE RECEIVER OPERATING CURVE (ROC) FOR RELIABILITY



Appendix 4. Questionnaire format sheet

Questionnaire prepared to collect epidemiological data of Bovine Viral Diarrhoea at Holeta, Ethiopia by Worku Birhanu

These questions are part of brief questionnaire for the purpose of collecting specific information on dairy cattle movement and transmission and also the health status, management and bio-security measures for BVDV survey. Information provided is confidential and will not be disclosed/ transferred to other party without the consent of the respondents. The interview might take 20-30 minutes to complete. Please answer the questions as accurate as possible. Thank you for your collaboration in advance.

Questionnaire number: _____

Interviewer's name----------Date (DD/MM/YYYY, EC) ----------/----------/---------

A. General information

1. Respondent's name----------Phone----------E-mail----------Fax----------
   Position;1) Manager 2) owner) Other----------
2. Address: Region: ----------Zone------City ------ Sub-city --------District-----Keble------
3. Farm location (Geo.cord): Elevation----------Alt---------- longitude----------
4. Farm name: ---------- herd size ---------- Farm code----------
5. Ownership of the farm; A) Private B) Government C) Cooperative
6. Farming system; A) Intensive B) Semi-intensive C) Extensive

7. Herd structure:

Herd composition	**Pure HF**	**HF Z cross**	**Jersey**	**J Z cross**	**Local**	**Total**
Calves (0-1yr)						
Heifers (1-2 yrs)						

Bullock/Steers						
Cows						
Bull/Oxen						
Total						

*HF-Holstein Frisian, Z- Zebus, JZ- Jersey Zebu cross,

B. History of cattle movement and Information required for transmission modeling

8. Date of farm establishment (----------)
9. Have you introduced cattle to your farm in the last three calendar years? A) Yes B) No
   If yes, how? A) Purchase B) Gift C) Borrowing of bull D) calf E) Others----------
10. How does the farm get replacement stock (multiple options possible)? A) AI, B) Own bull, C) Purchasing, D) Bull from other farm, E) Gift, F) Government breed improvement programme G) Others (specify)----------
11. Which service you use while your cows get heat? A) AI, B) own bull, C) Borrowed bull
12. Do you use a bull for the whole herds? A) Yes B) No
13. If yes, where you get the bull? A) Own bull B) Borrowing C) others specify?
14. Do you know the bovine Viral Diarrhea virus (BVDV) status of the herd you borrow the bull from? A) Yes B) No
15. Have you had any animal in your herd with chronic diarrhea/abortion during the last six months? A) Yes B) No
16. Have you had any animal in your herd with retain fetal membrane (RFM) during the last six months? A) Yes B0 No
17. Have you had any animal in your herd with reduced milk yield during the last six months? A) Yes B) No
18. Have you had any animal in your herd with repeat breeding during the last one calendar year A) Yes B) No
19. Number of caves born during the last 12 months (up to day of sampling) Sex: F----M---
20. Is there any deformity at birth? a) Yes b) No
21. If yes, what types of abnormalities you have seen? Specify----------
22. Number of dead calves during the last 12 months (up to day of sampling) at calving

23. Reason for death: a)Dead at birth/aborted b) Respiratory embarrassment c) Diarrhea d) Unknown cause e) Sudden death f) Others/Mention-----

24. What types of animal disease commonly affect your herd? ----------

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25. What are the major clinical signs (syndrome) a) Abortion b) Respiratory sign c) Diarrhea d) retain fetal membrane E) reduced milk yield

26. Is there any outbreak before? a) Yes b) No

27. Which age groups were more affected by the outbreaks? a) Young b) Adult c/ all groups

28. If yes what are the major clinical sign?

C. FARM MANAGEMENT /HUSBANDRY/HOUSING

28. What is the housing situation of calves? A) Separate pen, B) share same barn but no close contact (at least 3m apart) with others, C) mixed with others

29. Do sick animals have isolation pen? A) Yes, B) No

30.How is the cow layout/arrangement in the house, A) face to face, B) tail to tail, C) one row, D) other (specify)_____

31. Size of the house/barn? Floor surface area (LxW)? _____ (m^2)..

32. Wall condition? A) Solid, B) solid with few windows, C) half open D) full open

33. Do you have access to share the common of pen adult cow with calves? A) Yes B) No

D. Immunosuppressant factors

34. Do you practice regular de-worming of the herd? A) Yes, B) No

35**.** Did you vaccinate your cattle? A) Yes B) No

If yes, i) How? A) Regularly, B) when there is an outbreak

ii) For which diseases? A) FMD, B) LSD, C) Anthrax, D) Blackleg, E) Pastureullosis, F) Other (specify)____

36. How often your animals get health care /service? A) Regularly, B) only when ill health situation exist in the herd, others (specify) _____

37. How often you experience the incidence of viral disease (FMD, LSD, BVD, etc) in the cattle during the last one calendar year? A) Just once B) Twice C) Not at all

E. Evaluate the overall bio-security measure of the farm in 1-3 scales?

a) [**Scale 1 means poor:** share attendants, service providers including vets/AI technicians or facilities including bull; mix with others during watering/feeding; separate only by a partition allowing free air circulation.

b) Scale 2 means Good: no shar**e** attendants and facilities including bull; mixing is prohibited but may be suspected to use shared open air barn/grass land, facilities like vehicle for feed delivery or trough; separated from apparently health animals with a reasonable distance or barrier; calves from infected cows are kept separately and fed with heat treated or sourced from healthy cow only).

c) Scale 3 means very Good: no shar**e** attendants and facilities including bull; mixing is totally prohibited/no sharing at all; complete separated from apparently health animals with a reasonable distance or barrier; feeding and watering is completely separate; calves from infected cows are kept in separate room/place in which no means they can meet with infected cattle; and fed with heat treated or sourced from healthy cow only].

38. Ventilation

1) Poor (closed wall with few windows),

2) Satisfactory (above half of the two sides of the wall opened /meshed),

3) Very good (above half of the four sides of the wall opened/meshed, and through the roof)

39. Do you take any measures to minimize possibility of disease transmission? A) Yes B) NO

If yes, which measures? (Multiple options possible)

A) No visitors allowed,

B) Only AI technicians, vets or special guests are allowed

C) Visitor is subjected to use disinfectants at the entry and exit,

D) No access for wildlife, cats and/or dogs to the farm and feed storage,

D) The farm and feed storage has no access for wildlife but for dogs and/or cats,

F) No knowledge on bio-security and thus no any measure at all,

G) Restrictions on herd grazing/mixing with other herds

H) Practice of burying/ burning fetal membrane and dead bodies unhealthy

40. Do you have any suggestion on how to prevent/ control BVDV/infectious disease, please explain?

Appendix 5. Ethical Clearance

Animal Research Ethical Review Committee

Ethical clearance certificate