BOVINE VIRAL DIARRHEA VIRUS (BVDV) EXPOSURE IN DAIRY CATTLE IN SOUTHERN AND CENTRAL ETHIOPIA AND POTENTIAL ASSOCIATION WITH REPRODUCTIVE PERFORMANCE

BY

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Bovine Viral Diarrhea Virus (BVDV) Exposure in Dairy Cattle in Southern and Central Ethiopia and Potential Association with Reproductive Performance

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As members of the examining board of the final PhD open defense, we certify that we have read and evaluated the dissertation prepared by Kassaye Aragaw Lidete titled: "**Bovine viral diarrhea virus (BVDV) exposure in dairy cattle in southern and central Ethiopia and potential association with reproductive performance**" and recommend that it be accepted as fulfilling the dissertation requirements for the degree of Doctor of Philosophy in Veterinary Obstetrics and Gynecology.

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STATEMENT OF AUTHOR

I declare that this dissertation is my authentic and real work and that all source materials used for this thesis have been properly acknowledged. This thesis has been duly submitted in fulfillment of the requirements for a PhD degree at the College of Veterinary Medicine and Agriculture of Addis Ababa University, and is deposited at the University/College library to be made available to borrowers under rules of the library. I sincerely declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made. In all other instances, however, permission must be obtained from the author and/or the University.

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Dedication

To my late younger sister, Fantu

ABBREVIATIONS

ACE	Antigen capture ELISA
BVD	Bovine viral diarrhea
BVDV	Bovine viral diarrhea virus
CP	Cytopathic
CSA	Central statistical agency
DNA	Deoxyribonucleic acid
EARO	Ethiopian agricultural research organization
ELISA	Enzyme linked immunosorbent assay
FA	Fluorescent antibody
FAO	Food and agriculture organization of the United Nations
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MD	Mucosal disease
MLV	Modified live virus vaccine
NCP	Non cytopathic
OIE	World organization for animal health
ORF	Open reading frame
Pab	Polyclonal antibody
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Persistent infection
RNA	Ribonucleic acid
RT-PCR	Reverse transcription- PCR
TI	Transient infection
UTR	Untranslated region
VN	Virus neutralization
WHO	World health organization of the United Nations

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important viral pathogen of cattle with a worldwide distribution. Although infection with the virus is usually subclinical, it may cause wideranging clinical conditions in cattle. Its main economic significance results from its effect on reproductive performance of cattle. It causes embryonic mortality and absorption, abortion, stillbirth, fetal malformation and birth of weak calves depending on stage of pregnancy during infection. In cetain outbreaks it may cause severe morbidity and mortality in susceptible herds depending on the strain of the virus. Despite the economic importance of the virus in almost every major cattle producing countries of the world only few studies have so far been conducted in Ethiopia. Those few works were also mostly limited to serological studies in limited geographic areas. There is lack of reports regarding occurrence of active disease or outbreaks associating infection with clinical conditions and losses. There is also so far no published work on the genotype of the virus prevalent in the country. Therefore this study was designed with aims to estimate the seroprevalence, identify associated risk factors and assess association of BVDV serostatus with reproductive wastage in dairy cows in Ethiopia. The study also attempted to detect and characterize BVDV nucleic acid in samples collected from bovine abortions and sera collected from selected animals. Accordingly, sera were collected from 954 randomly selected cattle from 98 dairy herds in southern and central Ethiopia. These sera samples were

tested for BVDV antibodies using a commercial ELISA. Fetal tissue samples and placenta from 30 bovine abortions and sera from 26 selected cattle were also collected for detection of BVDV nucleic acid using reverse transcription (RT)-PCR. Among screened sera samples 20.9% (95%CI, 18.4, 23.6) were tested positive to BVDV antibodies. The herd prevalence was 50% (95%CI, 40.1, 59.9) and the intra-herd prevalence ranged between 2.6% and 100% (mean = 31.4%) in positive herds. Geographic region, herd size and animal arrangement in the farm had significant association with serostatus ($p < 0.05$). Cattle from southern Ethiopia and herds of large size had 2.8 (95%CI; 1.9, 4.2) and 2.6 (95%CI; 1.5, 4.6) times higher odds of being seropositive compared to their counterparts, respectively. Serostatus to BVDV was associated with history of anestrus, repeat breeding (RB), mastitis and extended calving interval (CI) ($p < 0.05$). Animals with history of extended CI and mastitis were 1.7 (95%CI; 1.0, 2.7) and 2.2 (95%CI; 1.5, 3.2) times more likely to be seropositive compared with those with normal CI and no history of mastitis, respectively. On the other hand, animals with history of anestrus and RB were less likely to be seropositive to BVDV compared to cattle with no such history. All samples tested with RT-PCR were negative for BVDV nucleic acid. Our study highlights the association of BVDV infection with some important reproductive performance traits and potential risk factors. The study also demonstrated high occurrence of reproductive health problems in dairy cattle in Ethiopia and that the reproductive performance of dairy cattle in the study herds is suboptimal.

Keywords: Bovine viral diarrhea virus, Dairy Cattle, Ethiopia, Reproductive Disorders, Reproductive Performance

1. INTRODUCTION

Ethiopia has huge cattle population estimated at 60.4 million, however only a small fraction (about 1.8%) are improved dairy (exotic or crossbred) animals (CSA, 2018). The local cows, which make the vast majority, have low genetic potential for milk production (Tegegne *et al.*, 2013; Shapiro *et al.*, 2017). As a result of this and other factors the milk produced in the country is way too small to satisfy the existing demand, making the country net importer of milk and dairy products (Yilma *et al.*, 2011).

The country generally produces no more than 4 billion litres of milk per year (Yilma *et al.*, 2011; Rockeman *et al.*, 2015; Tegegne, 2018). Per capita consumption is very low, estimated at about 20 - 40 liters a year (Yilma *et al.*, 2011; Tegegne, 2018). It is much lower than the world's average of 100 litres (FAO, 2010) and that of the 200 litres recommended by the WHO (Yilma *et al.*, 2011). At the current production rate, there is an annual shortage of about 18 billion liters to achieve the WHO's recommendation of 200 liters per capita consumption per year (Tegegne, 2018). As a result of economic growth, rising per capita income, urbanization and population growth (FAO, 2007) the gap between demand and supply is expected to widen further unless significant improvements are made in genetics, feeding, animal health and other management conditions (Shapiro *et al.*, 2017).

The national livestock sector strategy calls for a considerable increment in the number of crossbred cattle, supported by improved supply of feed and health management, if the demand for dairy products is to be satisfied with domestic production (Shapiro *et al.*, 2017). One of the methods used by many developing countries to improve milk production in a relatively short period of time is crossbreeding of locally adapted cattle breeds with improved dairy cattle breeds. However, the success of such crossbreeding programmes has varied depending on several factors, including the simultaneous implementation of reasonable standards of animal nutrition, disease control, husbandry and improved infrastructure status (Bane and Hultnas, 1977).

Crossbreeding of indigenous zebu cattle with improved European breeds was introduced to Ethiopia in the 1930s by the Italians. However, wide-scale cattle crossbreeding activities were started later by the Institute of Agricultural Research (IAR) and Chilalo Agricultural Development Unit (CADU) using Holstein-Friesian, Jersey, and Simmental sires that were

crossed with the local Horro, Borana and Arsi dams (Brannang *et al*., 1980; EARO, 2001). Since then, governmental and non-governmental organizations have made various efforts to improve the dairy sector by establishing dairy cattle improvement ranches and distributing crossbred F1 heifers to smallholder farmers (EARO, 2001; Belihu, 2002). Despite years of effort and commitment of large resources the crossbreeding program didn't appear to have met its objectives both in terms of number (proportion) of crossbred cattle and improvement in milk yield.

Reproductive performance and calf survival need to be improved if crossbreeding is to be successful as they determine the genetic gain over a period of time, by increasing the number of replacement heifers. It is necessary to have the knowledge about reproductive problems of the herds and their causes if reproductive performance of dairy herds is to be improved.

Earlier studies conducted in various parts of the country documented unsatisfactory reproductive performance of dairy (grade and crossbred cows) cows/herds characterized mainly by extended calving interval and prolonged age at first calving (Asseged and Birhanu, 2004; Lobago *et al*. 2006, 2007; Abraha *et al*., 2009; Fekadu *et al*., 2011). This is usually attributed to inadequate nutrition, diseases, inefficiency of breeding services and management factors (Shiferaw *et al*., 2003; Lobago *et al*., 2006; Fekadu *et al*., 2011; Yalew *et al*., 2011), though not always supported by reliable data.

Numerous genetic, environmental and management factors and certain clinical conditions are known to affect reproductive performance of dairy cows. Nutrition (dry matter intake), body condition in the postpartum period, metabolic stress, occurrence of reproductive problems such as cystic ovaries and uterine infections, and some management practices such as accuracy of heat detection, use of proper AI techniques affect reproductive performance (Dobson *et al*., 2007). Other factors such as milk yield and age of the cow, suckling and season of year, heat stress and certain clinical conditions such as lameness, mastitis, endometritis/metritis, hypocalcemia and ketosis impact fertility (Arthur *et al*., 1996; Dobson *et al*., 2007). Occurrence of some infectious diseases may also severely affect fertility of cattle (Arthur *et al*., 1996).

In one study reproductive diseases were identified to be the most frequent clinical health problems in dairy herds in Addis Ababa and surrounding towns (Lema *et al*., 2001). Among reproductive health problems retained fetal membranes (RFM), uterine infections, and dystocia

have been reported to be the most important problems in cows in Ethiopia (Shiferaw *et al*., 2005; Gizaw *et al*., 2007; Bitew and Prasad, 2011; Gashaw *et al*., 2011; Tesfaye and Shambel, 2013). Cows with reproductive disorders are likely to have poor reproductive performance (Shiferaw *et al*., 2005).

Specific infectious diseases that cause infertility in cattle include genital campylobacteriosis (due to *C. fetus*), brucellosis (*B. abortus and B. melitensis*), leptospirosis (*L. interrogns*), salmonellosis (usually due to *S*. Dublin), listeriosis (*L. monocytogenes*), *Haemophilus somnus*, bovine viral diarrhea (BVD, due to bovine viral diarrhea virus- BVDV), infectious bovine rhinotracheitis (IBR, due to bovine herpes virus 1- BHV1), *Trichomonas fetus, Neospora caninum* (Arthur *et al*., 1996) and Schmallenberg virus (SBV) (Gibbens, 2012).

Bovine viral diarrhea (BVD) is an economically important cattle disease (Houe, 2003; Bachofen *et al*., 2010) with a worldwide distribution (Radostits *et al*., 2007; OIE, 2008). It is caused by *Bovine viral diarrhea virus* (BVDV), which belongs to genus *Pestivirus* of family *Flaviviridae*. Depending on genetic and antigenic properties BVDV virus is divided into two species namely BVDV-1 and BVDV-2 (Fauquet *et al*., 2005; Vilcek *et al*., 2005).

Bovine viral diarrhea (BVD) was originally described in 1946 in New York State as an acute, rarely fatal, highly contagious disease of cattle with fever, diarrhea, mucosal lesions, and leucopenia (Olafson *et al*., 1946; Carman *et al*., 1998). The clinical manifestation of infection with BVDV varied from mostly subclinical infection to sometimes acute fatal disease, depending upon the type of infection (acute/transient or persistent infection), the immune status of the animal, virus strain and stage of gestation in pregnant cows (Radostits *et al*., 2007; Bachofen *et al*., 2010). Though loss may arise from reduced milk production and increased mortality especially in young stock, it is the reproductive wastage in the form of reduced fertilization, embryonic mortality, abortion, stillbirth, birth of weak calves, teratogenesis and birth of persistently infected (PI) calves, which is economically the most important (Anderson *et al*., 1990; Brownlie, 1990; Houe, 2003; Grooms, 2004).

Serological evidence of infection of cattle with BVDV in Ethiopia has been well documented. Some of these studies also attempted to epidemiologically assess the association between BVDV serostatus with some reproductive problems (Nigussie *et al*., 2010; Asmare *et al*., 2012; Aragaw *et al*., 2018; Asmare *et al*., 2018). Despite its well-established impact on cattle productivity and

knowledge of its wide existence in the country, through serological studies, there is lack of accessible published information on the occurrence of active disease/outbreak (clinical cases of BVD-MD) in the country in general and on associated losses of any kind (reproductive or otherwise) in particular. There is also so far no report on the genotype of the virus circulating in the country; neither through virus isolation nor the detection of antigen or nucleic acid. Considering the genetic heterogeneity of pestiviruses, molecular studies should have been undertaken to extend the serological findings as genetic and antigenic diversity may affect the epidemiology, diagnosis and control of the disease (Baule *et al*., 1997; Ridpath, 2005).

1.1. Hypothesis of the study

The hypothesis of the study is that BVDV infection is prevalent in dairy cows/herds in Ethiopia and is responsible for significant reproductive loss.

1.2. Objectives

In order to solve some of the above mentioned information gaps this study was conducted with the following objectives:

General objective

- To assess the occurrence and significance of BVDV infection in dairy cattle in central and southern Ethiopia

Specific objectives

- To estimate the seroprevalence of BVDV in dairy herds
- To identify factors associated with seroprevalence
- To assess association of BVDV serostatus with occurrence of reproductive problems and reproductive performance of dairy cows in the study areas
- To assess occurrence of BVDV nucleicacid in aborted fetuses

2. LITERATURE REVIEW

2.1. Etiology

Bovine viral diarrhea (BVD) is caused by *Bovine viral diarrhea virus* 1 (BVDV-1) and BVDV-2 of the genus *Pestivirus* of the family *Flaviviridae*. Besides BVDV-1 and BVDV-2, the genus *Pestivirus* consists of another two recognized species: *Classical swine fever virus* (CSFV) and *Border disease virus* (BDV) (Simmonds *et al*., 2011).

Pestiviruses are small enveloped viruses, with a single-stranded, positive-sense RNA genome; with genotypic as well as antigenic diversity (Fauquet *et al*. 2005; Bachofen *et al*., 2010). The virus genome is approximately 12.3–12.5 kb in length (Deng *et al*., 2012), and is flanked at either end by 5′ and 3′ untranslated regions (5′UTR, 3′UTR). The single intervening open reading frame (ORF) encodes a polyprotein of approximately 4000 amino acids, which is postranslationally cleaved by viral and cellular proteases to 11–12 structural and nonstructural proteins, dependent on the virus biotype (Meyers and Thiel, 1996; Deng *et al*., 2012). The first protein encoded by the viral ORF is the autoprotease N^{pro}, which cleaves itself from the downstream viral structural proteins (Wiskerchen *et al*., 1991; Stark *et al*., 1993). The ORF is preceded by an untranslated region (5′ UTR) of 381–386 nucleotides whose sequence is highly conserved among pestiviruses (Deng and Brock, 1993).

Pestivirus virions consist, together with the RNA, of four structural proteins, the nucleocapsid C protein and the envelope glycoproteins E^{rns}, E1 and E2. Eleven or twelve pestiviral proteins have been identified as products of polyprotein processing which occurs co- and post-translationally by viral and host cell proteases (Figure 1). In the hypothetical polyprotein, the proteins are arranged in the order N^{pro}/C/E^{rns}/E1/E2/p7/NS2-3/NS4A/NS4B/NS5A/NS5B; NS2-3 can be processed to yield NS2 and NS3 (Meyers and Thiel, 1996; Rice, 1996).

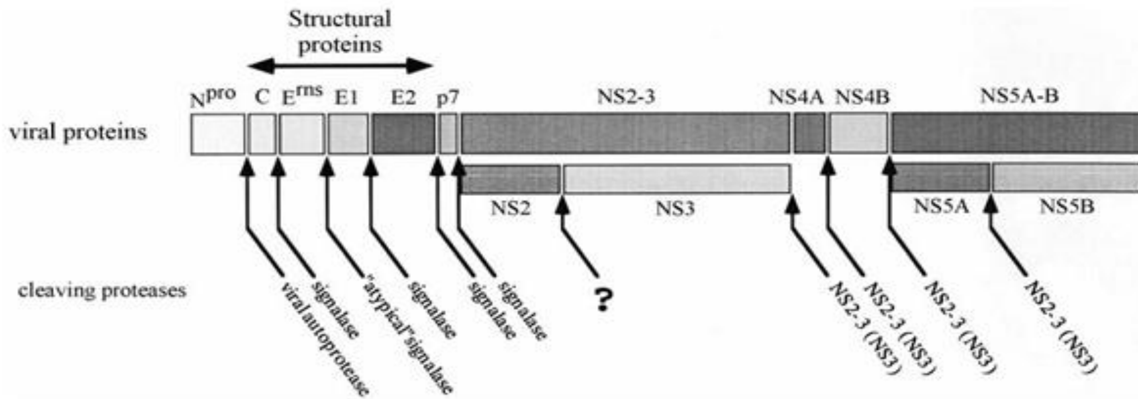

Figure 1. Genome organization of pestiviruses (Kummerer *et al*., 2000).

There are two genotypes (species) of BVDV namely BVDV-1 and BVDV-2 (Fauquet *et al*., 2005), each with subgenotypes (Flores *et al*., 2002; Vilcek *et al*., 2005). There are varying numbers of subgenotypes of BVDV in the literature, especially for BVDV-1 (Jenckel *et al*., 2014; Weber *et al*., 2014). Number of subgenotypes identified under each genotype is increasing with time; so far there are reports of up to 21 subgenotypes for BVDV-1 (1a-1u) and 4 subgenotypes for BVDV-2 (2a-2d) (Vilcek *et al*., 2001; Flores *et al*., 2002; Vilcek *et al*., 2005; Bachofen *et al*., 2010; Deng *et al*., 2012; Jenckel *et al*., 2014; Yesilbag *et al*., 2017). Subgenotypes are designated by small letter alphabets as BVDV-1a, BVDV-1b, etc. and BVDV-2a, BVDV-2b etc. Both genotypes of the virus are segregated into 2 biotypes namely cytopathic (CP) and non-cytopathic (NCP) depending on their ability to cause cytopathic effects in cell culture (Thiel *et al*., 2005; Bachofen *et al*., 2010).

BVDV-1 is the most widely distributed genotype worldwide (Fulton *et al*., 2005; Van Vuuren, 2005; Bachofen *et al*., 2010; Ridpath *et al*., 2010; Deng *et al*., 2012; Emran *et al*., 2014). Although BVDV-2 have been mainly identified in the US (Fulton *et al*., 2005) and Canada, several studies have reported their presence in Europe (Van Rijn *et al*., 1997; Wolfmeyer *et al*., 1997; Vilcek *et al*., 2001), South America (Canal *et al*., 1998; Gil *et al*., 1998; Flores *et al*., 2002), Asia (Nagai *et al*., 1998; Choi and Song, 2010) and Africa (Ularamu, 2010).

Viruses belonging to the BVDV-2 group were initially identified in severe outbreaks of acute BVD in North America in the late 1980s (Corapi *et al*., 1989; Carman *et al*., 1998). However, the

genotype since then has been isolated from subclinical or mild cases clinically not so different from those caused by most genotype 1 (Ridpath *et al*., 1994; Wolfmeyer *et al*., 1997; Bolin and Ridpath, 1998).

The diversity of the virus is identified through phylogenetic and antigenic characterization (Becher *et al*., 1999; Flores *et al*., 2000). Antigenic characterizations used include study using monoclonal (Mab) and polyclonal antibodies (Pab) and virus neutralization (VN) tests (Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). The phylogenetic methods used include sequencing of the stable 5′ untranslated region (5′ UTR), N^{pro} and E2 genetic regions of the virus (Becher *et al*., 1999; Vilcek *et al*., 2001; Nagai *et al*., 2004).

In addition to genomic differences, studies have shown antigenic differences between subgenotypes as demonstrated by differences in cross-neutralization (Pizarro-Lucero *et al*. 2006; Bachofen *et al*., 2008), monoclonal antibody binding (Bolin and Ridpath, 1998) and response of PI animals to vaccination (Fulton *et al*., 2003).

2.2. Epidemiology

2.2.1. Distribution

Bovine viral diarrhea virus is one of the most important and widespread viral pathogens of cattle (Radostits *et al*., 2007; OIE, 2008). The distribution of BVDV is worldwide (Baker, 1990; OIE, 2008). It has been reported from most major cattle producing parts of the world (Radostits *et al*., 2007; Walz *et al*., 2010).

BVDV infection once introduced into the cattle population of an area tends to remain endemic; however, the infection status of a single herd can change rapidly; that is, within 2-3 years, it can change from actively infected (active virus spread in the herd) to immune and then to a susceptible status once again (Viltro *et al*., 2002).

2.2.2. Prevalence

Infection with BVDV is common in cattle populations throughout the world, as indicated by the high prevalence of seropositive cattle (Walz *et al*., 2010). Studies carried out in different parts of the world have estimated the prevalence of antibody-positive cattle to be about 28.6%-82% (Duong *et al*., 2008; Gur, 2011; Raizman *et al*., 2011; Kulangara *et al*., 2015; Aragaw *et al*., 2018; Erfani *et al*., 2018; Ran *et al*., 2019). Herd-level seroprevalence of BVDV in cattle varies

from 43 to 80.7 % depending on geographic region (Sakhaee *et al*., 2009; Talafha *et al*., 2009; Saa *et al*., 2012; Almeida *et al*., 2013; Velasova *et al*., 2017; Aragaw *et al*., 2018; Hou *et al*., 2018). In contrast, the prevalence of PI is much lower and is generally between 0.5 and 2% at animal level in the cattle population in different countries of the world (Taylor *et al*., 1995; Brock, 2003; Peterhans *et al.,* 2003; Ahmad *et al*., 2011). A recent metanalysis of data published from around the world has estimated the herd level pooled worldwide PI prevalence at 18.88% (Scharnböck *et al*., 2018). The current BVDV epidemiological situation demonstrates that several countries that implemented BVDV intervention programmes have successfully reduced PI prevalences at animal level. Sweden, Finland, Norway have completely eradicated BVDV (as of May 2018) (Scharnböck *et al*., 2018).

Prevalence is mainly affected by presence of PI animals on the farm (Houe, 1999), production system/type, age of the animals, biosecurity measures adopted by the farm, herd size and density of cattle population in the area (Houe, 1999; Mockeliūnien *et al*., 2004; Talafha *et al*., 2009; Handle *et al*., 2011; Saa *et al*., 2012; Scharnböck *et al.,* 2018).

2.2.3. Source of infection

The most important sources of BVDV infection are PI animals (Radostits *et al*., 2007). The virus is introduced into a susceptible herd mainly by introduction of PI animals or pregnant animals carrying a PI fetus (Houe, 1999). PI animals are efficient transmitters because they constantly shed the virus in large quantities over prolonged periods of time in their body secretions and excretions and the spread to susceptible cattle in contact is rapid (Dufell and Harkness,1985; Brownlie *et al*., 1987b). Virus may also be shed for a few days in small amounts by acutely/transiently infected (TI) animals (Brownlie *et al*., 1987b). Acutely infected animal shed the virus during viremia. In general virus is shed through saliva, ocular and nasal discharge, feces, urine and milk (Radostits and Littlejohns, 1988). Bovine virus diarrhea virus may be excreted in semen in both acutely infected and PI bulls, although the virus titers in the latter is a lot higher (Paton *et al*., 1989; Kirkland *et al*., 1991). Aborted materials and discharges from reproductive tract of an infected cow can be potent sources of the virus (Radostits *et al*., 2007).

Introduction to a herd is also possible through contact between herds at communal grazing, through fences of adjacent farms, contact between animals at market and shows. Fomites have

also been incriminated as vehicle of transmission between herds. The virus may also be introduced through semen used for artificial insemination (Houe, 1999; Gethmann *et al*., 2015).

Apart from BVDV presence in PI cattle, there is evidence that following apparent recovery from transient infection (TI), BVDV may maintain prolonged or chronic infections within immunoprivileged sites such as in tissues of the ovary, testes, central nervous system, and in circulating white blood cells (Givens and Marley, 2013). These apparently recovered animals can remain infectious for BVDV-naïve cattle for months post-infection (Collins *et al*., 2009).

Although cattle are the natural host, infection by BVDV has been demonstrated in numerous other species. Moreover, PI with BVDV is documented in alpacas, mouse deer, mountain goats, white-tailed deer, sheep, and goats. The fact BVDV can infect and cause PI in a variety of other species that are in natural contact with cattle poses a risk for infection of pestivirus-susceptible cattle populations (Walz *et al*., 2010; Bachofen *et al*., 2013; Brodersen, 2014). It has been proved that under experimental conditions, PI animals transmit infection approximately to 60% susceptible animals within 24 h (Littlejohns, 1985; McGowan *et al.,* 1993).

2.2.4. Transmission

BVDV is usually transmitted by both horizontal and vertical means. Although the most common route of BVDV transmission is direct contact between an infected and a susceptible animal, it can also be transmitted indirectly through different types of vehicles (Lindberg, 2003; Laureyns *et al*., 2010). Infected cattle shed BVDV in body fluids and excretions including nasal discharge, saliva, semen, urine, feces, tears, milk, and uterine flushing, each of which could allow wide dissemination of the virus (Thurmond, 2005; Lanyon *et al*., 2014).

Direct contact with a PI animal (especially nose-to-nose contact) is the most efficient mode of transmission of the virus under natural circumstances (Cook *et al*., 1990; Traven *et al*., 1991; McGowan *et al*., 1993; Niskanen *et al*., 1996; Walz *et al*., 2010) as these animals continuously shed large amounts of virus in the environment (Coria and McClurkin, 1978; Straver *et al*., 1983; Brock *et al*., 1991). Also direct contact with acutely infected animals can transmit the virus, although less efficiently (Meyling *et al*., 1990). Virus is excreted in smaller amounts from acutely infected animals and for only a few days during the acute infection (Brownlie *et al*., 1987b).

Several ways of indirect, vehicle or vector transmission of BVDV have been demonstrated, i.e. reusing needles and nose tongs (Gunn, 1993), rectal gloves (Lang-Ree *et al*., 1994), by using live or contaminated vaccines (Liess *et al*., 1984b; Luken *et al*., 1991), or through cattle trade and vehicles (Gethmann *et al*., 2015). Transmission can also occur through blood feeding flies (Tarry *et al*., 1991). However, in general, pestiviruses are relatively easily inactivated, and their infectivity outside the host is of short duration (Hafez and Liess, 1972; Duffel and Harkness, 1985; Liess, 1990).

Infections with BVDV can also be transmitted through infected semen and fluids used for embryo transfer (Fray *et al*., 2002). Bovine virus diarrhea virus is excreted in semen in both acutely infected and PI bulls, although the virus titers in the latter is a lot higher (Paton *et al*., 1989; Kirkland *et al*., 1991; Grooms, 2004). Accordingly, semen from a PI bull is much more efficient in the transmission of BVDV as compared to semen from acutely infected bull (Meyling and Jensen, 1988; Kirkland *et al*., 1997). Transmission occurs during natural breeding or artificial insemination (AI) of cows (Rikula *et al*., 2008; Newcomer *et al*., 2014).

The possibility of airborne transmission has not been proven and remains controversial, but transmission by air is considered possible over several meters by some authors (Bitsch and Runsholt, 1995). BVDV can spread between herds mainly by cattle movement, live vaccines, semen and embryos, visitors, including veterinaries and AI technicians (Lindberg and Alenius, 1999).

Vertical transmission of BVDV occurs when the virus is transmitted transplacentally from the infected dam, whether the dam is transiently or persistently infected, to her offspring (Kennedy, 2005). Infection of susceptible pregnant cows with the NCP virus before the development of fetal immunocompetence results in the birth of PI cattle (Lanyon *et al*., 2014).

Transmission between sheep and cattle, both ways, has been demonstrated (Carlsson, 1991; Carlsson and Belak, 1994; Paton *et al*., 1995), although the most common direction of transmission is believed to be from cattle to other species (Graham *et al*., 2001; Lindberg, 2003).

2.2.5. Maintenance of the virus in a herd

The only way that BVDV infection can persist in a herd for an extended period of time without being re-introduced, is if one or more seronegative animals are in early pregnancy at the same

time as there are PI animals in the herd. In any other case, self-clearance will occur (Lindberg and Alenius, 1999). If PI females reach breeding age, offspring will be PI (McClurkin *et al*., 1979, Straver *et al*., 1983). Affected families may thus develop and provide a means to maintain the virus in a herd (Littlejohns and Walker, 1985).

PI animals remain infected for life and shed large quantities of virus, thereby they assure the persistence of BVDV in the cattle population even in the absence of animals susceptible to TI (Bachofen *et al*., 2010).

2.2.6. Host range

Cattle are the natural host for BVDV (Walz *et al*., 2010). While the virus mainly infects cattle, it has been detected in pigs, and a wide range of domestic and wild ruminant hosts (Brodersen, 2014; Yesilbag *et al*., 2017).

Evidence for PI has been proven in at least seven species (sheep, pigs, alpaca, white-tailed deer, eland, mouse deer, and American mountain goat) (Bachofen *et al*., 2013). A review on reports of infection with BVDV in different species of animals (domestic and wild) demonstrated that there exists evidence of BVDV infection in 7 of the 10 families comprising the mammalian order Artiodactyla including Antilocapridae, Bovidae, Camelidae, Cervidae, Giraffidae, Suidae, and Tragulidae, representing over 50 species (Passler and Walz, 2009). This may pose a risk for infection and re-infection of in-contact cattle populations (Krametter-Froetscher *et al*., 2010; Brodersen, 2014).

Pigs infected with BVDV exhibited clinical symptoms similar to classical swine fever (CSF), or subclinical infections (Terpstra and Wensvoort, 1988). Lambs infected congenitally with BVDV demonstrated clinical signs of border disease (Carlsson and Belak, 1994).

2.3. Pathogenesis and Clinical Manifestations

BVDV infections are either transient (acute) or persistent (Bachofen *et al*., 2010). When infected early in gestation with NCP virus in utero, animals become immunotolerant to the infecting viral strain and may be born persistently infected (PI) (Adler *et al*., 1996). In contrast, transient infection (TI) occurs when postnatal immunocompetent cattle are infected with BVDV (Baker, 1995; Hansen *et al*., 2010).

The clinical manifestations and pathogenesis of BVDV infections are dependent on host factors such as the age, immune status, concurrent infections with other pathogens of the animal, and reproductive status and stage of gestation in the cow at the time of infection, and type of infection (transient versus persistent) as well as the genotype, strain and biotype of the virus. The epidemiological situation – endemic versus non-endemic - may also influence the clinical outcome of the infections (Corapi *et al.*, 1989; Baker, 1990; Brodersen and Kelling, 1998; Bachofen *et al.*, 2010; Walz *et al.*, 2010).

The complex pathogenesis of pestivirus infections is manifested by a wide variety of clinical signs observed during BVDV infection, including digestive disorders, reproductive disorders and respiratory tract infections. Clinically BVD is a very diverse condition ranging from asymptomatic or mild and transient signs of upper respiratory tract infection to severe acute disease with signs associated with the digestive, hematopoietic, reproductive or respiratory organ systems, often exacerbated by super-infection with other pathogens (Sandvik, 2005).

In the USA, various cases of BVDV infection were classified into syndromes based on the predominant clinical manifestation (Saliki, 1996, cited by van Vuuren, 2005): reproductive disease comprising abortion, repeat breeding, stillbirth, weak calves; acute/peracute BVD in animals of all ages often resulting in death; classical BVD represented by gastro-enteritic disease, pyrexia and respiratory disease; hemorrhagic syndrome with bloody secretions and petechial hemorrhages; mucosal disease (MD) characterized by gastroenteritis, digital erosions and ulcers in PI animals; and respiratory disease including pyrexia, bronchopneumonia and weakness.

2.3.1. Acute infection

Cattle with acute infection usually recover and eliminate the virus within 2 weeks post-infection (Baker, 1995; Hansen *et al.*, 2010). The pathogenesis of acute infection has not been clearly described. It is likely that the initial infection is within the oronasal mucosa and that spread from this site is systemic. Virus may be isolated from nasal swabs during the first few days postinfection but only in low titer. There is preliminary evidence that certain field strains appear to be well adapted to growth in the nasal mucosa (Brownlie, 1990). Those capable of rapid growth within the oronasal mucosa may account for the limited oculonasal discharge and shallow ulcerations seen in some of the acute infections (Baker, 1987). Systemic spread of

infection may occur as free virus in serum or as virus associated with the cells within the buffy coat fraction of blood (Truitt and Schechmeister, 1973). BVDV is lymphotrophic, and acutely infected cattle are immunosuppressed as a result of reduction in circulating immune cells and diminished function of immune cells. Cells of both the innate and adaptive immune responses are affected. Leukopenia occurs in most acutely infected cattle, but the severity of leukopenia can be influenced by BVDV strain (Walz *et al*. 2001; Kelling *et al*., 2002; Liebler-Tenorio *et al*., 2003). Lymphopenia (T-lymphocytes and B-lymphocytes) and neutropenia are the major hematologic abnormalities (Ellis *et al*., 1988; Kelling *et al*., 2002). Removal of BVDV-infected leukocytes by the immune system, destruction of immune cells by BVDV, and increased trafficking of immune cells into tissue sites of viral replication are all responsible for leukopenia. There is lymphoid depletion in the thymus, spleen, lymph nodes, and gut-associated lymphoid tissues (Peyer's patches), and the severity might also be strain dependent (Walz *et al*., 2001). Diminished function in immune system cells has also been described during acute BVDV infection, and affected cells include lymphocytes, neutrophils, and monocytes and macrophages (Roth *et al*, 1981; Roth and Kaeberle, 1983; Welsh *et al*., 1995).

Although great majority of BVDV infections go unnoticed due to mild nature of the disease, it is clearly evident from clinical observations that BVDV can, under certain circumstances, cause disease. Acute virulent infections in immunocompetent animals, characterized by high morbidity and mortality, have occurred in different parts of the world (Perdrizet *et al*., 1987; David *et al*., 1994; Carman *et al*., 1998; Friedgut *et al*., 2011).

Acute infection in non-immune non-pregnant animals

In susceptible, non-pregnant animals the infection is in most cases subclinical (Houe, 1995) but can, depending on genotype and strain also produce severe disease where animals succumb to the infection (Perdrizet *et al*., 1987; Pellerin *et al*., 1994; Friedgut *et al*., 2011; Gethmann *et al*., 2015).

Subclinical BVD: This is usually a clinically unrecognizable (subclinical, inapparent) infection with the development of serum neutralizing antibodies and elimination of the virus from normal immunocompetent animals. Subclinical infection accounts for the high percentage of normal animals (70-90%) that are serologically positive (Ames, 1986). Cattle undergoing subclinical infections may have a mild elevation of body temperature, leukopenia about days 3 to 7 post-

infection and occasionally a mild nasal discharge (Carlson *et al*., 1957) that is followed by the development of specific neutralizing antibody (Baker, 1990).

Mild transient clinical disease: A mild transient clinical disease characterized by inappetence for a few days, depression, fever, mild diarrhea, oculonasal discharge, occasionally oral lesions (erosions or shallow ulcerations), transient leukopenia and recovery in a few days may occasionally occur. In lactating cows, milk production may decrease (Radostits *et al*., 2007). Clinical infections generally occur in cattle ranging in age from 6 months to 2 years (Blood *et al*., 1983). In susceptible herds, morbidity may be high but mortality is low to none. The incubation period is 5 to 7 days and is followed by transient fever and leukopenia (Dufell and Harkness, 1985). Viremia occurs 4 to 7 days after infection (Brownlie *et al*., 1987b) and in some cases may persist up to 15 days (Dufell and Harkness, 1985). In calves, the infection is often associated with respiratory and gastrointestinal (GI) symptoms such as coughing and diarrhea (Traven *et al*., 1991; Loken, 1992; Baker, 1995; Potgieter, 1997). Such symptoms can also be a result of secondary, or concurrent infections (Brodersen and Kelling, 1998; Elvander *et al*., 1998; Klingenberg *et al*., 1999; Fulton *et al*., 2000; Klingenberg, 2000) since BVDV acts as an immunosuppressive agent by impairing immune functions mainly associated with the cellular response (Potgieter, 1995; Adler *et al*., 1996; Bruschke *et al*., 1997).

In adult bulls, an acute infection may be associated with a transient impairment of semen quality (Paton *et al*., 1989; Kommisrud *et al*., 1996; Kirkland *et al*., 1997). There are indications that virus may persist and replicate in testicular tissue for more than 6 months (Givens *et al*., 2002).

Severe acute BVD: Infection may yet cause severe BVD in some outbreaks. This is a severe highly fatal form of BVD. The disease has been described in many countries (David *et al*., 1994; Carman *et al*., 1998); it is often highly fatal, occurs in immunocompetent seronegative cattle in postnatal life (Kummerer *et al*., 2000; Wentz *et al*., 2003) and is characterized by severe depression, reduction in milk yield, fever up to 42.0^{o}C, loss of appetite, pneumonia, oculonasal discharge, profuse watery diarrhea, dysentery, conjunctivitis, oral ulceration, corneal edema, sudden death and abortion in pregnant cows in all age groups of different types of cattle (Carman *et al*., 1998; Radostits *et al*., 2007). Morbidity rates may be up to 40% and crude mortality rates may reach 25% (Radostits *et al*., 2007). The condition is mostly associated with BVDV-2 (Carman *et al*., 1998; Gethmann *et al*., 2015; Friedgut *et al*., 2011) although BVDV-1 was also

rarely found responsible for a similar severe disease (Liebler-Tenorio *et al*., 2000; Ridpath *et al*., 2007; Lunardi *et al*., 2008). Inadequate biosecurity of animals imported into the herd, and the failure to vaccinate for BVDV or an inadequate BVDV vaccination program are common risk factors associated with outbreaks. In affected herds, all ages of cattle are affected including calves, yearlings and adults. Mortality is highest in the young age groups (Radostits *et al*., 2007). Such an outbreak in Quebec, Canada resulted in the death of 32,000 out of 143,000 (22.4%) animals in the 1993 veal calf crop (Pellerin *et al*., 1994).

Thrombocytopenia and hemorrhagic syndrome: Some severe acute BVD outbreaks (and experimental infections) were associated with thrombocytopenia and hemorrhagic signs (Corapi *et al*., 1989; Friedgut *et al*., 2011; Gethmann *et al*., 2015). This disease is regarded as a distinct form of severe acute BVD and termed hemorrhagic syndrome (Ridpath *et al*., 2006). It is characterized clinically by high fever, very high mortality rates, drop in milk production, severe thrombocytopenia, leukopenia, anemia, mucosal lesions, bloody diarrhea, epistaxis, petechial and ecchymotic hemorrhages and prolonged bleeding from injection sites or insect bites (Corapi *et al*., 1989; Rebhun *et al*., 1989; Bolin and Ridpath, 1992; Carman *et al*., 1998; Jenckel *et al*., 2014). Type 2 BVDV isolates are most commonly associated with the hemorrhagic syndrome (Radostits *et al*., 2007). Thrombocytopenia has been reported as a feature of peracute BVDV infection (Carman *et al*., 1998), where in platelet counts are reduced to below 25 000/µl (Radostits *et al*., 2007). The cause of thrombocytopenia is not completely understood, however necrosis of megakaryocytes, reduced production of thrombocytes by megakaryocytes, increased consumption of thrombocytes in the periphery, and functional defects of thrombocytes have all been suggested as contributing factors (Walz *et al*., 1999).

Other manifestations of acute BVDV infection

Immunosuppression: There is evidence that postnatal BVDV infections of cattle can cause immunosuppression and enhance the development of other infectious diseases (Potgieter, 1995). The consequence of immunosuppression is an increased susceptibility to other infectious disease agents, and the bovine respiratory disease complex is an example where BVDV plays an important role in polymicrobial disease (Walz *et al*., 2010).

BVDV infection may potentiate or enhance the pathogenicity of co-infecting pathogens, such as parainfluenza virus type 3, infectious bovine rhinotracheitis virus, coronavirus, rotavirus, bovine

respiratory syncytial virus, *Pasteurella* spp., *Salmonella* spp., *Actinomyces pyogenes,* coccidia and helminths (Ames, 1986; Baker, 1987; Potgieter, 1988; Brodersen and Kelling, 1998). If management and environmental conditions are suboptimal and animals are in contact with a variety of potential pathogens, BVDV may enhance development of a secondary disease. Even modified live BVDV vaccines have been demonstrated to be immunosuppressive (Roth and Kaberle, 1983).

Data suggest that NCP BVDV can shut down production of several key chemokines in infected macrophages, including type-I interferon (IFN) and tumor necrosis factor alpha (TNFα) production, that play crucial roles in the immune response to infection (Adler *et al*., 1996; Burr *et al*., 2012). Lymphocytes from BVDV-infected animals have impaired memory responses to BVDV and other antigens (Lamontagne *et al*., 1989). Alsaad *et al*. (2012) found a significant leukopenia and lymphopenia in BVD affected calves.

The pathology and hence the clinical manifestation of mixed infections is highly dependent on the nature of the second pathogen. In the case of *Pasteurella haemolytica,* for example, there is a fibrinopurulent pneumonia and pleuritis (Potgieter *et al*., 1984).

Respiratory disease: BVDV may have a role in bovine respiratory disease because of its immunosuppressive effects on the host (Potgieter, 1977; Howard *et al*., 1987; Howard *et al*., 1989; Rebhun *et al*., 1989). Despite the fact that BVDV is generally considered to be lymphotrophic and enterotrophic, it has been identified in outbreaks of respiratory disease, usually in association with other pathogens. In one study BVDV was the virus most often isolated from pneumonic lungs in cases of shipping fever in feedlot cattle and was usually found in association with *Pasteurella haemolytica* (Reggiardo, 1979). Another study reported BVDV to be the most frequent virus found in association with multiple virus infection in outbreaks of respiratory disease in calves (Richer *et al*., 1988). A seroepidemiologic study demonstrated an association between antibody titres to BVDV (as well as other viruses) and the treatment of respiratory disease (Martin and Bohac, 1986). These findings suggest that BVDV may be involved as an initiator in bovine respiratory disease. Results of experimental attempts to reproduce respiratory disease with BVDV have been variable (Baker, 1995).

Fulton *et al*. (2000) demonstrated involvement of BVDV-1 and 2 in acute respiratory disease of calves with pneumonic pasteurellosis. In a fatal outbreak in Israel, acute BVDV infection caused

mainly respiratory distress in calves aged 10 to 90 days (Friedgut *et al*., 2011). Respiratory signs have been most frequent findings in cattle affected with BVDV in South Africa (Kabongo and Van Vuuren, 2004; Fulton *et al*., 2005).

Diarrhea of neonatal calves: The role of the virus causing diarrhea in calves under a few weeks of age is uncertain. Naturally occurring cases of acute neonatal diarrhea due to infection with the virus in immunocompetent calves under 6 weeks of age have been reported only rarely. Calves born with PI status may be unthrifty and be affected with chronic diarrhea and pneumonia as young calves. However, if the virus causes diarrhea in calves the pathogenesis is not clear. Calves born from cows free of the infection are not likely to be exposed to the infection. Immunocompetent dams provide colostral immunity to their calves, which should protect them against viremia due to BVDV for 6 months or longer (Bolin and Ridpath, 1995).

Many reports indicated that severe acute BVD in adult cattle are mainly associated with GI signs (diarrhea), whereas it is manifested clinically by respiratory signs in calves (Friedgut *et al*., 2011).

Effect on the ovaries: Field outbreaks of NCP BVDV are commonly associated with conception failure and an increased incidence of early abortion (Roeder and Drew, 1984; Virakul *et al*., 1988; Larsson *et al*., 1994). Fray *et al*. (1999, 2002) showed that viremia has deleterious effects on follicular function. Viremia during the follicular phase may reduce the fertility of cattle by disrupting the capacity of the ovulatory follicle to form a competent corpus luteum, thereby compromising early embryo development and maternal recognition of pregnancy (Fray *et al*., 2002). Viremias induced experimentally during the follicular phase have resulted in a 50% decrease in pregnancy rate and a deterioration in the quantity and quality of embryos recovered after superovulation (McGowan *et al*., 1993; Kafi *et al*., 1997). Viremia induced during the first 2–6 days of the estrous cycle suppressed estradiol secretion from the first dominant postovulatory follicle, although the mechanism was unresolved (Fray *et al*., 1999). Viremia during the luteal phase can retard follicle development (Grooms *et al*., 1998).

Pestiviruses, and BVDV in particular, have a broad tissue tropism infecting many cell types including thecal, granulosa and luteal cells, oocytes (Booth *et al*., 1995; Grooms *et al*., 1996; Fray *et al*., 1998), pituitary gonadotrophs (Anderson *et al*., 1987) and central nervous tissue

(Hewicker-Trautwein *et al*., 1995). Clearly, NCP BVDV has the potential to disrupt cell function within one or more components of the hypothalamic–pituitary–ovarian axis (Fray *et al*., 2002).

Effect of venereal infection on reproductive performance of cows: Acute infections of the urogenital tract of seronegative cattle with BVDV can produce clinical disease and may be a greater cause of loss to the national herd than results from the persistently viremic animal. The virus can infect both ovarian and testicular tissues and can be recovered from semen of acutely infected bulls (Ramsey and Chivers, 1953; Whitemore *et al*., 1978). The semen is often of poor quality (Whitmore *et al*., 1981) and has the potential to spread infection to seronegative heifers (Meyling and Jensen, 1988). However, the pathogenesis of urogenital infection during acute disease is poorly described (Brownlie, 1990).

Significantly reduced conception rates have been observed in seronegative cattle exposed to the virus at the time of breeding, by serving cows with PI bulls (McClurkin *et al*., 1979) and experimentally by intra-uterine administration of BVDV (Archbald, 1974; Archbald and Zemjanis, 1977; Whitmore *et al*., 1981; Grahn *et al*., 1984). Seronegative cows inseminated with infective semen generally fail to conceive until they develop an immune response to the virus (McClurkin *et al*., 1979). The adverse effect of this virus on conception is attributed to fertilization failure (Grahn *et al*., 1984).

Thus, BVDV may cause a herd problem characterized by repeat breeding if the infection is by the venereal route in seronegative cows. The most notable finding would be an increase in the number of services per conception. Such a problem will be transient in nature (until an immune response occurs), and it may be difficult to incriminate BVDV if there are no other concurrent signs of infection in the herd (Baker, 1995).

BVDV has been associated with ovaritis in infertile heifers. It was possible to re-isolate BVDV from the ovaries many months after they had seroconverted with no evidence of persistent viremia (Ssentongo *et al*., 1980).

Acute infection in non-immune pregnant cows and congenital infection

Clinical effect of BVDV infection in an immunocompetent pregnant heifer or cow would be similar to that described above, except for its effect on the embryo or the fetus. The main importance of BVDV infection during pregnancy is the outcome if transplacental spread of the

virus to the conceptus occurs. In sero-negative dams, fetal infection can follow from either acute or persistent viremias (Brownlie, 1990). However, BVDV rarely infects the fetuses of sero-positive cattle. Maternal antibodies appear well able to prevent the access of virus through the placentome. Whether maternal antibody prevents the virus from becoming viremic has not been determined.

The virus shows affinity to rapidly dividing cells and a growing fetus is therefore a favored site of replication. In non-immune pregnant animals, the virus infects the conceptus, irrespective of the time of gestation, with effectively 100% probability (Duffell and Harkness, 1985).

However, the exact route by which the virus reaches the fetus is unclear (Lindberg, 2003). Whether, following acute or PI, the virus infects the fetus by either direct cell to cell transmission or systemic spread is not clear. The time taken for the passage of virus from dam to fetus is variable but it has been recorded that abortions due to BVDV can occur within 10-18 days after intramuscular infection (Rebhun *et al*., 1989). Abortions can take place several months after fetal infection (Brownlie, 1990).

During acute infection the virus invades the placentome, replicates and may cross to the fetus without producing lesions (Cay *et al*., 1989). In sheep, BVDV has been shown to damage the maternal vascular endothelium within 10 days of infection and the resulting cellular debris is ingested by the fetal trophoblast (Barlow, 1972). This could be a mechanism of virus transfer from dam to offspring but may also account for the placentitis that leads to the high level of abortion following BVDV infection. It is well recorded that early embryonic death, infertility and "repeat breeder" cows are often the sequel to pestivirus infection during pregnancy (Van Oirschot, 1983). In a herd infected with BVDV, the conception rates were reduced from 78.6% in the immune cows to 22.2% in infected cattle (Virakul *et al*., 1988).

The specific outcome of the fetal infection depends on the age of the fetus at the time of infection and the biotype of the infecting virus. Therefore a wide range of reproductive failures can be seen in infected herds (Roeder *et al*., 1986). The possible outcomes include failure to conceive, fetal resorption, abortion, mummification, congenital malformations and the birth of calves weak and undersized, stillborn calves, calves PI with BVDV or normal calves seropositive to the virus (Figure 2) (Carlsson *et al*., 1989; Oberst, 1993; McGowan and Kirkland, 1995; Fray *et al*., 2000).

Abortions may appear at any time during pregnancy and are not necessarily associated with the time of infection (Lindberg, 2003).

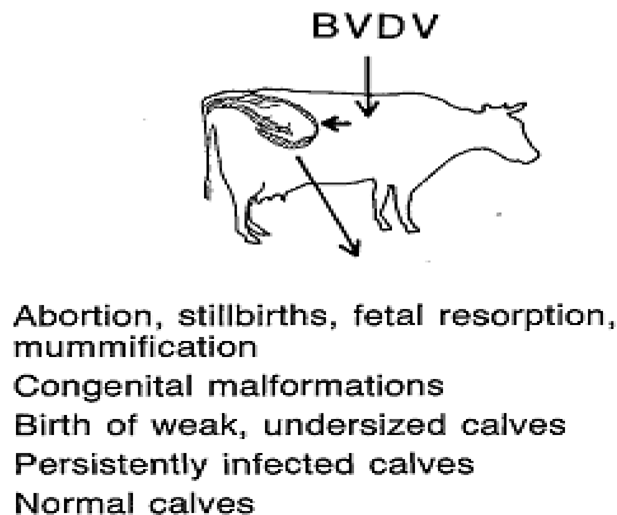

Figure 2. Possible outcomes of fetal infection with BVDV (Baker, 1990)

Failure in fertilization and early embryonic loss

Early studies suggested that interruption of normal fertilization or embryonic death may be the mechanism for decreases in conception rates associated with acute BVDV infection (Grooms, 2004). Failure of fertilization may follow inflammation of the oviducts in infected cows. The oviducts have important functions in bovine fertility including the transport, storage, and capacitation of spermatozoa, the pick-up of the newly ovulated oocyte by the infundibulum and the transport, maturation and fertilization of the oocyte. The secretory products of the oviducts should also provide an optimum environment for the sustenance of the spermatozoa, oocytes, and the early embryo that is undergoing cleavage (Senger, 2003; Rodriguez-Martinez, 2007). BVDV infection was associated with salpingitis in infected non-pregnant cows (Archbald *et al*., 1973). Generally, inflammation of the oviducts can interfere with the secretive and other physiologic functions of the oviducts, thereby compromising the ideal environment required for oocyte and sperm transport, and for fertilization (Oguejiofor *et al*., 2019).

Infection with BVDV may also have harmful effects on sperm–oocyte integrity and interaction at the time of fertilization (Garoussi and Mehrzad, 2011). Archibald *et al*. (1973) provided evidence that BVDV may interfere with early embryonic development. More recent studies have also

provided evidence of viral interference with endometrial functions during exposure to infection and also in the period of early pregnancy (Oguejiofor *et al*., 2019).

Embryonic and fetal loss: There is uncertainty about the pathogenesis of infection during the first 30 days of pregnancy. There is good evidence that BVDV will reduce the conception rate during this period (Virakul *et al*., 1988) and that the virus will replicate freely in the maternal placenta (Parsonson *et al*., 1979). However, there is also the view that limited transplacental infection occurs during this early stage (Whitemore *et al*., 1978) because the contact between maternal epithelium and fetal trophoblast is not sufficiently intimate for vertical transmission until the 'bridge' formation at around 30 days (Kendrick, 1971; Barlow, 1972). There is little doubt that fetal infection will occur after this 30-day period and the outcome depends on whether the virus establishes during the first (up to about 110-120 days), the second (to about 180-200 days), or third trimester (to full term, about 280 days) (Brownlie, 1990).

BVDV does not appear to be a major cause of early embryonic death (Whitmore *et al*., 1981; Potter *et al*., 1984) but fetal infection from 50 to 100 days of gestation may result in fetal death followed by abortion, stillbirth or mummification (Casaro *et al*., 1971; Kendrick, 1971; Kahrs, 1973; Done *et al*., 1980; Stober, 1984). Fetal death can follow directly from viral invasion but damage of the maternal placenta may contribute by disrupting its vascular supply of nutrients. Experimental infections during this period have shown that more than 30% of fetuses are aborted (Brownlie *et al*., 1986). Expulsion of the fetus may occur up to several months after infection (Kahrs, 1981). The overall incidence of abortion caused by BVDV appears to be low (2-7%) but in non-immune herds a higher percentage may be encountered (Kahrs, 1980; Ernst *et al*., 1983; Stober, 1984; Ames, 1986). Transplacental infections do not always cause abortion (Baker, 1990). Experimental infection of cattle during the first trimester of pregnacy with the CP biotype does not give abortions and there is some doubt whether this biotype can even establish in the early fetus (Brownlie *et al*., 1989).

Infection during the first two trimesters can result in abortions (Gillespie *et al*., 1967) whereas infection during the first trimester can also produce calves that remain persistently viremic for life. Calves infected during the last trimester are able to mount an active immune response (Brown *et al*., 1979).

If the fetus is infected after it has become immunocompetent, it will develop antibodies (Howard, 1990). However, despite the ability to mount an immune response, the growing fetus is negatively affected and these animals can be weak at birth, ill-thrifty and therefore more susceptible to other infections (Larsson *et al*., 1994; Moennig and Liess, 1995). The pregnant dam, if non-PI, develops antibodies in response to the infection. If a PI is established in the fetus, her immune response will be further triggered and antibody levels will continue to rise until the production of colostrum starts, shortly before parturition (Meyling and Jensen, 1988; Brownlie *et al*., 1998). This phenomenon can be used for diagnostic purposes, to identify dams at risk of giving birth to PI calves (Lindberg *et al*., 2001).

Abortion had been noted in several herds as BVDV was first described and continues to be an economically important aspect of BVDV infections in the present day (Brodersen, 2014). Experimental transplacental fetal infection with BVDV was first demonstrated in 1969 (Ward *et al*., 1969). Although the mechanism of fetal infection is not clear, evidence suggests that BVDV may cross the placenta by causing vasculitis on the maternal side of placentation allowing for access to the fetal circulation (Fredriksen *et al*., 1999). Fetal loss due to in utero BVDV infection is often determined by the gestational age at the time of infection (Carlsson *et al*., 1989; McGowan and Kirkland, 1995). Fetal death following infection of susceptible dams can occur at any point during gestation, although they are most common during the first trimester (Kahrs, 1968; Cassaro *et al*., 1971; Kendrick, 1971; Done *et al*., 1980; Duffell and Harkness, 1985; Roeder *et al*., 1986; Sprecher *et al*., 1991). However, BVDV should not be ruled out in cases where late term abortions predominate. Depending on the time of infection, fetal reabsorption, mummification, or expulsion can occur (Casaro *et al*., 1971; Done *et al*., 1980). In the later stages of gestation (125–285 days of gestation), immunocompetence and organogenesis are usually complete. Although abortions and the birth of weak calves have been attributed to infection with BVDV late in gestation (Ward *et al*., 1969), fetuses infected during this time period are normally able to mount an effective immune response to BVDV and effectively clear the virus. These calves are usually normal at birth and have precolostral neutralizing antibodies to BVDV (Grooms, 2004).

In a field investigation where BVDV was introduced into a susceptible herd as a point source, an abortion rate of 21% occurred during the subsequent 6 months (Roeder *et al*., 1986). In endemically infected herds without BVDV control programs (vaccination, biosecurity, test and

removal), it has been estimated that 7% of fetal deaths may be attributable to infection with BVDV (Rufenacht *et al*., 2001). In diagnostic lab surveys in the United States, BVDV has been isolated from 0.1% to 27.2% of submitted abortion cases (Grooms, 2004). BVDV was determined to be the etiology in 4.1% of abortions submitted to the Ontario (Canada) Ministry of Agriculture from 1993–1995 (Alves *et al*., 1996). In the United Kingdom, BVDV was isolated from 27% of examined abortion cases (Murray, 1990, 1991).

Hormonal imbalances may play a role in fetal loss, as there are elevated prostaglandin levels in the dam during BVDV infection that result in lysis of the corpus luteum (the main source of progesterone in the cow) (Brodersen, 2014).

Fetal death usually follows 10 to 27 days postexposure with expulsion of the fetus occurring up to 50 days later (Murray, 1991). Because of the delay between fetal death and subsequent diagnosis of abortion, fetal and placental lesions seen are usually nondiagnostic, and BVDV virus isolation is not always successful (Baker, 1987). Under experimental conditions or when aborted fetuses are expelled soon after death, lesions observed include conjunctivitis, peribronchiolar and interalveolar pneumonia, and nonspecific myocarditis. Placental lesions consist mainly of vasculitis, edema, congestion, and hemorrhage, with some degeneration and necrosis (Jubb *et al*., 1985; Murray, 1991).

Congenital defects/Teratogenesis: Viruses that establish in the early fetus during organogenesis can have the distinction of causing bizarre malformations that permanently affect the animal (Brownlie, 1990). Infection of the fetus between approximately 100 to 150 days of gestation can result in a variety of congenital defects (Dufell and Harkness, 1985). During this stage of gestation, organogenesis is being completed and the immune system is becoming fully functional (Grooms, 2004). Infection during this period may result in damage and destruction of stem cells, resulting in congenital defects at birth (Baker, 1990).

When the lesions induced by BVDV infection are particularly severe, the fetus will die and be aborted. However, it is evident that the NCP biotype can replicate in the early fetus, often causing damage to selected tissues but not sufficient to cause death. Such calves are born with a variety of clinical signs that range from apparently normal to weak, unthrifty calves or occasionally braindamaged calves (Brownlie, 1990). Transplacental infection with BVDV may also result in retardation of fetal growth (Done and Terlecki, 1980; Dufell and Harkness, 1985).

This is manifested by the birth of weak, undersized calves, which may die shortly after birth (Baker, 1990).

The pathogenesis of this wide range of lesions is unlikely to be due to a single defect. The virus appears unrestricted in its choice of cells in which to replicate. It has, however, a preference for mitotically active cells, particularly those of the central nervous system (CNS) and lymphoid tissues (Brown *et al*., 1973; Done *et al*., 1980; Ohmann, 1988; Fernandez *et al*., 1989). Although not clear, the combination of direct cellular damage by virus and inflammatory responses to virus have been proposed as pathogenic mechanisms (Castrucci *et al*., 1990). BVDV causes significant intrauterine growth retardation in many tissues of the fetus, particularly in the CNS and the thymus (Done *et al*., 1980) and a direct cytolytic effect has been suggested for the hypoplasia in the germinal layer of the cerebellum (Brown *et al*., 1973) and other tissues (Cay *et al*., 1989). Congenital anomalies involving the CNS are most common following fetal infection with BVDV. These include cerebellar hypoplasia, microencephalopathy, hydrocephalus, hydranencephaly (Badman *et al*., 1981), and porencephaly (Hewicker-Trautwein and Trautwein, 1994). Hypomyelination of the CNS, which is often associated with thymic hypoplasia, has also been observed (Binkhorst *et al*., 1983; Anderson *et al*., 1987). At birth, calves that have cerebellar hypoplasia show extreme difficulty in becoming ambulatory. Those that can stand are ataxic, resulting in tremors, wide-based stance, and stumbling gait. The defects are usually severe enough that compensation does not occur and the calves either die or are euthanized (Baker, 1987). Fetal cerebellar effects have been seen following infection as early as the 79th day and as late as the 150th day of gestation (Brown *et al*., 1973). Severity of cerebellar lesions increase with the age of the fetus when infected (Brown *et al*., 1973).

Other teratogenic effects that have been associated with BVDV infection include cataracts (Bielefeldt-Ohmann, 1984; Wohrmann et al., 1992), microopthalmia (Kahrs *et al*., 1970; Scott *et al*., 1973; Brown *et al*., 1975), optic neuritis (Bielefeldt-Ohmann *et al*., 1984), retinal degeneration (Scott *et al*., 1973), thymic hypoplasia (Done *et al*., 1980), hypotrichosis/alopecia (Baker, 1987; Kendrick, 1971), curly hair coat (Larsson *et al*., 1991), hyena disease (Espinasse *et al*., 1986), deranged osteogenesis (Constable *et al*., 1993), mandibular brachygnathism (Scott *et al.,* 1972), and growth retardation (Baker, 1987; Done *et al*., 1980; Constable *et al*., 1993). Table 1 summarizes congenital defects associated with infection of fetus with BVDV.

Table 1. Congenital defects associated with fetal bovine viral diarrhea infection (Grooms, 2004)

Defects involving the central nervous system	Defects involving the ocular system	Other defects
Cerebellar hypoplasia	Cataracts	Thymic hypoplasia
Microencephalopathy	Microopthalmia	Hypotrichosis/alopecia
Hydrocephalus	Retinal degeneration	Deranged osteogenesis
Hydranencephaly	Optic neuritis	Mandibular brachygnathism
Porencephaly		Growth retardation
Hypomyelination		

Normal calves born seropositive to BVDV: Infection with BVDV in the later stages of gestation, after the development of immunocompetence, rarely causes congenital malformations. Calves may be normal at birth and have neutralizing antibodies to BVDV (Casaro *et al*., 1971; Muscoplat *et al*., 1973; Ohmann *et al*., 1982). However, calves congenitally infected with BVDV may be at more risk for experiencing a serious postnatal health event. In a study attempting to define the impact of congenital BVDV infections on large dairy farms, Munoz-Zanzi *et al*. (2003) showed that calves born with BVDV neutralizing titers were twice as likely to experience a severe illness during their first 10 months of life compared with calves born free of BVDV neutralizing titers.

Immunotolerance: Another outcome of fetal infection during the first trimester is the establishment of a viremia that persists for life (Kahrs, 1973; Cay *et al*., 1989). Although the exact mechanism of immunotolerance is unclear, it is generally felt that circulation of virus during the period of gestation when immunocompetence is developing (90–120 days) is a prerequisite for persistence. Viral proteins are recognized as self- antigens with resulting negative selection of BVDV specific B and T lymphocytes during their ontogeny. Persistent BVDV infection in cattle appears to arise from specific B- and T-lymphocyte immunotolerance (Coria and McClurkin, 1978; McClurkin *et al*., 1984) that results in an absence of neutralizing and nonneutralizing antibodies to the persistent virus (Donis and Dubovi, 1987). It is this immunotolerance, reflected by the lack of specific antibody to the persisting virus, that allows the virus to persist in the blood and tissues for the lifetime of the animal. These calves are born PI with BVDV and remain so for life (Bolin *et al*., 1985b, McClurkin *et al*., 1985).

Only the NCP biotype can establish a PI of the fetus if the dam is infected between approximately days 40 and 120 of its development (Bachofen *et al*., 2010). PI animals are the key transmitters of the infection since they shed virus continuously, in large amounts and in all bodily fluids (Coria and McClurkin, 1978; Meyling and Jensen, 1988; Brock *et al*., 1991; Kirkland *et al*., 1991).

Several groups have recently attributed the PI to the ability of NCP BVDV to interfere with the induction of type-I interferon (IFN) production in macrophages (Charleston *et al*., 2001; Schweizer and Peterhans, 2001; Baigent *et al*., 2002, 2004). This lack of IFN induction and the failure to induce apoptosis might be advantages for the survival of the virus (Schweizer and Peterhans, 1999, 2001).

2.3.2. Persistent infections

BVDV has the unique ability to produce PI animals when infecting fetuses in the first gestation phase before the fetus becomes immunocompetent (Ohmann, 1988; Meyling *et al*., 1990). PI is a condition in which a congenitally infected calf is immunotolerant to the infecting strain of BVDV and hence remains viremic for its life and constantly shed virus into the environment (Baker, 1990). The identity of PI animals is based on the recovery of NCP virus on successive occasions and the lack of antibody to the persisting virus (Brownlie, 1990). Immunotolerance is specific to the infecting NCP strain of BVDV, and postnatal PI animals can respond immunologically to heterologous strains of BVDV (Collins *et al*., 1999).

In the case of PI, clinical signs in PI animals can be differentiated pathologically into mucosal disease (MD) and non-MD cases (Bachofen *et al*., 2010). MD is correlated to the appearance of the CP virus that arises as a result of mutations or recombination during viral RNA replication from the persisting NCP virus (Deregt and Loewen, 1995; Neill and Ridpath, 2001; Lackner *et al*., 2004). PI, immunotolerant cattle are also at risk for development of MD if superinfection with a homologues CP strain occurs. In addition to MD, PI calves suffer from a multitude of health and performance problems (Baker, 1990). However, they may also be clinically healthy. PI cows that reach adult age can conceive (McClurkin *et al*., 1979). If so, the infection will be transmitted to the fetus, and thus, the offspring will always be PI (Baker, 1987).

Non-MD cases: The clinical signs in PI animals that have not (yet) developed MD can encompass a wide spectrum of symptoms, ranging from the apparently normal healthy animal to subclinical disorders, ill-thrift and growth retardation. In an endemic situation, clinical cases of BVDV PI animals fall roughly in two distinct categories, with lung-centred pathology occurring mainly in young animals and mucosal pathology mainly in older animals (Bachofen *et al*., 2010). Chronic or recurrent intestinal and/or pulmonary symptoms are frequently observed but occasionally dermatological, neurological or haematological disorders are the only signs of a PI (Braun *et al*., 1996; Taylor *et al*., 1997a). In many cases the clinical and necropsy findings do not allow a clear differentiation between MD and non-MD cases (Bachofen *et al*., 2010).

PI calves may have a death rate of 50% in the first year of life (Dufell and Harkness, 1985) and may be smaller at birth, have difficulty in standing and sucking, have a slower rate of growth (Bachofen *et al*., 2010), can show signs of CNS defects, such as muscular tremors, incoordination and blindness, and may die within days of birth or be culled from the herd for being a "poor doer". A curly haired coat is characteristic of some PI calves (Taylor *et al*., 1995; Taylor *et al*., 1997a, OIE, 2008). PI calves may be predisposed to infection by other microorganisms which are manifested most often as enteritis and pneumonia (Barber *et al*., 1985; Werdin *et al*., 1989; Bachofen *et al*., 2010). Immunosuppression has been reported in PI calves (Roth *et al*., 1981; Roth *et al*., 1986; Potgieter, 1988), which may account for the increased susceptibility to diseases such as enteritis and pneumonia.

There is considerable variation in the signs and pathology described for persistently viremic cattle. Their clinical appearance can range from normal to grossly abnormal (Bachofen *et al*., 2010). It is speculated that the age, size, and timing of viral challenge for the early fetus might be responsible for this variation. The pathogenesis of the grossly abnormal calf reflects the viral tropism for the CNS, lymphoid and epithelial cells. Within the CNS, the predilection sites for viral persistence are the cerebral cortex and the hippocampus (Fernandez *et al*., 1989). Lesions in such tissues are often more severe when the fetus is infected during the second trimester (Scott *et al*., 1973; Binkhorst *et al*., 1983) and account for the depression and incoordination seen in some newborn calves. Frequently these calves fail to survive and grossly abnormal brain lesions, such as cerebellar hypoplasia can be seen at post-mortem (Brown *et al*., 1973; Done *et al*., 1980).

Lesions within the lymphoid tissues, apart from the reduced size of organs, such as the thymus (Done *et al*., 1980), are not so evident. The gross changes, seen in the Peyer's patches of the small intestine during MD, are not observed (Brownlie *et al*., 1984). However, there are cellular changes that are said to account for the immunosuppression seen in persistently viremic animals. There is a reduction in the recirculating B-cells (Muscoplat *et al*., 1973) and also in T-cells (Reggiardo and Kaeberle, 1981). It has been estimated that 4.4% of blood leukocytes, 5.4% of T-cells and 2.1 % of B-cells are infected with the virus (Bolin *et al*., 1987).

Several epithelial tissues sustain BVDV replication. BVDV antigen can be demonstrated within the keratinocytes of the tongue, skin and labia (Ohmann, 1983) and this may account for the erosive oral lesions which characterize clinical disease (Brownlie, 1990).

Mucosal disease

Mucosal disease (MD) is the most dramatic form of BVDV associated clinical disease because of the severity and characteristics of lesions (Walz *et al*., 2010). MD was first reported in 1953 and described as a fatal condition of cattle, characterized by widespread lesions of the alimentary mucosal surfaces and lymphoid tissues (Ramsey and Chivers, 1953; Brodersen, 2014).

MD occurs when cattle that are immunotolerant and viremic with NCP virus, become superinfected with a CP strain of BVDV which shares close homology with the persistently infecting non-cytopathic strain (Brownlie *et al*., 1984; Bolin *et al*. 1985c; McClurkin *et al*., 1985). The CP virus may arise by a molecular event, such as mutation, from the persisting NCP virus, or as a result of recombination between the persisting NCP strain and an exogenous CP strain (Kummerer *et al*., 1998; Tautz *et al*., 1998; Becher *et al*., 2001). Mucosal disease has been described as a sequel to vaccination with modified-live virus vaccine (Liess *et al*., 1984a). These vaccines are mostly derived from CP virus strains and may be acting as the superinfecting challenge (Brownlie, 1990). The isolation of both biotypes, NCP and CP, proves a PI animal to suffer from MD (Bachofen *et al*., 2010).

MD is a sporadic form of BVDV infection in cattle generally between the age of six months and two years. The disease is characterized by severe clinical signs, low morbidity and high case fatality. Cohorts of PIs that originate from the same strain of BVDV often succumb to MD in a narrow window of time. This occurs when a PI develops a mutation of the NCP BVDV resulting

in a CP BVDV, which is then subsequently spread to PI cohorts (Walz *et al*., 2010). The course of the disease can be either acute (acute fatal MD), with a duration of 2 days to 3 weeks, or chronic (chronic or delayed/late onset MD) with animals lingering up to 18 months (Lindberg, 2003). The clinical variations of MD are attributable to the antigenic relationship between the PI NCP strain and the superinfecting CP strain (Bolin, 1995).

Chronic disease may occur as a result of superinfection of persistently viremic animals with CP isolates that have partial antigenic "homology" with the persisting virus (Brownlie *et al*., 1987a). Recombinations with heterologous CP strains and/or mutations in the exogenous CP strain have been suggested as possible reasons for development of late-onset type of MD (Ridpath and Bolin, 1995; Fritzemeier *et al*., 1997; Sentsui *et al*., 2001). If the CP strain is homologous to the NCP strain, the PI animal does not produce neutralizing antibodies to it (Lindberg, 2003).

Clinically MD cases exhibit fever (40-41°C), anorexia, drooling of saliva wetting hair around the mouth, massive mucosal erosions throughout the GI canal and profuse watery diarrhea which occurs 2-4 days after the onset of clinical illness leading to progressive wastage and death (Baker, 1990; Radostits *et al*., 2007). The feces are foul smelling and may contain mucus and variable quantities of blood. Occasionally, small tags of fibrinous intestinal casts are present. Straining at defecation is common and the perineum is usually stained and smeared with feces (Radostits *et al*., 2007).

Lesions of the oral cavity mucosa consist of discrete, shallow erosions which become confluent, resulting in large areas of necrotic epithelium becoming separated from the mucosa. These erosions occur: inside the lips, on the gums and dental pad, on the posterior part of the hard palate, at the commissures of the mouth and on the tongue. The entire oral cavity may have a 'cooked' appearance with the grayish colored necrotic epithelium covering the deep-pink, raw base. Similar lesions occur on the muzzle and may become confluent and covered with scabs and debris. Although the oral lesions are significant in the identification of the disease, they may be absent or difficult to see in up to 20% of the affected animals (Radostits *et al*., 2007).

There is usually a mucopurulent nasal discharge associated with some minor erosions on the external nares and similar lesions in the pharynx. Lacrimation and corneal edema are sometimes observed. Lameness occurs in some animals and appears to be due to laminitis, coronitis and

erosive lesions of the skin of the interdigital cleft, which commonly affect all four feet (Radostits *et al*., 2007).

In chronic cases, the animals show similar symptoms, but in a more protracted form. Also, apart from GI symptoms like intermittent diarrhea and chronic bloat, dermatological symptoms like erosive lesions on the skin and laminitis may develop (Lindberg, 2003). There may be intermittent bouts of diarrhea, inappetence, progressive emaciation, rough dry hair coat, chronic bloat, hoof deformities and chronic erosions in the oral cavity and on the skin. Shallow erosive lesions covered with scabs can be found on the perineum, around the scrotum, preputial orifice and vulva, between the legs and at the skin-horn junction around the dewclaws, in the interdigital cleft and at the heels, and there may be extensive scurfiness of the skin. The failure of these skin lesions to heal is an important clinical finding suggesting chronic MD. Chronic cases will sometimes survive for up to 18 months during which time they are unthrifty and ultimately die from chronic inanition (Radostits *et al*., 2007).

The pathology of MD is characterized particularly by the erosive mucosal lesions and destruction of the lymphoid tissue in the GI tract (Jubb *et al*. 1985; Liebler *et al*., 1995). It has been demonstrated that the CP biotype has a particular tropism for the gut-associated lymphoid tissue (Clarke *et al*., 1987) and that this biotype rapidly homes to the Peyer's patch tissue (Brownlie *et al*., 1988). Although, the reason for the gross lesions visible over the Peyer's patches is likely to be a result of the direct lytic action of the virus, there is preliminary evidence that a synergism between the two biotypes is required for the full expression of MD (Clarke *et al*., 1989).

The lesion that develops after the destruction of the lymphoid tissue in the Peyer's patches and the collapse of its overlying intestinal mucosa is the prominent lesion in MD. This gives the characteristic erosions along the small intestine. In studies on the sequential development of MD it was evident that these Peyer's patch lesions occurred in the course of superinfection with CP virus and coincided with clinical disease (Brownlie *et al*., 1988). However, there are animals that die without clinical diarrhea. This would suggest that the diarrhea and any resulting dehydration was not an essential part of the syndrome. The cause of death is not fully understood (Brownlie, 1990).

2.3.3. Herd level manifestations of BVDV infection

At the herd level, BVDV infection typically results in an increased incidence of reproductive disorders and in impaired calf health (Houe and Meyling, 1991; Larsson *et al*., 1994; Moerman *et al*., 1994; Fray *et al*., 2000). Examples of reproduction parameters in which the effect of BVDV infection has been shown are conception rates, pregnancy rates, abortion rates and time to first calving (Houe *et al*., 1993; McGown *et al*., 1993; Rufenacht *et al*., 2001; Valle *et al*., 2001). Venereal infection in a herd is characterized by increased incidence of repeat breeding (Baker, 1990). Increases in the incidence of treatments of retained placenta and silent heat have also been reported, as well as an increase in the risk of infectious diseases in adult animals, such as mastitis (Niskanen *et al*., 1995). Also, a negative association between milk yield and BVDV infection has been reported (Barber *et al*., 1985; Moerman *et al*., 1994; Lindberg and Emanuelson, 1997). In herds relying on the production of animals for slaughter, important causes of production losses include growth retardation in sick non-PI and PI animals and increase in the prevalence of infectious diseases subsequent to immunosuppression (Kelling *et al*., 1990; Klingenberg *et al*., 1999; Taylor and Rodwell, 2001). In feedlot cattle BVDV was associated with increased incidence of respiratory disease (Martin and Bohac, 1986).

2.4. Diagnosis

2.4.1. Samples for BVDV diagnosis

The types of samples collected for diagnosis of BVDV differ according to the type of infection (acute versus persistent), the intended laboratory test/procedure and whether the sample is collected from live or dead animal (ante-mortem versus postmortem).

The samples collected for virus isolation (VI) from live animals include, whole blood in ethylene diamine-tetraacetic acid (EDTA), citrate or heparin (for Buffy coat or to be used as it is), serum, swab (ocular, nasal, tracheal, rectal), milk (bulk milk for herd diagnosis), skin biopsy (usually ear notch sample) and semen (Hamel *et al*., 1995; Carman *et al*., 1998; Fulton *et al*., 2003; OIE, 2008; VanderLey *et al*., 2011). If the sampling is done at necropsy lymph nodes, kidneys, liver, lung, thymus, spleen and intestinal mucosal scrapings (Hamel *et al*., 1995; Belknap *et al*., 2000) can be collected for VI. BVDV has also been isolated from umbilicus of fetus (Belknap *et al*., 2000).

Skin biopsies (usually ear notches) are commonly used for diagnosis of PI in live animals using immunohistochemistry (IHC) (Clarke *et al*., 1987; Njaa *et al*., 2000; VanderLey *et al*., 2011). For diagnosis of PI cattle postmortem, almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. However BVDV has been detected with IHC from other tissues including cardiocytes, epithelium of kidney and lung, lamina propria and tunica muscularis of small intestine, epithelium of oral cavity, histocytes and epithelium of thymus, retina and vascular smooth muscle of heart, kidney and lung. Sparse staining has been seen in brain and spinal cord (Haines *et al*., 1992; Njaa *et al*., 2000). IHC can also be done on Buffy coat collected from peripheral blood sample collected in EDTA (Baszler *et al*., 1995).

For detection of BVDV antigens using antigen capture ELISA (ACE) whole blood collected on EDTA (to be used as it is or for collection of Buffy coat or peripheral blood leukocyte lysate), plasma, serum (Kampa *et al*., 2007; OIE, 2008) and skin biopsies (usually ear notches) (OIE, 2008; Amin *et al*., 2014) may be used. Skin biopsies and nasal swab samples were found reliable samples for detection of PI infection as compared to serum, and vaginal/preputial, conjunctival, rectal and oral swabs (VanderLey *et al*., 2011). Other tissue samples may be examined with ACE if the diagnosis is made postmortem (Kampa *et al*., 2007).

Samples used for serology (antibody detection) are usually sera; but bulk milk samples may be used for serological detection of BVDV in a herd (Kampa *et al*., 2007; OIE, 2008; Raizman *et al*., 2011).

Blood samples collected on EDTA (for separation of Buffy coat), serum, swab samples, somatic cells within bulk milk, and almost any tissue during postmortem may be collected for detection of BVDV genome (RNA) using reverse transcriptase polymerase chain reaction (RT-PCR) (OIE, 2008; Amin *et al*., 2014).

2.4.2. Virus isolation

Virus isolation (VI) in cell cultures followed by fluorescent antibody (FA) staining with BVDV-specific antibody is the standard method of detection of BVDV-infected cattle (Saliki *et al*., 1997). It is regarded as the reference test for virological diagnosis of BVDV and is a good indicator of the presence of live and infectious virus (Brock, 1995).

Continuous cell lines usually Madin-Darby bovine kidney (MDBK) cells and several primary and secondary cell cultures of bovine turbinate, calf testis epithelial, kidney, lung, spleen, fetal muscle, esophageal, embryonic tracheal and goat synovial membrane cells have been used for isolation of BVDV (Ellis *et al*., 1995; Fray *et al*., 2002; Fulton *et al*., 2003; Lindberg, 2003; Nagai *et al*., 2004; Carman *et al*., 2005; OIE, 2008; Bachofen *et al*., 2013; Amin *et al*., 2014; Jenckel *et al*., 2014).

There are many variations of procedure in use for VI. All should be optimized to give maximum sensitivity of detection of a standard virus preparation. This may include one or more *in-vitro* passage(s). Conventional methods for VI are used, with the addition of a final immune-labeling step (fluorescence or enzymatic) to detect growth of NCP virus (OIE, 2008).

To identify PI animals, VI on primary bovine cells, followed by immuno-enzyme staining is regarded as the "gold standard" method (Kampa *et al*., 2007). VI, although highly specific, is time consuming, expensive, and suffers from suboptimal sensitivity due primarily to the autolyzed condition in which many specimens are submitted for diagnosis (Ellis *et al*., 1995). There have been significant numbers of false negative results with the VI method (Ellis *et al*., 1995). Moreover, standard VI is laborious and not practically applicable to large numbers of samples (Saliki *et al*., 1997). FA staining is required to identify NCP strains, which constitute about 90% of BVDV field isolates (Dubovi, 1992).

Buffy coat cells, whole blood, washed leukocytes, serum or skin biopsies are suitable for isolation of the virus from live animals, while various tissues can be used from post-mortem cases (OIE, 2008). To confirm a diagnosis of PI, animals should be retested after an interval of at least 3 weeks (OIE, 2008).

Samples for VI need to be processed appropriately. Tissue samples are homogenized in minimum essential medium which may contain antibiotics and fungicides, centrifuged and the supernatant inoculated onto appropriate cell culture (Baszler *et al*., 1995; Ellis *et al*., 1995; Evermann and Ridpath, 2002). In order to get leukocytes for inoculation, blood may be centrifuged (with or without mixing with phosphate buffered saline (PBS)), the buffy coat separated and washed with PBS before inoculation on cell culture (Corapi *et al*., 1989; Polak and Zmudzinski, 1999). Swabs are eluted in the aforementioned medium and inoculated onto appropriate cell culture at an appropriate dilution (Evermann and Ridpath, 2002).

Studies reported use of highly variable incubation time for inoculated cells. Some studies used 3 to 7 days incubation with daily microscopic examination for cytopathogenic effect (Corapi *et al*., 1989; Ellis *et al*., 1995; Hamel *et al*., 1995). Others used 2 to 7 days incubation with up to 3 blind passages incubated for a regular interval with daily examination for cytopathic effect (Baszler *et al*., 1995; Polak and Zmudzinski, 1999; Evermann and Ridpath, 2002; Fray *et al*., 2002).

The presence of BVDV in cell cultures is detected usually either by direct or indirect immunofluorescent staining (Vickers and Minocha, 1990; Kabongo and Vanvuuren, 2004). Inoculated cell cultures, at the end of incubation or earlier when cytopathic effect is observed, are processed and examined with fluorescent antibody technique (usually indirect fluorescent antibody technique) to detect presence of the virus. Both monoclonal and polyclonal antibodies have been used as primary antibodies, although use of the latter is more common. Fluorescein isothiocyanate (FITC) conjugated anti-IgG antibodies specific to the species of animal in which the primary antibodies were produced is used as secondary antibody (Corapi *et al*., 1989; Hamel *et al*., 1995; Polak and Zmudzinski, 1999; Evermann and Ridpath, 2002; Fray *et al*., 2002). Cytoplasm fluorescence is observed using an ultra violet (UV) microscope (Ellis *et al*., 1995; Polak and Zmudzinski, 1999).

2.4.3. Antigen detection

Antigens of BVDV can be detected in various samples using immunofluorescence (fluorescent antibody test), immunohistochemistry (IHC) and antigen capture ELISA (ACE) (Hewicker-Trautwein *et al*., 1990; Vickers *et al*., 1990; OIE, 2008).

Immunofluorescence

Immunofluorescence (IF) method is a specialized type of IHC, which uses a florescent dye to visualize antibody binding under a florescent microscopy. The commonly used florescent dyes include florescein isothiocyanate (FITC), tetraethylrhodamine isothiocyanate (TRITC) or Rhodamine. There are two major types of IF staining methods: Direct method and indirect method.

Both direct and indirect fluorescent antibody tests can be used to detect presence of BVDV antigens in tissues (Vickers *et al*., 1990; Kabongo and Vanvuuren, 2004) and cultured cells. The

procedure can be conducted on formalin-fixed, paraffin-embedded tissue sections or cryosections (Ellis *et al*., 1995). The tissues are affixed to microscope slides and processed appropriately. Fluorescent staining is performed using polyclonal or monoclonal primary antibodies (Vickers *et al*., 1990; Ellis *et al*., 1995). Secondary antibodies in case of indirect fluorescent antibody test are fluorescein-conjugated anti-IgG antibodies specific to the species in which the primary antibodies were raised. For the direct method, slides are stained for the presence of BVDV antigen using fluorescein-labelled BVDV specific antibody (Kabongo and Vanvuuren, 2004). The stained tissues are examined using an ultraviolet microscope (Ellis *et al*., 1995).

Significant numbers of both false negatives and false positives have been reported for BVDV infection with the immunofluorescence method (Ellis *et al*., 1995).

Immunohistochemistry (IHC)

IHC is a method for demonstrating the presence and localization of antigens (usually proteins) in tissue sections and cells by the use of an antibody with specificity for an antigen. These are subsequently visualized by a marker such as an enzyme forming a color precipitate (such as 3', 3'-diaminobenzidine (DAB)). IHC staining is now a main ancillary technique used in the clinical disease diagnosis, especially in paraffin embedded tissues (Makki, 2016).

IHC is a method for detection of intracellular viral antigen and is the test of choice for demonstration of virus in tissues (Hewicker-Trautwein *et al*., 1990; Haines *et al*., 1992), particularly where suitable monoclonal antibodies (mAbs) are available. Using ear notches as specimen, this method can be used to screen for PI animals (Njaa *et al*., 2000). Many diagnostic laboratories rely on IHC assays as a primary means of screening calves for PI status (Cornish *et al*., 2005).

For PI animals almost any tissue can be used. Skin biopsy and buffy coat from peripheral blood collected in EDTA anticoagulant are appropriate samples from live animal. In the case of postmortem diagnosis several tissues may serve as a sample (heart, kidney, lung, small intestine, skin, lymph nodes, thyroid gland, thymus, epithelium of oral cavity, stomach, placenta, retina) (Carman *et al*., 2005).

The appropriately processed sample is mounted on a microscope glass slide. The primary antibody (anti-BVDV mAb or pAb) is placed on the slide and allowed to react (incubated). After

washing the slide in PBS, the secondary antibody/antiserum, which is species specific anti IgG (against the species in which the primary antibodies were raised) conjugated to an enzyme, (e.g. horseradish peroxidase (HRP) or alkalin phosphatase (AP)) is applied on the tissue section and allowed to react. Then after washing the slide in PBS a substrate (containing a chromogen) such as diaminobenzidine tetra hydrochloride or Nova red for peroxidase and fast-red for AP is added and allowed to react. Finally the slide is washed in PBS, counterstained (usually with Mayer's hematoxyline) and examined under light microscope. Sections are considered positive if specific intracytoplasmic stainings are observed (Haines *et al*., 1992; Ellis *et al*., 1995; Carman *et al*., 2005; Cornish *et al*., 2005).

Antigen capture ELISA (ACE)

Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published (Entrican *et al*., 1995) and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Both monoclonal- and polyclonal-based systems are described (OIE, 2008).

ACE is used to detect BVDV antigen in tissue, blood, serum, plasma and peripheral blood leukocyte lysate samples (OIE, 2008). It has been successfully used to identify PI animals in control programmes (Kampa *et al*., 2007). It might not be suitable for diagnosis of acute BVDV infection as viremia is transient in these animals (OIE, 2008). It is economical, rapid, specific, sensitive, independent of cell culture facilities and convenient to test large number of samples (Lindberg, 2003). It is comparable to IHC in terms of sensitivity and specificity (Cornish *et al*., 2005; Kampa *et al*., 2007).

The most commonly used antigen capture ELISA are developed using monoclonal antibodies against E^{rns} and NS2-3 a structural and nonstructural proteins of the BVD virus, respectively (OIE, 2008). The E^{rns} is secreted from infected cells during virus replication and can be detected directly in serum which allows user friendly and high throughput testing (Brownlie *et al*., 2000; Kuhne *et al*., 2005) as compared to previous forms which rely on extraction of viral antigen from the buffy coat of whole blood samples (Lindberg, 2003). E^{rns} ELISA is comparable in performance with IHC, but with several advantages compared to it (Kampa *et al*., 2007). The NS2-3-capture ELISA detects BVDV in leukocytes and tissue samples using specific affinity

mAb against the NS2-3 protein, and has been successfully used to identify PI animals in BVDV control programmes in some countries (Sandvik and Krogsrud, 1995; Synge *et al*., 1999).

2.4.4. Serology/ Antibody detection

Antibody to BVDV can be detected in cattle sera by a standard virus neutralization (VN) test or by ELISA, using one of several published methods (Howard *et al*., 1985; Katz and Hanson, 1987; Edwards, 1990; Paton *et al*., 1991). Control positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid (OIE, 2008).

Virus neutralization

Virus neutralization (VN) test is the gold standard for antibody detection (Edwards, 1990). It is sensitive and specific (Lindberg, 2003).

Most laboratories use highly cytopathogenic, laboratory-adapted strains of BVDV for VN tests, because it makes the test easier to read, although immune-labelling techniques, that allow simple detection of the growth or neutralization of noncytopathogenic strains, are now available. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle population. Two widely used CP strains are ‘Oregon C24V’ and ‘NADL’. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralization test that uses type 1 strain of the virus, and vice versa (Fulton *et al*., 1997). It is important that BVDV-1 and BVDV- 2 be used in the test (OIE, 2008).

The test serum is serially diluted in two-fold (usually in the range of 1:2 to 1:152) in cell culture media or PBS, and a specified titer of the CP BVDV virus (usually NADL) is added to it. Each dilution is prepared in duplicates and inoculated into appropriate cell culture. The inoculated culture is incubated under favorable condition for specified period (usually 3 to 5 days). Finally the monolayer cells are microscopically examined for CPE. The virus neutralizing antibody titer is calculated from the reciprocal of the serum dilution that neutralized the CP BVDV (Polak and Zmudzinski, 1999; Fray *et al*., 2002; Raizman *et al*., 2011). Results are expressed as the reciprocal of the serum dilution at which 50% neutralization of virus will occur (Raizman *et al*., 2011).

Although VN is sensitive and specific it is cell culture dependent and labor demanding as compared to the ELISAs. Therefore, the latter are regularly used when a large sample throughput is required (Lindberg, 2003).

ELISA

ELISAs are also used for BVDV antibody detection. There are two principal types: indirect and competitive. In indirect ELISAs, antibodies are trapped by immobilized antigen, and detected using enzyme-conjugated species-specific antiglobulins and a chromogenic substrate. The optical density (OD) is then measured, which will be higher in a positive sample than in a negative. In competitive ELISAs (also called blocking ELISAs), virus-specific antibodies in the sample block the binding of conjugated virus-specific antibodies to fixed viral antigen. In contrast to the indirect ELISA, a positive sample in a blocking ELISA will yield a weaker signal than a negative sample (Lindberg, 2003).

To estimate the prevalence of BVDV antibodies, ELISA is the most frequently used diagnostic technique in serum and/or milk samples (Beaudeau *et al*., 2001; Eiras *et al*., 2012). Detection of antibodies from bulk tank milk (BTM) is considered an inexpensive and reliable alternative for monitoring the disease that in turn can be applied for control strategies within dairy herds, as the test can provide information about the status of a large group of animals (lactating cows) or individual milk samples (Beaudeau *et al*., 2001; Stahl *et al*., 2002). Commercial ELISA tests currently used in BTM have almost equal sensitivity (81%) and specificity (91%) compared to the ones applied in serum and have the advantage that the sampling method is less invasive, faster and easier to perform in large herds (Thobokwe *et al*., 2004).

2.4.5. Molecular diagnosis

RT-PCR

Molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) are used for detection of infection or phylogenic characterization of the infecting BVDV (Hamel *et al*., 1995; Fulton *et al*., 2003).

Detection of RNA by RT-PCR includes four different steps; extraction of RNA, reverse transcription to cDNA, primer-directed amplification and lastly detection of amplified products

(McGoldrick *et al.,* 1999). Primers should be selected in conserved regions of the genome (OIE, 2008). The 5′-UTR provides meaningful phylogenetic inferences (Vilcek *et al.,* 2001) as this region has the highest degree of sequence conservation and is efficiently amplified by RT-PCR. It is also the most frequently analyzed portion of the genome.

RNA for detection with PCR can be extracted from buffy coat, whole blood, serum, other tissue samples or milk (Hamel *et al*., 1995; Choi and Song, 2010; Walz et al., 2010; Emran *et al*., 2014).

Molecular techniques have the advantage of being highly sensitive having a special value where low-level virus contamination is suspected (Harasawa, 1995) whereas insensitive to toxic substances in the specimen and to the presence of interfering antibodies. The RT-PCR technique is sensitive enough to enable the detection of PI lactating cows in a herd of up to 100 animals or more, by testing the somatic cells within bulk milk (Radwan *et al*., 1995; Drew *et al*. 1999). However they are extremely sensitive to sample contamination which can lead to false positive results (Belak and Ballagi-Pordany, 1993). The development of closed analysis systems, where BVDV nucleic acid amplification and detection is made in the same tube, has reduced this problem (Mahlum *et al*., 2002).

Today, quantitative (real-time PCR) and multiplex assays (multiplex PCR) have been developed where virus can be quantified and where both genotypes, or additional viral agents can be assayed within the same sample (Gilbert *et al*., 1999; Onodera *et al*., 2002). A multiplex PCR can be used to amplify and type virus from cell culture, or direct from blood samples, by producing different sized PCR products (Gilbert *et al*., 1999). Real-time RT-PCR incorporate the use of DNA fluorescently labeled probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate pestiviruses in real time (McGoldrick *et al*., 1999).

For phylogenetic analysis, BVDV viruses are usually typed based on comparison of genomic sequences from the 5′- untranslated region (5′-UTR), N^{pro}, E2 and NS2-3 genomic regions (Becher *et al*., 1999; Vilcek *et al*., 2001; Vilcek *et al*., 2005; Flores *et al* 2002).

As both PI and TI animals are viraemic, it is necessary to perform two antigen tests at an interval of at least three weeks in order to distinguish PI from TI animals (Larson *et al*., 2005).

2.5. Treatment, Control and Prevention

There is no specific treatment for any of the diseases associated with the BVDV (Radostits *et al*., 2007). Therapy for acute BVDV infection is non-specific. Depending on the severity of disease, hydration should be maintained with oral or IV fluids and electrolytes. Antimicrobial therapy may be considered in the presence of or for the prevention of secondary bacterial infections. Because the virus can cause immunosuppression, treatment with corticosteroids would be contraindicated but non-steroidal anti-inflammatories would be a therapeutic consideration (Baker, 1990).

Due to variations in the losses that BVDV infections induce in the cattle industry and the differences in risk factors in different areas, decisions on selection of control strategies should always rely on thorough epidemiological investigations in the same areas as in which the programme is going to be applied (Houe, 2003).

Strategy for the control of BVDV in a herd depends on its BVDV infection status. Where the herd is free from BVDV, measures which prevent introduction of the virus (biosecurity measures) should be instituted; in an already infected herd efforts aimed at reducing the impact of the disease and exposure of animals to the virus should be made. Therefore, for a regional or national control programme, initial serological herd tests such as bulk milk tests and spot tests (sample of animals in a certain age) are used to determine the herd's BVDV status. Non-infected herds are monitored and certified as being free by repeated sampling, and infected herds undergo a virus elimination programme aimed at removing PI animals (Lindberg, 2003; Radostits *et al*., 2007). It must be remembered, however, that control measures that are only used at the individual herd levels are of limited usefulness, as control measures on one farm may be foiled by lack of control on neighboring farms. A herd not eliminating infected animals will give a higher risk for infection of any neighbors that have performed an elimination strategy. Therefore control strategies instituted on a regional or national level rather than the herd level may be more effective (Houe, 2003).

The ultimate goal of BVDV prevention and control measures is to eliminate the potential for the birth of calves, PI with the virus (Brock, 2004). Identifying and eliminating PIs should be a major focus when attempting to control and prevent BVDV as these animals are the major source of infection that would result in the birth of PI calves. By eliminating PIs, the major source of

virus capable of causing TI in pregnant dams and subsequent fetal infections is eliminated (Grooms 2004). Therefore, when developing a successful BVDV prevention and control program, 3 aspects should be considered: (1) identification and elimination of PI animals, (2) vaccination/immunization programs and biocontainment (protocols to minimize the negative impact of infection), and (3) implementing biosecurity measures to prevent BVDV exposure of susceptible cattle (Radostits *et al*., 2007). Each of these three principles has been applied to BVDV control and greater success can be expected when used simultaneously in BVDV control programs (Brock, 2003; Lindberg, 2003). Several European countries have successful eradication programs (Grom and Barlic-Maganja, 1999; Greiser-Wilke *et al.*, 2003; Sandvik, 2004),

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, 2003).

2.5.1. Identification and elimination of PI animals

Cattle PI with BVDV are the primary source of virus spread within and between farms (Grooms, 2004), although TI animals can, to a lesser extent, also serve as a reservoir (Walz *et al*., 2010). For BVDV to establish in a herd there should be contact between PI animals and susceptible pregnant cows at early stage of gestation. Therefore by eliminating PIs, the major source of virus capable of causing TI in pregnant dams and subsequent fetal infections, which may result in PI calves, is eliminated (Grooms, 2004) which would help to control the spread of the virus and ultimately eliminate it from the herd (Radostits *et al*., 2007). Removing PIs from the population removes the source of infection and reduces the disease reproduction rate to the point that the virus cannot survive and the disease is controlled (Segura-Correa *et al*., 2010).

Herd monitoring for PI animals can be done with pooled whole blood or serum samples for PCR testing. By pooling samples, the expense of screening herds with a low prevalence of PI animals is minimized. The PCR test is ideal for pooled sample testing for PI animals because it is sensitive enough to detect minute amounts of virus. A single PI animal can be detected in pools of 200 to 250 negative samples. If the initial pool is PCR-positive, it must be split and retested to differentiate viremic and non-viremic animals. Once the viremic animals are identified, they

must be classified as TI or PI with either a subsequent PCR, virus isolation, or using the IHC testing of a skin biopsy sample in three weeks (Larson *et al*., 2004).

Following the successful detection and removal of PI animals, 'self-clearance' or elimination of all evidence of the infection from the herd will occur (Radostits *et al*., 2007). While programmes to eradicate BVDV are primarily based on detection and removal of PI animals (Lindberg and Alenius, 1999), the focus on bovines alone may be problematic because BVDV is known to also infect other wild and domestic species of the artiodactyla, which may potentially play a role in the epidemiology of the infection (Doyle and Heuschele, 1983; Van Campen *et al*., 2001).

2.5.2. Prevention of introduction of infection into herd (biosecurity)

In a consensus statements of the American College of Veterinary Internal Medicine Walz *et al*. (2010) made recommendations as to the biosecurity measures to be taken to prevent introduction of the virus into a herd. Accordingly, all purchased cattle should be isolated and tested for PI status before entry into the herd. Isolation of new additions for 3 weeks before entry into the resident herd should prevent transmission of BVDV from acutely infected animals. Most lapses in herd biosecurity involve purchasing PI cattle or purchasing pregnant cattle with unknown BVDV status of the fetus. Purchased pregnant cattle should be isolated and their offspring tested to ensure that they are free of BVDV. Semen should only be used from bulls that have been tested for BVDV infection. For purebred herds marketing valuable embryos and livestock, testing of embryo transplantation recipients for PI status is essential. Exposure of cattle to other ruminants at exhibitions should be limited, and animals should be quarantined for 3 weeks before reentry into the breeding herd. Further biosecurity principles include elimination of fence-line contact with neighboring livestock and sanitation of equipment and people entering the farm (Walz *et al*., 2010).

In many cases introductions can be guaranteed, as far as is reasonably possible, to be free of infection by selecting animals which have convincing titers of serum antibody or are negative and are derived from a totally negative herd. In other cases, antibody negative introductions should be examined for virus or held for a period of on-property quarantine in close contact with a few serologically negative test animals which are subsequently examined for antibody (Radostits *et al*., 2007).

Cattle producers purchasing pregnant heifers to expand their herds must be aware of the possibility their fetuses may already be PI. At that stage there is no simple test which will identify those heifers which are pregnant with a PI fetus. Calves from these purchased heifers of unknown vaccination history should be considered infected until proven otherwise (Radostits *et al*., 2007). Imported livestock should be subject to tests for BVDV and antibodies in the herd of origin and importation of virus positive or pregnant antibody positive animals should not be allowed (Lindberg and Alenius, 1999).

Infected semen from PI bulls can be avoided by testing bulls twice for virus in blood before entering the AI centers. In addition, bulls should undergo a quarantine period before entering an AI center in order to overcome the acute phase of the infection and no longer to shed virus. However, an acutely infected bull may still pose a threat as it can escape both virus and antibody detection tests, enter the quarantine facilities and initiate an acute infection among other bulls which may still shed the virus when released from quarantine. In addition, virus may continue to be shed in semen after the end of the viremia (Kirkland *et al*., 1991). Therefore, regular testing of bulls at AI centers for seroconversions and for virus in semen may also be essential (Houe, 1999).

Transmission via embryo transfer is possible, but can be avoided using recommended washing procedures, even when embryos are transferred from PI donor cows (Wentink *et al*., 1991; Bak *et al*., 1992; Brock *et al*., 1997). Attention should be paid to the use of fetal calf serum used for embryo transfer as it may be contaminated with BVDV (Houe, 1999).

2.5.3. Immunization programs and bioconfinement

Vaccination

The most important subpopulation to protect from exposure to BVDV is pregnant cattle, especially those in early gestation as these may give birth to PI calves which would perpetuate the virus in the herd and also may result in reproductive loss (Radostits *et al*., 2007). There are two ways to protect heifers/naïve cows from reproductive failure: 1. Exposure to natural infection. This produces a strong, long-lasting immunity (Potgeiter, 1995). 2. Vaccination. This offers the advantage of controlled, safe and strategic protection against BVDV (Galletti, 2007).

To be effective, vaccination against BVDV infection should protect against viremia, block infection of target cells of the reproductive and lymphatic systems to avoid occurrence of fetal infection and immunosuppression (Kelling, 2004). However, the main objective of vaccination against BVDV is to prevent transplacental infection and thus the establishment of new PI (Van Oirschot, 2001). An important strategy for successful control is vaccination of the breeding female at least several weeks before breeding (Smith and Grotelueschen, 2004). The aim of a vaccination program is therefore to ensure that all breeding females have antibodies to the virus before they become pregnant. It is important to emphasize that vaccination should be done at least 3 weeks before breeding so that the breeding females become seropositive to the virus before conception. This is necessary regardless of the type of vaccine used (Radostits *et al*., 2007).

Vaccines are an important component to BVDV prevention, and their effectiveness has been to limit transmission and clinical disease rather than completely prevent infections with BVDV, as has been demonstrated in experimental and field studies using either inactivated or modified-live BVDV vaccines (Carman *et al*., 1998; van Oirschot *et al*., 1999). Vaccination against BVDV should protect against viremia and prevent dissemination of virus throughout the host, including preventing infection of the reproductive tract and fetus. The focus for vaccine efficacy has shifted from protection against clinical disease to protection against fetal infection. Protection against fetal infections after BVDV vaccination varies, being influenced by use of inactivated or modified-live vaccine, the timing of challenge, and the degree of homology between vaccine and challenge strains. Fetal protection is superior when animals are challenged with strains from the same genotype (Walz *et al*., 2010).

In regions where BVDV is endemic and re-exposure is likely it remains prudent to continue vaccination after eliminating the virus from the herd (Smith and Grotelueschen, 2004). Vaccination and test-and-cull approaches for BVDV eradication are, therefore, not mutually exclusive, as long as safe (killed) vaccines are used and it is ensured that the herd's BVDV status can be monitored. However, the message has to be recognized that biosecurity is the first line defense and that vaccination is back-up protection. Also in schemes based entirely on test-and-cull, vaccination could be a helpful tool to break the vicious circle in infected herds. However, it should be regarded as therapy - a time limited measure - and not as prophylaxis (Lindberg, 2003).

An important issue with the use of BVDV vaccination is the ability of immune response stimulated by one virus strain to crossprotect against heterologous BVDV strains and prevent fetal infection. Several field studies suggest that immunologic protection against heterologous BVDV challenge may be incomplete with respect to fetal protection (Kelling *et al*., 1990; Bolin *et al*., 1991; Van Campen *et al*., 2000). Results of vaccine fetal protection studies have been mixed and are often dependent on the challenge model. Therefore, it is not known if variations among subgenotypes is significant enough to impact detection or the protection afforded by vaccination. Some other studies suggest clinical significance of antigenic differences as was evidenced by the failure of vaccines and diagnostics based on BVDV-1strains to control and detect, respectively, BVDV-2 strains (Deregt, 2005). The recognition of genomic and, particularly, antigenic difference between the 2 BVDV species led to the redesign of vaccines and diagnostics (Ridpath, 2005). Considering the implications of the genomic diversity in the diagnosis, epidemiology and control of BVDV infections it is possible that regional control programs would be improved by taking into consideration the BVDV sub/genotypes present in a particular region in the design of diagnostics and vaccines (Baule *et al*., 1997; Grooms, 2004; Ridpath et al., 2010).

It is difficult to eradicate BVDV by using vaccines alone, possibly due to (i) removal of PI animals must be completed before vaccination, because vaccination as a successful stand-alone strategy to eradicate BVDV had never been reported in the literature, (ii) a critical vaccination coverage rate should be reached to prevent new PIs, (iii) farmers using vaccines often neglect to maintain or implement biosecurity measures, (iv) farmers use vaccines incorrectly, e.g., by applying vaccination after insemination, (v) in general, vaccines are not proven to be fully efficient in the prevention of in-utero transmission of the virus, and (vi) the risk of live BVDV vaccine becoming contaminated with other viruses (Scharnböck *et al*., 2018).

BVDV vaccines

Vaccines available for BVDV include modified live-virus (MLV) vaccines and inactivated-virus (killed) vaccines. The MLV vaccines usually contain a single strain of attenuated CP BVDV. Several inactivated vaccines contain both CP and NCP strains of the virus (Radostits *et al*., 2007).

Modified live vaccines contain a live but attenuated strain of the virus, and generally give a better immunological response than killed vaccines. The latter consist of virus that has been inactivated, together with an immuno-stimulating additive- an adjuvant (Van Oirschot, 2001). Modified live vaccines are capable of producing transplacental infections in pregnant animals and MD in PI cattle, if they are used incorrectly. They have also been shown to have the same immunosuppressive properties as wild strains (Roth and Kaeberle, 1983). It should also be noted that MLV vaccines when administered to susceptible pregnant cows can cause fetal infections with the development of congenital defects (Baker, 1990). Killed vaccines are safer to use, but require repeated dosing in order to provide adequate protection (Lindberg, 2003). An inactivated vaccine provides fetal protection in dams vaccinated twice before AI (Brownlie *et al*., 1995).

BVDV is a virus that exhibits substantial variation and although the main antigenic epitopes are known, it is difficult to produce vaccines that are able to prevent infection with heterologous genotypes and subtypes within these (Hamers *et al*., 2001). Also, it has not been possible to satisfy the needs for broad and high degree of protection with an ability to differentiate between natural infection and vaccination (Van Oirschot, 1999).

BVDV-1 vaccine strains have been historically contained only BVDV-1a and BVDV-1b isolates. The question arises as to whether these vaccines can protect against infection with all of the highly diverse BVDV-1 isolates. Preliminary observations has suggested that cells infected with a virus strain belonging to a particular BVDV-1 subgenotype were incompletely recognized by antibodies prepared even against a strain of the same subtype (Vilcek *et al*., 2001), confirming antigenic differences (Vilcek *et al*., 2005).

There is a tendency to move towards the development of non-replicating vaccines (similar to classical killed vaccines), because of safety issues. New types of non-replicating vaccines are e.g. subunit vaccines, recombinant subunit vaccines, peptide vaccines, DNA vaccines and some vector vaccines (Lindberg, 2003).

2.6. Economic Effects

BVD causes economic losses through, reduction in milk production, reproductive losses, unthrifty calves, susceptibility to other infections, growth retardation, early culling, increased mortality among young stock, respiratory disorders, death among animals acquiring acute

infection and death from MD (Houe, 2003; Lindberg, 2003). In addition to production losses economic losses due to BVDV include expenditure for treatment of sick animals (Fourichon *et al*., 2005).

Losses associated with reproductive failure are generally considered to be the most important economically (Anderson *et al*., 1990; Baker, 1990; Ellis *et al*., 1995; Carman *et al*., 1998; Al-Afaleq *et al*., 2007), and occur due to reduced conception rate, embryonic mortality, abortion, congenital defects, stillbirths and birth of weak calves which die immediately postpartum (Houe, 2003; Lindberg, 2003; Radostits *et al*., 2007).

Important factors that affect the magnitude of the losses are the initial herd immunity, the number of animals in different stages of gestation at the time of the infection and the virulence of the virus (Lindberg, 2003). Introduction of the infection into a totally susceptible population invariably causes extensive losses until a state of equilibrium is reached (Houe, 1999). New infections in naive herds can be associated with extreme, but transient, reproductive losses. As a large proportion of the adult animals become immunized the losses will change in nature, from mainly reproductive losses to losses due to impaired calf health (Lindberg, 2003). Large losses due to fetal infection occur during the first 2-3 years following introduction of infection to a susceptible herd (Houe, 1999). Infection with highly virulent BVDV strains causing severe clinical signs and death after acute infection gives rise to substantial economical losses (Houe, 1999). Economic losses in an acute severe BVD outbreak in severely affected dairy herds in Ontario Canada were estimated at USD 40,000-100,000 per herd due to lost animals, decreased milk production and abortion (Carman *et al*., 1998). The economic losses are high when epidemics of fatal mucosal disease occur (Radostits *et al*., 2007). However, there have also been descriptions of BVD infections that combined with other infections could cause much higher losses (Houe, 2003).

The economic losses associated with BVDV infection at herd and national levels have been estimated in several countries, especially in countries undergoing control/eradication program (Duffell *et al*., 1986). Most estimation of the losses at the national level ranged between 10 and 40 million USD per million calvings. But these figures are rough estimates due to the many uncertainties in the parameters used in the calculations and should be considered conservative

estimates as in most cases they included direct losses such as abortions and deaths whereas indirect effects such as increased risk of getting other diseases were not included (Houe, 2003).

2.7. BVDV Infection in Ethiopia

Even though the disease is known to incur heavy economic losses to the cattle industry, has a worldwide distribution and known to exist in other African countries; there are only a few published works on BVD in the country. Even these works are limited to demonstrating the presence of antibodies against BVDV in sera samples of cattle collected from some parts of the country. In a case-control study to assess the association of some pathogens with reproductive problems in commercial dairy cattle in central and southern Ethiopia Asmare *et al*. (2012) found 11.7% of the sera samples positive for BVDV. A similar result (11.5%) was reported by a study conducted on cattle sera collected from central and western Ethiopia (Nigussie *et al*., 2010). Successive studies documented 32.6% and 69.0% (Aragaw *et al*., 2018) and 51.7% and 95.6% (Tadesse *et al*., 2019) individual animal level and herd prevalence, respectively in dairy cattle in different parts of the country.

A recent study on cattle from different production systems in the country failed to detect BVDV antigens in 882 ear-notch samples (Yitagesu et al., 2021). Apart from this work there is so far no accessible published work involving virus isolation, antigen or viral genome detection. Therefore there is lack of information regarding the genotypes and subgenotypes of BVDV circulating in the country. There is also no published work showing association of BVDV infection with clinical conditions including reproductive problems. As a result it is not possible to appreciate the economic significance of BVDV infection in Ethiopia.

3. MATERIALS AND METHODS

3.1. Study Area

This study was conducted in southern and central Ethiopia between May 2019 and March 2020. Blood samples were collected from dairy cattle in Hawassa, Wolita Sodo, Wondo Genet and Arsi Negele towns and Alage TVET college from southern Ethiopia, and Addis Ababa, Debre Berhan, Bishoftu (Debre Zeit) and Tulefa towns in central Ethiopia.

Addis Ababa, Debre Berhan, Tulefa and Bishoftu represent the Great Addis milkshed, while Hawassa, Wolaita Sodo, Wondo Genet, Arsi Negele and Alage the Hawassa-Shashemene milkshed. The Great Addis milkshed, located in the central part of the country, is the most developed milkshed and is leading the dairy development in the country. The Hawassa-Shashemene milkshed, on the other hand, is located in the southern part of the country where there is significant market potential. However still much has to be done to develop dairy production in the area (Land O'Lakes Inc., 2010; Zijlstra *et al*., 2015).

Hawassa, Wondo Genet, Arsi Negelle and Alage lie within the central part of the Ethiopian Rift Valley. The altitude of the Ethiopian mid Rift Valley ranges from 1500m to 2500m a.s.l., and the average annual rainfall ranges from 600mm to 1500mm. The mean minimum and maximum temperatures are 11.4°C and 26°C, respectively (EMA, 1999; Sisay and Baars, 2002). Wolaita Sodo on the other hand is located at about 383 kms south of Addis Ababa at a latitude and longitude of 6° 54′ N and 37° 45′ E with an elevation between 1600 and 2100 m a.s.l. The area receives a mean annual rainfall of 1200 – 1300 mm and mean annual temperature of 17°C – 19°C. The area has a bimodal rainfall pattern: long rainy period from July to October and short rainy period from March to May. The average annual rainfall is 1014mm (Lemma *et al*., 2010).

Addis Ababa, the capital of Ethiopia, is located in the central highlands at a latitude and longitude of 8°58'N and 38°45'E with an elevaltion between 2200 and 2600 m altitude, with a subtropical highland climate. The city receives mean annual rainfall of 1300 mm in bimodal distribution, with 11°C and 24°C as mean minimum and maximum temperatures, respectively. Bishoftu is located 47 km southeast of Addis Ababa, at an altitude of about 1850 m a.s.l. The annual rainfall is about 800 mm, of which 84% is in the long rainy season from June to September. The annual average minimum and maximum temperature are 12.3°C and 27.7°C, respectively (Beyene *et al*., 2009; Datiko *et al*., 2013).

Tulefa and Debre Berhan are located about 70 and 130 km northeast of Addis Ababa at an altitude of about 2800 m a.s.l. The climate is characterized by a long rainy season (June to September) accounting for 75% of the annual rainfall, a short rainy season (February/March to April/May) and a dry season (October to January). The area receives about 945 mm rainfall annually. The average monthly minimum temperature ranges from 2°C in November to 8°C in August, while the average monthly maximum temperature ranges from 18°C in September to 23°C in June, with mean relative humidity of 60% (Tibbo, 2000; 2006).

3.2. Study Animals

The study included smallholder and commercial dairy herds of different size located in urban and peri-urban areas, herds owned by higher learning institutions and a breeding herd. The study herds were composed mostly of Holstein-Friesians (HF) and HF-Zebu crosses with variable blood levels, while only one herd (a breeding herd) consisted Jersey cows only. The animals were kept under intensive management whereby they spend the whole or most of the day indoors. Most of the herds were vaccinated against foot and mouth disease, lumpy skin disease, anthrax and blackleg, while none of the studied herds were vaccinated against BVDV as there is so far no practice of vaccinating cattle against BVDV in the country. For practical purpose herds with 10 or more cows were defined as large herds, while those with smaller size as small herds.

3.3. Study design

The study was a cross-sectional observational study comprising 3 components:

1. Seroprevalence study of BVDV,
2. Survey to identify risk factors and reproductive problems, and evaluate reproductive performance
3. Detection of BVDV in aborted fetuses and selected sera samples

3.3.1. Seroprevalence study

The study was a cross-sectional study with a two stage random sampling scheme. The sample size for each geographic region (GR) included in the study, *i.e.* central Ethiopia and southern Ethiopia, was calculated with 32.6% estimated prevalence (Aragaw *et al.*, 2018), desired 95% confidence level and 5% accepted error (Thrusfield, 2005). The minimum required sample size

was calculated as 338 per GR (a total 676 for the two GR) and allocated to the selected towns proportional to their dairy cattle population size. The final number of samples collected from each GR, however, was slightly higher (493 and 465 from southern and central regions, respectively). Accordingly, a total of 954 dairy cattle were sampled from 98 herds from southern and central parts of the country. These GR were chosen for their dairy cattle population and convenient proximity to the University and the Laboratory where the study was conducted. Towns were also selected purposively for their dairy cattle population. Herds in each town were randomly selected from the list of dairy farms in the areas obtained from the respective departments of agriculture. About 20 to 50% of female cattle over 6 months old were randomly sampled from each herd until the predetermined sample size for that town was achieved. Only females over 6 months old were bled to avoid cross-reactions with colostral antibodies.

3.3.2. Survey

Animal and herd level data were collected for each and every animal and herd selected for the seroprovalence study using semi-structured questionnaires administered to owners or attendants having the relevant knowledge about the herd in order to identify risk factors and reproductive problems, and assess reproductive performance. Data were also collected through direct observation and from farm records where these were available.

3.3.3. Detection of BVDV in aborted fetuses and selected sera samples

Tissues from aborted fetuses and placentae were aseptically collected from 30 bovine abortions from Hawassa and Arsi Negele towns during May 2019 to May 2020 and preserved in 75% ethanol. The alcohol-preserved samples were kept at room temperature until processed for reverse transcription (RT)- PCR. Sera from 26 animals with high chance of beig positive for the virus were collected from two farms with veryhigh seroprevalence of BVDV (>85%). The sera samples were kept frozen at -20°C until/before processed within 4 days of sampling.

3.3.4. Molecular characterization of BVDV acquired during the study

We were to genetically characterize BVDV to be acquired during the study. The plan was to characterize BVDV that would be detected in aborted materials and from sera collected from selected animals. For the later a total of 26 sera were collected from two dairy farms which had a very high seroprevalence (>85%) as established by this study. Nine of the sera samples were

collected from seronegative animals while the remaining 17 samples were collected from poorly performing young animals of unknown serostatus.

3.4. Samples and Data Collection

3.4.1. Sero-prevalence study

Blood samples (about 10 ml) were taken from the coccygeal or jugular vein of each animal into plain vacutainer tubes and were left undisturbed overnight at room temperature to clot and contract. Sera were harvested into cryovials and transported to the Microbiology Laboratory of the Faculty of Veterinary Medicine of Hawassa University where they were stored at -20 ^{o}C. They were then transported to the National Veterinary Institute (NVI) in Bishoftu (Debre Zeit), Ethiopia, for further analyses. Cold chain was maintained during transportations and storage.

3.4.2. Survey

Epidemiological data, to define possible factors influencing the occurrence of BVDV infection and to explore the potential impact of the disease on dairy cattle fertility, were collected through a semi-structured questionnaire and examination of farm records when these were available. Some information was also obtained by direct observation of the farms. The information collected included: general data: number of cattle, number of cows, number of calves; animal-related data: age, breed, parity; herd-related data: reproductive management (natural or artificial insemination), housing of young animals (separate/with adult cattle), grazing (yes/no), water source (public water supply/well/pond), origin of replacement stock (external, same farm, mixed), separation of cows at calving (yes/no), arrangement of animals in the farm (head-to-head, head-to-tail, tail-to-tail, one row), calf-dam separation (before or after suckling colostrum); reproductive problems: anestrus (yes/no), repeat breeding (yes/no), abortion (yes/no), weak calf (yes/no), calf mortality (yes/no), dystocia (yes/no), fetal membrane retention (yes/no), mastitis (yes/no) and metritis (yes/no); reproductive performance indices: calving interval (normal, extended [>15m]), calving to first service interval (normal, extended [>90 d]), age at first calving (normal, prolonged [>30 m]) and number of services per conception.

3.4.3. Detection and characterization of BVDV

A total of 154 tissue samples were collected from 30 bovine abortion cases for detection of BVDV RNA. Samples of kidneys (n = 30), spleen (20), brain (27), liver (30), lungs (30) and

placenta (17) from the abortion cases were collected. Tissue samples were collected using sterile utensils and placed in wide-mouth sterile McCartney bottles containing 75 % ethanol. These alcohol preserved samples were stored at room temperature until processed. Sera samples, on the otherhand, were placed in sterile cryovials and stored frozen at -20 ^{o}C. In addition to the aborted materials, sera were also collected from highly likely BVDV positive animals for possible detection and characterization of the virus. Accordingly, sera were collected (for the second time) from seronegative animals (n = 9) and from animals with history of poor performance (n = 17), but of unknown serostatus, in two farms with very high BVDV seroprevalence (>85 %) for viral nucleic acid detection.

3.5. Laboratory Examination

3.5.1. Serological examination

The sera samples were screened for the presence of antibodies (Ab) against BVDV using indirect ELISA kit (IDEXX BVDV Total Ab) (IDEXX BVDV Total Ab, IDEXX Switzerland GmbH, Liebefeld-Bern, Switzerland) at the National Veterinary Institute (NVI) in Bishoftu, Ethiopia, following the manufacturer's instructions. The sensitivity and specificity of the test is 96.3 and 99.5%, respectively (BVDV Total Ab Test Brochure, IDEXX).

The test depends on binding of BVDV Ab of the sample to antigen on the plates. Captured BVDV Ab were detected by anti-bovine horseradish peroxidase conjugate after a substrate/chromogen solution was added. Color development indicates the presence of BVDV Ab in the test sample (positive result). The absorbance of the samples and controls was measured and recorded at 450 nm using ELISA micro-plate reader. The presence or absence of BVDV antibodies in the sample was determined by the sample to positive (S/P) ratio for each sample. The S/P ratio was calculated by using the absorbance obtained with the test sample and a positive control, corrected for the absorbance of the negative control, using the following formula: S/P = (Sample A(450) - NCx) / (PCx - NCx)), where NCx and PCx are negative and positive control means, respectively. Samples with S/P ≥ 0.3 were considered as positive

3.5.2. Molecular examination

Processing of specimens: After cleaning the tissues by washing three times in phosphate buffered saline (PBS), a 10 % tissue homogenate was prepared from approximately 1 g pooled

tissue containing liver, kidneys and lungs, and spleen and placenta (when these were available) from one animal, in 9 ml of PBS using a sterile pestle and mortar. Accordingly, a total of 30 pooled tissue homogenates were prepared. The suspension was centrifuged at 3,500 rpm for 10 min to remove insoluble components and the supernatant was decanted into 2 ml micro-centrifuge tubes for storage at -20 °C until used. RNA was extracted from the supernatant.

Viral RNA extraction: Viral RNA was extracted from the tissue homogenates and directly from the sera samples. Viral RNA was isolated using a commercial kit (RNeasy®Mini Kit, QIAGEN®) according to the manufacturer's instructions. Briefly, 350 µl of Buffer RLT was added to 350 µl tissue homogenate/serum in 1.5 ml Eppendorf microcentrifuge tube and centrifuged at 15,000 x g for 3 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and 350 µl 70 % ethanol was added to the lysate and mixed well by pipetting. Then 700 µl of the lysate, including any precipitate that may have formed, was transferred to RNeasy®spin column and centrifuged at 13,000 rpm for 30 s, followed by three washing steps. Firstly 700 µl buffer RW1 was added to the RNeasy®spin column and centrifuged at 13,000 rpm for 30 s. Secondly 500 µl RPE buffer was added to RNeasy®spin column and centrifuged at 13,000 rpm for 30 s and finally another 500 µl RPE buffer was added to RNeasy®spin column and centrifuged at 13,000 rpm for 2 m. The column was then placed in a new clean 2 ml tube and centrifuged at 13,000 rpm for 1 m. The column was finally placed in a new clean 1.5 ml Eppendorf tube, 50 µl of RNase-free water added onto the membrane and centrifuged at 13,000 rpm for 1 m to elute the RNA.

Conventional RT-PCR: The tissue samples collected from abortions and sera samples from selected animals were tested for BVDV nucleic acid using RT-PCR. A commercially available single-tube RT-PCR kit was used for all assays (QIAGEN® OneStep RT-PCR KIT, QIAGEN GmbH, Hilden, Germany). The RT-PCR was performed in a final volume of 25 µl containing 20 µl of BVDV master mix prepared using QIAGEN® OneStep RT-PCR KIT (QIAGEN GmbH, Hilden, Germany) containing 4µl RNase free water, 5µl RT-PCR buffer 5x (Tris Cl, KCl,(NH4)2SO4, 12.5 mM MgCl2, dithiothreitol; pH 8.7, 20 C), 1µl 10mM dNTPs, 5µl Q solution 5x, 2µl each primer, 1µl OneStep RT-PCR enzyme mix and 5µl of the template (RNA extract). The PCR primer pairs used for detection of BVDV were previously described (Hamel *et al*., 1995) and were obtained/purchased from Sigma-Aldrich, Darmstadt, Germany. The primers

were derived from the conserved sequences within the BVDV 5′-untranslated region. The sequences of the BVDV-specific primers were 5′-AGGCTAGCCATGCCCTTAGT-3′ (primer BVD-99, forward) and 5′-TCTGCAGCACCCTATCAGG-3′ (primer BVD-342, reverse). The PCR reaction was performed with the following thermocycling conditions in a programmable thermal cycler (2720 Thermal Cycler, Applied Biosystems): the RNA was transcribed to cDNA at 50 °C for 30 min followed by a denaturation cycle at 95 °C for 15 min. The amplification programme included 35 cycles of three steps each comprising of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 15 s and product extension at 72 °C for 30 s, final elongation at 72 ^{o}C for 10 min and hold at 4 ^{o}C.

The PCR products were detected by electrophoresis on 1.5 % agarose gel in Tris-acetate (TAE) buffer at 120 V for 80 min. Gels were run with appropriate DNA ladders (Thermo-Scientific) starting at 100 bp on bench-top apparatuses (Bio-Rad). All PCR reactions included positive and negative controls. Amplification products were stained with GelRed® (Biotium) and photographed using UV transilluminators. The sizes of the PCR products were estimated compared to the migration pattern of the DNA ladder. Samples revealing a band of 244 bp were considered positive for BVDV RNA.

3.6. Data Management and Statistical Analyses

Data were stored in MS Excel. The database included individual animal biodata and herd-related information and individual animal BVDV serostatus. All statistical analyses were performed using Stata version 13.0 (Stata Corp., College Station, TX, USA) statistical software. Descriptive statistics (proportion, mean, count) was used to summarize and present the data. The apparent prevalence (AP) of Ab to BVDV was estimated using the ratio of positive results to the total number of cattle/herds examined. The true prevalence (TP) was estimate by adjusting the AP for specificity (Sp) and sensitivity (Se) of the ELISA test using the formula, $TP = (AP - (1 - Sp))/(1 - [(1 - Se) + (1 - Sp)])$ (Dohoo *et al*. 2009). Associations between individual animal serostatus and potential risk factors were assessed using multivariable logistic regression analysis. The predictor variables were checked for collinearity before being entered into the model. Collinearity between independent variables was verified in a cross-tabulation using Goodman and Kruskal's Gamma statistic, with a cut point of gamma at $\geq + 0.6$ or $\leq - 0.6$. For those variables with collinearity, one of the two was excluded from the multivariable analysis

according to biological plausibility (Dohoo *et al*., 2009). Confounding was checked for by monitoring the change in regression parameters and was considered to be present if parameters changed by 25% or more upon inclusion of exclusion of a factor. In the development of the multivariable logistic regression model, the backward elimination technique was used and the statistical significance (p <0.05) of individual predictors to the model was assessed using the Wald's test and likelihood ratio test. The models were finally assessed using the Hosmer and Lemeshow method for goodness of fit and the receiver operating curve (ROC) for reliability (Dohoo *et al*., 2009). Association between BVDV serostatus and history of occurrence of reproductive problems (abortion, repeat breeding, anestrus, calf mortality, weak calf, RFM, metritis, mastitis) was assessed using univariable logistic analysis. A p value less than or equal to 0.05 was considered for statistical significance.

4. RESULTS

Sera samples, for BVDV antibody detection, were collected from 954 cattle in 98 dairy herds from central and southern Ethiopia, and animal and herd level data were collected from these animals and herds to assess association with the BVDV serostatus of the animals. These data were also used to estimate the occurrence of reproductive problems and evaluate the reproductive performance of dairy cattle in the study areas.

4.1. Reproductive problems

Of the study cows 13.4, 26.5, 41.1, 10.2 and 7.3% respectively had an abortion, retained fetal membranes (RFM), mastitis, uterine infection (abnormal vulvar discharge) and dystocia at least once in their life. About 19% of the cows lost at least one calf during their life, whereas 34.0% and 40.3% of cows encountered anestrus and repeat breeding (RB), respectively, during their reproductive life (Table 2).

Table 2. History of occurrence of reproductive problems in dairy cows in central and southern Ethiopia

Reproductive problem	No. of observations	No. (%) affected	95% CI
Abortion	710	95 (13.4)	11.1, 16.1
Retained fetal membranes	680	180 (26.5)	23.3, 29.9
Mastitis	705	290 (41.1)	37.5, 44.8
Uterine infection	688	70 (10.2)	8.1, 12.7
Dystocia	686	50 (7.3)	5.6, 9.5
Anestrus	720	245 (34.0)	30.6, 37.6
Repeat breeding	715	288 (40.3)	36.7, 43.9
Calf mortality	683	132 (19.3)	16.5, 22.5

4.2. Reproductive performance

Calving interval (CI), calving to first service interval (CFS), age at first calving (AFC) and number of services per conception (NSC) were used as measures of reproductive performance in heifers and cows in this study. The mean time interval between the two most recent calvings of the cows (CI), the mean CFS for the most recent calving, the mean AFC and the mean NSC for the most recent pregnancy were found to be 458.8 days, 130.9 days, 32.3 months and 2.13, respectively (Table 3).

Table 3. Mean (±SD) reproductive performance indices of dairy cows in central and southern Ethiopia

Reproductive traits	No. Obs.	Mean (±SD)	Min	Max
Calving interval (days)	398	458.8 (±125.0)	319	1095
Calving to first service interval (days)	457	130.9 (±115.4)	30	730
Age at first calving (months)	499	32.3 (±6.9)	22	63
Number of services per conception	601	2.1 (±1.3)	1	10

4.3. Seroprevalence

Of 954 sera samples assessed 20.9 % (95% CI: 18.4, 23.6) were observed to be positive for anti-BVDV antibodies. Of the 98 herds evaluated, 50 % (95%CI: 40.1, 59.9) had at least one seropositive animal, and the percentage of seropositive animals in individual seropositive herds varied between 2.6 % and 100 % (mean, 31.4 %; median, 25%). The true individual animal level prevalence of BVDV was calculated to be 21.3 %. The percentage of cattle with positive sera was 30.1 % in southern and 11.2 % in central region. Sera showed positivity in 28 (52.8%) of the 53 herds from southern and in 21 (46.6 %) of the 45 herds from central region (Table 4). Seropositive animals were detected in all the 9 towns included in the study with proportion ranging from 2.6 % in Wondo Genet to 90.6 % in Allage. The proportions of seropositive animals per town are shown in Figure 3.

Table 4. Animal and herd-level seroprevalence of BVDV in dairy cattle in central and southern Ethiopia

Geographic region	Animal level			Herd level		
	No. tested	No. Pos.	Pre. (95% CI)	No. tested	No. Pos.	Pre. (95% CI)
Central Ethiopia	465	52	11.2 (8.6, 14.4)	45	21	46.7 (32.4, 61.4)
Southern Ethiopia	489	147	30.1 (26.2, 34.3)	53	28	52.8 (39.2, 66.0)
Overall	954	199	20.9 (18.4, 23.6)	98	49	50.0 (40.1, 59.9)

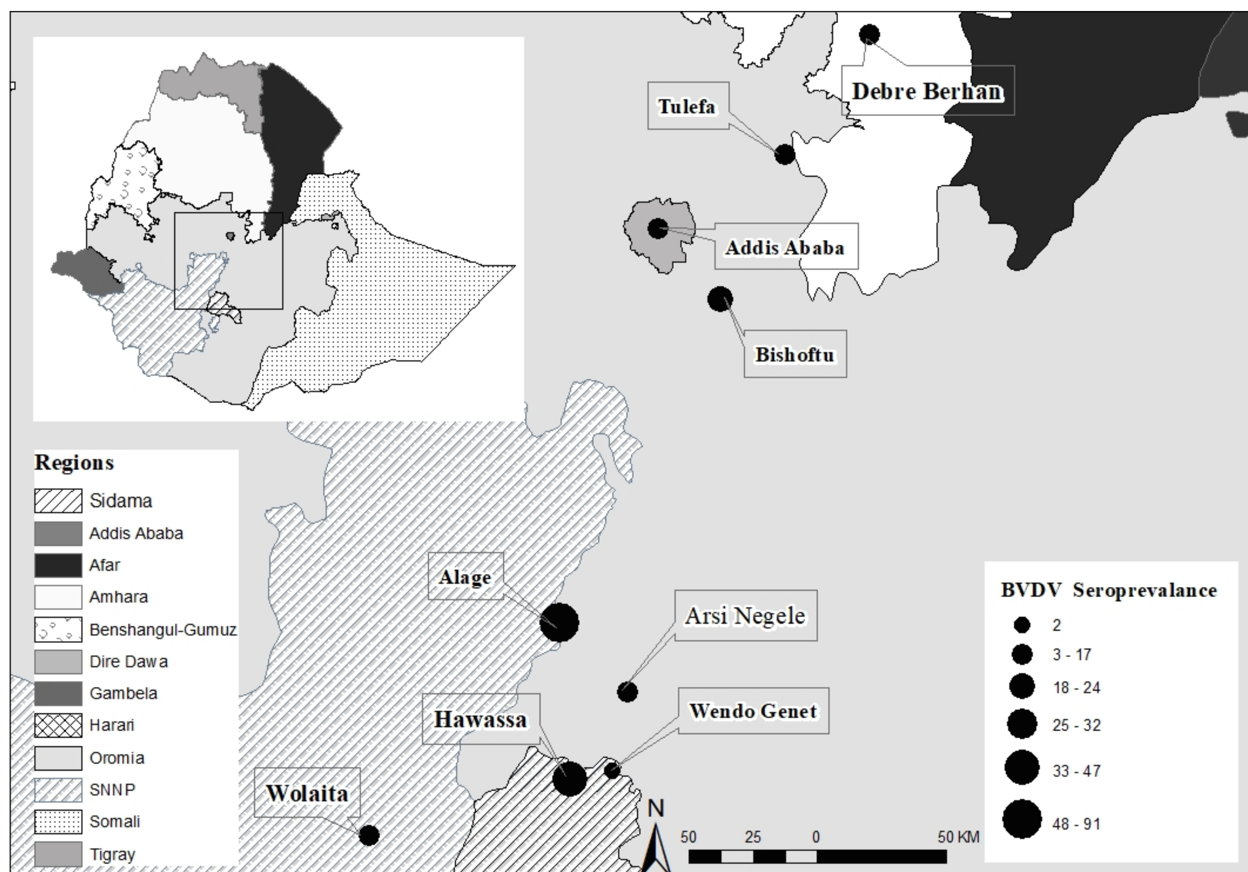

Figure 3. Proportional dot map showing seroprevalence of BVDV in nine study sites

4.3.1. Association with risk factors

Geographic region in which the dairy farm was located and herd size were significantly associated with serostatus of animals to BVDV ($p < 0.05$). Dairy cattle in southern Ethiopia had significantly higher seroprevalence (OR, 2.8; 95%CI, 1.9, 4.2) compared to cattle in central

Ethiopia, while animals raised in large herds with 10 or more cows had 2.6 (95%CI, 1.5, 4.6) times the odds of seroreaction than animals raised in smaller herds. Cattle in farms with a head-to-head arrangement had significantly higher seroprevalence (OR = 1.7; 95%CI, 1.1, 2.7) compared to farms in which animals were arranged in a tail-to-tail style. On the other hand age and breeding method were not associated with BVDV serostatus ($p > 0.05$) (Table 5). Variables: parity of the cow, water source of the farm, housing of young animals (separate/with adult cattle), practice of quarantine by the farm, isolation of sick animals, use of calving pen, separation of cows near calving, calf dam separation (before or after suckling colostrum), and origin of replacement stock for the farm (own/own and other herds) were not used in the multivariable logistic regression analysis due to strong collinearity with other variables considered in the study (gamma ≤− 0.60 or ≥0.06). Variables used in the analysis were preferred to the corresponding collinear variables because of biological plausibility.

Table 5. Results of multivariable logistic regression analysis of risk factors by BVDV serostatus in dairy cattle in central and southern Ethiopia

Variable	Category	No. Examined	No. (%) Positive	95% CI	OR (95% CI)	*p* value
Age	Young (≤2.5 years)	205	44 (21.5)	16.4, 27.6	1	
	Adult (>2.5 years)	728	153 (21.0)	18.2, 24.1	1.2 (0.8, 1.7)	0.473
Geographic region	Central	465	52 (11.2)	8.6, 14.4	1	
	Southern	489	147 (30.1)	26.2, 34.3	2.8 (1.9, 4.2)	0.000
Herd size	Small (<10 cows)	239	26 (10.9)	7.5, 15.5	1	
	Large (≥10 cows)	715	173 (24.2)	21.2, 27.5	2.6 (1.5, 4.6)	0.001
Type of reproduction	AI	313	67 (21.4)	17.2, 26.3	1	
	Natural/Bull	116	28 (24.1)	17.2, 32.8	0.7 (0.4, 1.3)	0.289
	Both	524	104 (19.8)	16.6, 23.5	0.8 (0.5, 1.2)	0.301
Animal arrangement	Tail to tail	292	41 (14.0)	10.5, 18.5	1	
	Head to head	458	131 (28.6)	24.6, 32.9	1.7 (1.1, 2.7)	0.014
	Head to tail	63	3 (4.8)	1.5, 13.9	0.4 (0.1, 1.3)	0.117
	One row	137	24 (17.5)	12.0, 24.8	1.5 (0.7, 2.9)	0.274

AI, artificial insemination

4.3.2. Association with reproductive problems

A univariable logistic regression analysis using BVDV serostatus as predictor variable for history of occurrence of reproductive problems in individual animals demonstrated that anestrus,

repeat breeding, mastitis and calving interval (CI) were significantly associated with BVDV seropositivity ($p < 0.05$). Accordingly, animals positive for BVDV antibodies were more likely to have a history of extended CI (OR, 1.7; 95%CI, 1.0, 2.7) and mastitis (OR, 2.2; 95%CI, 1.5, 3.2) compared with seronegative ones. However, seropositive animals were less likely to have history of anestrus and repeat breeding (OR, 0.6; 95%CI, 0.4, 0.9 each). On the other hand, serostatus was not significantly associated with history of occurrence of abortion, calf mortality, weak calf, retained fetal membranes, metritis, extended calving to service interval and extended age at first calving ($p > 0.05$) (Table 4).

Table 6. Results of univariable logistic regression analysis of association between BVDV serostatus (as a predictor variable) and reproductive problems in dairy cows in central and southern Ethiopia

Reproductive problem	BVDV Sero Status	No.	No. (%) affected	95% CI	OR (95% CI)	*P* value
Abortion	Neg	561	77 (21.3)	18.2, 24.7	1	
	Pos	149	18 (18.9)	12.2, 28.2	0.9 (0.5, 1.5)	0.600
Weak calf	Neg	554	44 (20.8)	17.8, 24.1	1	
	Pos	146	12 (21.4)	12.5, 34.2	1.0 (0.5, 2.0)	0.913
Calf mortality	Neg	540	109 (21.8)	18.5, 25.4	1	
	Pos	143	23 (17.4)	11.8, 24.9	0.8 (0.5, 1.2)	0.271
Anestrus	Neg	567	207 (24.2)	20.6, 28.3	1	
	Pos	153	38 (15.5)	11.5, 20.6	0.6 (0.4, 0.9)	0.007
Repeat breeding	Neg	560	238 (24.6)	20.7, 28.9	1	
	Pos	155	50 (17.4)	13.4, 22.2	0.6 (0.4, 0.9)	0.022
RFM	Neg	541	143 (20.4)	17.1, 24.2	1	
	Pos	139	37 (20.6)	15.3, 27.1	1.0 (0.7, 1.5)	0.965
Mastitis	Neg	558	207 (15.4)	12.2, 19.2	1	
	Pos	147	83 (28.6)	23.7, 34.1	2.2 (1.5, 3.2)	0.000
Metritis	Neg	541	56 (21.5)	18.5, 24.9	1	
	Pos	147	14 (20.0)	12.2, 31.1	0.9 (0.5, 1.7)	0.769
Extended CI (>15m)	Neg	310	95 (21.0)	16.5, 26.2	1	
	Pos	99	42 (30.7)	23.5, 38.8	1.7 (1.0, 2.7)	0.031
Extended CSI (>90 d)	Neg	380	164 (17.2)	13.1, 22.3	1	
	Pos	82	37 (18.4)	13.6, 24.4	1.1 (0.7, 1.8)	0.745
Prolonged AFC (>30 m)	Neg	398	183 (20.1)	15.7, 25.3	1	
	Pos	107	53 (22.5)	17.6, 28.3	1.2 (0.8, 1.8)	0.513

RFM, retained fetal membrane; CI, calving interval; CSI, calving to service interval; AFC, age at first calving

4.4. Detection of BVDV RNA in aborted materials and selected sera

Niether the 30 pooled samples of tissues from aborted fetuses and placenta nor the 26 sera collected from selected animals were found positive for BVDV RNA.

5. DISCUSSION

5.1. Reproductive performance

Productive life, milk yield, reproductive performance, and health of primiparous cows are closely related to their AFC (Gabler *et al*., 2000; Ettema and Santos, 2004). The 32.3 months AFC obtained in this study compares favorably with 31.5 m and 32.7 m recorded for 50% and 75% Boran-Friesian crosses at a ranch in central Ethiopia (Haile-Mariam *et al*., 1993). But it was significantly lower compared to about 40 m to 47 m reported for crossbred dairy cattle from various locations in Ethiopia (Shiferaw *et al*., 2003; Abraha *et al*., 2009; Haile *et al*., 2009; Fekadu *et al*., 2011; Yalew *et al*., 2011). Though the AFC observed in our study looks good compared to previous reports in the country, it is however over 8 months higher compared to the recommended target of ≤24 months of age at first calving (Stephens and Rajamahendran, 1998). A substantial delay in the attainment of sexual maturity has a profound influence on the total cost of raising dairy replacements, due to an additional, non income-generating period of the cow (Meyer *et al*., 2004). Nutrition is a very important factor that determines pre-pubertal growth rates, reproductive organ development, and time of onset of puberty and subsequent fertility (Noakes *et al*., 2001).

The observed CI (458.8 days), in our study, was comparable to 422 - 446 days (Haile *et al*., 2009) and 475 days (Yalew *et al*., 2011) reported for dairy cows in Ethiopia. However it was shorter compared to 516 - 561.3 days recorded for crossbred cattle from different parts of the country (Haile-Mariam *et al*., 1993; Shiferaw *et al*., 2003; Lobago *et al*., 2007; Fekadu *et al*., 2011). The CI we have observed is 2 to 3 months longer compared to the 12 to 13 months CI generally considered to be economically optimal for dairy cows (Rajamahendran *et al*., 2001). Shorter CI would result in more calves being born, increase the lifetime milk production per cow and maximize income (Hossein-Zadeh, 2013). Efficient and accurate estrous detection, proper semen handling techniques, timely artificial insemination (AI) and good management of postpartum anestrus would help to shorten CI (Rajamahendran *et al*., 2001; Baruselli *et al*., 2004; Graves, 2017).

Our observation of 130.9 days CFS is comparable to the 141.98 (Shiferaw *et al*., 2003) and 141 days (Lobago *et al*., 2007) recorded for crossbred dairy cows in central Ethiopia. But it is much higher compared to 67 days reported from Iran for Holstein cattle (Ansari-Lari *et al*., 2010).

Reducing days to the first service usually translates into improved reproductive performance, as measured by other traits such as average days open and CI (DeJarnette, 2004). Although the present estimate is within the range of the previous reports in the country, it is inadequate compared to the optimum calving to conception interval (CCI) recommended (75–85 days) to achieve the target 12-month CI (Baruselli *et al*., 2004). The high occurrence of postpartum anestrus observed in the current study may partly explain the extended CFS noted in this study. Lobago *et al*. (2006) attributed the prolonged CCI they observed in dairy cows in different production systems in central Ethiopia to inadequate nutrition, poor health services and managerial problems.

The 2.1 NSC observed in the present study is comparable to the NSC of 2 obtained by Asseged and Birhanu (2004) in central Ethiopia. However, several studies in the country recorded lower NSC as compared to ours: 1.61-1.81 (Haile-Mariam *et al*., 1993); 1.75% (Shiferaw *et al*., 2003); 1.6 (Lobago *et al*., 2006) and 1.69 (Fekadu *et al*., 2011). The optimum NSC is considered to range between 1.6 and 1.8 (Rodzki, 2011, cited by Borkowska *et al*., 2012). Higher NSC could be attributed to failure to detect heat in time, poor AI technique and certain diseases.

5.2. Reproductive problems

In the present study, 13.4% of the cows experienced abortion in their reproductive lifetime. Although numerous questionnaire surveys reported the prevalence of abortion in dairy cattle in the country only a few explicitly indicated whether it refers to the occurrence in the entire reproductive life of the cow or restricted only to one pregnancy. Nonetheless Bitew and Prasad (2011) recorded a similar abortion rate (13.9%) in western Ethiopia. However, our observation is higher than the 5.7% (Shiferaw *et al*., 2005), 5.9% (Haile *et al*., 2010) and 8.0% (Yalew *et al*., 2011) abortion prevalence reported in crossbred dairy cows in central Ethiopia. The annual abortion rate in dairy farms is recommended not to exceed 2 to 5% (Yaeger, 1993). Abortion over 5% should be a major concern at it results in considerable economic losses through reduced lifetime milk production (associated with longer CI); loss of calf (loss of potential female herd replacements); expenses associated with rebreeding; and premature culling of cows (Peter, 2000).

In this study, 34.0% of the cows were found to have been affected, once or more, with postpartum anestrus during their reproductive life. Our result agreed with a 38.6% prevalence of

postpartum anestrus reported in crossbred dairy cows in central Ethiopia (Shiferaw *et al*., 2005). Haile *et al*. (2010), on the other hand, observed a much lower rate (10.1%) in the Addis Ababa milkshed. A study in India identified anestrus to be responsible for 65% of infertility cases in crossbred cattle (Kutty and Ramachandran, 2003). It should also be noted that silent estrus and missed heat are counted as anestrus. Using milk progesterone assay Lobago *et al*. (2007) identified 67.4% of the examined dairy cows in central Ethiopia to have a delayed postpartum (>55 days) resumption of ovarian activity.

A large proportion (40.3%) of dairy cattle involved in this study had a history of occurrence of RB. Our result is very high compared to previous reports from Ethiopia: 1.3% in Jimma (Gashaw *et al*., 2011), 3% in Bedelle (Bitew and Prasad, 2011) and 6.2% in Addis Ababa milkshed (Haile *et al*., 2010). Repeat breeding is one of the most important causes of infertility in cattle. An Indian study identified RB, caused by ovulatory disturbance and reproductive tract infections, in 25% of infertility cases in crossbred cattle (Kutty and Ramachandran, 2003).

The finding that 26.5% of the cows in the current study experienced retained fetal membranes compares favorably with the findings of Bekele *et al*. (1991) (7.1% to 28.8%) in central highlands of Ethiopia, while several other studies found a lower prevalence of RFM (5.4% - 19.2%) in different parts of the country (Shiferaw *et al*., 2005; Lobago *et al*., 2006; Haile *et al*., 2010; Bitew and Prasad, 2011; Gashaw *et al*., 2011). A high prevalence of RFM, exceeding 10%, should be a concern as the condition is usually related to reproductive tract infection and subsequent infertility (Bruun *et al*., 2002; Shiferaw *et al*., 2005; Lobago *et al*., 2006).

Signs of uterine infection (abnormal vulvar discharge) were observed in 10.2% of the cows included in the study. This finding is within the range of many earlier reports (2.8% - 16.9%) from various parts of the country (Shiferaw et al., 2005; Lobago *et al*., 2006; Bitew and Prasad, 2011). Some reviews also put the prevalence of metritis (LeBlanc *et al*., 2011) and endometritis (Sheldon and Owen, 2017) at around 10 to 20%. The risk factors most frequently associated with uterine infection include dystocia, stillbirths, retained fetal membranes, deficiencies in hygiene and metabolic imbalances around parturition (Sheldon and Owen, 2017).

The observation of 7.3% of cows experiencing dystocia in their entire calvings does seem acceptable in view of earlier cross-sectional observations as high as 6.6% in local and crossbred

cattle in various parts of Ethiopia (Haile *et al*., 2010; Bitew and Prasad, 2011; Gashaw *et al*., 2011).

The current study demonstrated that 41.1% of the study cows developed clinical mastitis in their life. Our finding is higher than reports of earlier studies on clinical mastitis in different parts of Ethiopia ranging between 3.4% and 37.0% (Dego and Tareke, 2003; Biffa *et al*., 2005; Lakew *et al*., 2009; Mekibib *et al*., 2010; Abebe *et al*., 2016).

Of the cows included in the survey 19.3% have lost at least a calf to death in their life. In studies conducted in central Ethiopia Lobago *et al*. (2006) and Wudu *et al*. (2008) found more or less similar high calf mortality rates of 17.4% and 22.3%, respectively. However, much lower calf mortality rates (about 9 %) were observed in different production systems in the country (Megersa *et al*., 2009; Yitagesu *et al*., 2021).

Interpretations and comparisons of results of occurrence of reproductive problems obtained in this study should take into consideration the potential differences in the study approaches used among the studies. Our study, for example, recorded the occurrence of the event of interest for the life of the animals while others might have only considered the occurrence of the condition during the most recent pregnancy, calving, or postpartum period.

5.3. Seroprevalence

The herd level prevalence (50.0%) observed in our study is lower than 69.0% (Aragaw *et al*., 2018) and 95.6% (Tadesse *et al*., 2019) reported earlier in Ethiopia and 92% reported in Cameroon from West Africa (Handel *et al*., 2011). Several reports from different parts of the world also described higher herd prevalence of BVDV than our study (Reinhardt *et al*., 1990; Sakhaee *et al*., 2009; Talafha *et al*., 2009; Saa *et al*., 2012; Hou *et al*., 2018). There are, however, a few reports of lower herd prevalence (Loken *et al*., 1991; Almeida *et al*., 2013). The 20.9 % individual animal seroprevalence observed in our study is within the 10.7% - 32.9% range reported by earlier studies in the country (Nigussie *et al*., 2010; Aragaw *et al*., 2018). A more or less comparable seroprevalence have been reported in dairy cows in Norway (18.5%) (Loken *et al*., 1991), India (24.7%) (Kulangara *et al*., 2015), and Saudi Arabia (26%) (Mahmoud and Allam, 2013). There are however several reports of higher individual seroprevalence of BVDV from Africa (Ghirotti *et al*., 1991; Handel *et al*., 2011) and other parts of the world (Garoussi *et*

al., 2009; Marques *et al*., 2016). The difference in seroprevalence might be due to variability in geographic regions involved in the studies, environmental factors, cattle density, management practices, control measures in place and prevalence of PI animals among the studies.

5.3.1. Risk factors for serostatus

Seroprevalence varied between the two geographic regions. Consistent with our observation previous works in Ethiopia (Nigussie *et al*., 2010) and elsewhere in the world (Obando *et al*., 1999; Nikbakht *et al*., 2015) have reported difference in seroprevalence of BVDV among geographic locations within a country. Variation in agro-ecology, cattle density and management practices can account for such differences. Cattle from larger herds were more exposed to BVDV than those from smaller herds. Similar observations made by several researchers demonstrated association between large herd size and high BVDV seroprevalence (Talafha *et al*., 2009; Mockeliuniene *et al*., 2004; Almeida *et al*., 2013; Aragaw *et al*., 2018). Large herds commonly have rearing conditions which increase the risk of exposure of susceptible seronegative animals in early pregnancy to PI animals - the most important factor in perpetuating the infection in a herd (Radostits *et al*., 2007). Cattle in farms with a head-to-head arrangement had higher seroprevalence than cattle arranged in tail-to-tail style. This type of arrangement may facilitate transmission as it puts the animals' heads relatively closer to each other. Nose-to-nose contact is considered to be the most effective route of transmission of BVDV (Walz *et al*., 2010). Studies commonly report higher seroprevalence of BVDV in older animals (Garoussi *et al*., 2009; Nigussie *et al*., 2010; Handel *et al*., 2011) which is frequently attributed to cumulative infection with age as infected animals remain antibody positive for life (Houe, 1995). On the contrary, our result showed no statistically significant association between age and BVDV serostatus. The lack of difference among the age groups in our study may suggest active circulation of the virus during the study period exposing the young population to the infection.

5.3.2. Association of occurrence of reproductive problems with serostatus

In contradiction with the common knowledge that associates BVDV with reproductive problems (Kirkbride, 1992; Valle *et al*., 2001; Grooms, 2004; Robert *et al*., 2004; Al-Afaleq *et al*., 2007; Walz *et al*., 2010), our study failed to demonstrate association between BVDV serostatus and indicators of reproductive losses considered in the study: abortion, weak calf, calf mortality, RFM, metritis, prolonged calving to service interval and AFC. Moreover, higher prevalence of

anestrus and RB were observed in seronegative animals compared with their seropositive counterparts. Though it was difficult to explain, we made a similar observation in an earlier study showing higher risk of RB in seronegative animals (Aragaw *et al*., 2018). Some studies also failed to demonstrate association between history of occurrence of reproductive problems (abortion and RB) and BVDV seropositivity (Loken *et al*., 1991; Erfani *et al*., 2018). The lack of association between reproductive losses and BVDV serostatus may partly be explained by the fact that most of our data were obtained through the less precise recall information, as most farms do not keep animal reproduction records. It should also be noted that serological study doesn't enable one to identify the temporal sequence of exposure to the agent vis-à-vis the occurrence of reproductive events of interest.

Seropositive animals had significantly higher proportion of cows with prolonged CI than seronegative cows. This corroborates previous observations, which demonstrated longer CI in BVDV infection (Niskanen *et al*., 1995; Burgstaller *et al*., 2016). It is possible that BVDV can cause extended CI as it can interfere with ovarian function, impede fertilization and may cause embryonic and fetal loss (Oguejiofor *et al*., 2019). There was however no difference in the rate of occurrence of extended calving to service interval between seropositive and seronegative animals. Contrary to our finding Robert *et al*. (2004) reported increased risk of late return to service in BVDV infected cows. In agreement with reports of increase in the risk of infectious diseases in BVDV infected herds (Niskanen *et al*., 1995) our study found higher risk of mastitis in seropositive than seronegative cows. Our study provides fresh additional serological evidence about the prevalence and risk factors of BVDV in dairy cattle in central and southern Ethiopia.

5.4. Molecular study

BVDV nucleic acid was not detected in tissue samples collected from 30 bovine abortions and sera collected from 26 selected animals thought to be relatively more likely to be found positive to the virus. Contrary to our finding a large survey of bovine abortions and stillbirths in the US implicated BVDV in 4.54% of the cases (Kirkbride *et al.,* 1992). In a more or less similar observation to our study, a recent study by Yitagesu *et al*. (2021) failed to detect BVDV antigens in 882 ear-notch samples collected from different production systems in Ethiopia. A study in Cameroon also couldn't demonstrate antigen positive animals (Handel *et al*., 2011).

Caution should be taken in the interpretation of the results of this study regarding detection of BVDV RNA in aborted fetuses, as the number of observations was small. It should also be noted that the tissue samples for PCR test were preserved in 75% ethanol, for lack of facility to freeze the samples immediately in the field, which is not among the best preservatives for RNA detection (Rissanen *et al*., 2010; Camacho-Sanchez *et al*., 2013). Morover, some of the fetuses might have been dead for sometime before expelled, and hence autolyzed and inappropriate for the test. Results concerning association of BVDV serostatus with occurrence of reproductive problems and indices of reproductive performance should also be interpreted with caution as virtually all small farms and most large farms donot keep production and reproductive record of their animals that most of the data collected during this study were based on the error prone recall information obtained from owners or attendants. It should also be noted that reproductive problems have multiple causes.

The limitations of this study, therefore, were: relying largely on recall information regarding the occurrence of reproductive problems and reproductive events used in the estimation of reproductive performance indicators, and failure to establish causal relationship between BVDV and abortion because we couldn’t detect the RNA of the virus in the tissue of the aborted fetuses we have examined, for reasons stated above. As a result, despite our aim, we couldn’t identify the genotype/s of the virus circulating in dairy cattle in the study area.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study demonstrated that dairy cows in the study areas experience considerable level of reproductive health problems that are known to severely affect reproductive efficiency. The reproductive performance indices observed in the study were also far from being optimum.

Results of our study also suggest that BVDV might be contributing to the suboptimal productivity of dairy cattle herds in Ethiopia through reduced reproductive performance and increased susceptibility to infections. It also highlights the variation in BVDV status within Ethiopian dairy herds.

Dairy cattle and dairy herd production and reproductive performance recording and monitoring schemes should be developed and promoted so that designing and implementation of appropriate, specific corrective intervention strategies would be possible at an animal and herd level. Reproductive management, along with improved husbandry practices, should be practiced to improve the reproductive and productive performance of dairy herds in the study area. Further studies of larger size and better depth (e.g. involving investigation of large number of cattle abortions) are needed to establish the role of BVDV, and other pathogens, in bovine infertility in the country. It is also necessary to isolate and identify the genotype of BVDV circulating in cattle populations of the country as it has epidemiological, diagnostic and control implications.

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8. APPENDICES

8.1. Questionnaire for collection of individual animal data

Individual Animal Data Collection Sheet

Farm Name:____

Owner's Name: ____

Town: _______________________ **Date:**______________________________

Individual animal data:

ID _______________ Sex __________ Age____________

Date of Birth_________________ Breed____________ Parity_________________

BCS ____________ Pregnancy status of the cow (Open/Pregnant/Unknown) __________

Date of last Calving (Stillbirth or Abortion)___________________ Date of first service (since last calving) _________

Date of last service ________________________________ How many services since last calving___________

No. of service for the last pregnancy ___________ Service: AI/Bull

Source: Own / Purchased Age at first calving:____________________

History of reproductive problems- individual animal:

Anestrus: Yes / No

Repeat breeding: Yes/ No (failed to conceive after 3 services)

Abortion: Yes / No (expulsion of dead fetus up to 260 days of pregnancy)

Term at abortion : I / II / III

Frequency of abortion:______

Retained Fetal Membranes (RFM): Yes/ No

Frequency of RFM:_______

Mastitis: Yes / No

Weak calves: Yes/ No

Calf mortality: Yes / No

Malformed calf: Yes / No

Metritis (abnormal discharge): Yes/ No

Stillbirth: Yes / No (expulsion of dead fetus after 260 days of pregnancy)

Dystocia: Yes / No

The last calving to service interval ________ The last calving interval _________________

No. of pregnancies ____________ No. of calving ___________

8.2. Questionnaire for collection of herd related data

Herd Level Data Collection Sheet

Date ___________________________________

Farm information

Region ________________________ Zone ________________ District ________________________

Altitude ___________________ Climate _______________

Farm Name___________________ Town _________________ Kebelle ________________________

Name of Interviewer ____

Name and Responsibility of the interviewee___________________________________

Type of farm (Urban-Peri Urban/Commercial/Smallholder/ Breeding/Research/University) ______________

Herd size____________________ No. Sampled _____

Breed of cattle in the farm • Local • Friesian • FriesianX • Jersey • JerseyX • Other

Other livestock species in the farm • Equine • Sheep • Goats • Pigs

No. of calves (<1yr):___________ Heifers:__________ Adult cows (2-8yrs): ____

No. of old cows (>8yrs) :_________ No. of lactating cows_________ No. of bulls____________

No. of pregnant cows__________ Minimum milk production per day per cow (litre)____________

Maximum milk production per day per cow (liter) ________________

Does the herd have any contact with another herd? (Yes/No)___________

Type of housing • Tie stall barn (Area_____m2) • Free stall barn (Area ________m2)

Floor type • Concrete • Wood • Earth

Is there Bedding in laying areas? Yes/No Does the house have drainage? Yes/No

Does the house have sufficient ventilation? Yes/No

Hygienic status of the house: Clean/ Not clean Clean=>75% of animals clean, dry floor, ventilated (no strong ammonia)

History of occurrence of reproductive problems in the farm (in the last 5 years):

Anestrus: Yes/ No Repeat breeding: Yes / No Abortion: Yes/ No Stillbirth: Yes / No

Retained Fetal Membranes (RFM): Yes / No Dystocia: Yes/ No Mastitis: Yes / No

Weak calves: Yes / No Calf mortality: Yes / No Malformed calf: Yes /No Metritis: Yes / No

Management risk factors- farm level

Do you use Calving pen?: Yes / No Separation of cows at calving: Yes/ No

Does the farm have calf pen?:Yes/No Separation of calves from dams: At birth/ Later

Source of replacement stock: _Own / Outside/Both   Breeding: AI / Bull / both

If Bull: Own/Outsourced   Have you ever culled any cows?: Yes/No

Main reason of culling over the last five years ____

____

How do you feed calves? ____

What are the main cattle feed types used by your farm?

- Crop residue
- Improved pasture
- Industrial byproducts (Oilseed cake, bran, molasses etc.)
- Hay
- Silage
- Other (Specify)

Is feeding of milking cows different from other cows? Yes/No

If yes how?____

Does the farm practice Grazing: Yes / No   Cows are kept in the house: the whole day/part of the day

How/what do you feed pregnant cows? ____

Source of water for animals (Tap/Well/Pond/River/Spring) ___________________________________

Housing type (Roof only/3 wall/4 wall)____

Ventilation: Sufficient/ Not sufficient

Housing of young animals: Separate / with adult cattle

Arrangement of animals in the farm: Tail to Tail/Tail to Face/Face to Face

Do you vaccinate your animals? Yes/No

If yes to which diseases? ____

Isolation of sick animals: Yes/No____________   Quarantine of incoming animals: Yes / No

Do you have Veterinary service available?: Yes/No; If yes Public/ Private/Both_________

Do you have dependable AI service? Yes/No   (Dependable= available whenever demanded)

Presence of dogs on the farm: Yes/No   Access of dogs to the farm: Yes/No

Presence of cats on the farm: Yes/No   Access of cats to the farm: Yes/No

Access of wild animals like hyenas to the farm: Yes/No

How do you dispose aborted material?____

How do you dispose dead animals?____

What are the Most important constraints of the farm? ____

____