ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

# A STUDY ON THE PREVALENCE OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN FEEDLOT AND DAIRY FARMS IN AND ADOUND ADAMA, EAST SHOA ZONE, ETHIOPIA

BY
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JUNE, 2009
DEBRE ZEIT, ETHIOPIA

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A thesis submitted to the Faculty of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirements of the degree of Master of Science in Tropical Veterinary Microbiology

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# A STUDY ON THE PREVALENCE OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN FEEDLOT AND DAIRY FARMS IN AND ADOUND ADAMA, EAST SHOA ZONE, ETHIOPIA


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**ABBREVIATIONS**

| | |
|---|---|
| µl | micro litter |
| $^0C$ | Degree Celsius |
| AAU | Addis Ababa University |
| Ab | Antibody |
| AC-ELISA | Antigen Capture- Enzyme Linked Immuno Sorbent Assay |
| BDV | Boarder Disease Virus |
| BVDV | Bovine viral diarrhea Virus |
| CD4 | Cluster of Differentiation |
| CI | Confidence Interval |
| CMI | Cell Mediated Immunity |
| CNS | Central Nervous System |
| Cp | Cytopathic |
| CSA | Central Statistical Agency |
| CSFV | Classical Swine Fever Virus |
| DNA | Deoxy Ribo Nucleic Acid |
| FVM | Faculty of Veterinary Medicine |
| gp | Glycoprotein |
| IHC | Immunohistochemistry |
| Kp | Killo base |
| m.a.s.l | Meter Above Sea Level |
| Mabs | Monoclonal Antibodies |
| MHC | Major Histo-compatibility Complex |
| Ml | Milliliter |
| MLV | Modified Live Virus |
| MoARD | Ministry of Agriculture and Rural Development |
| MTVI-ELISA | Micro-Titer Virus Isolation-Enzyme Linked Immunosorbent Assay |
| NAHDIC | National Animal Health Diagnostic and Investigation Center |
| Ncp | Non Cytopathic |
| NCR | Non Coding Regions |

| | |
|---|---|
| NS | Non Structural |
| OD | Optical Density |
| OIE | Office International des Epizoitics |
| ORF | Open Reading Frame |
| PCR | Polymerase Chain Reaction |
| PI | Persistently Infected |
| RNA | Ribo Nucleic Acid |
| RT-PCR | Reverse Transcriptase–Polymerase Chain Reaction |
| USD | United States Dollars |
| VN | Virus neutralization |

## ABSTRACT

A cross sectional study to assess BVDV prevalence and determine the risk factors and to compare the ELISA diagnostic results of milk and serum values in 40 feedlot and 60 dairy farms was carried out in Adama area, East Shoa Zone. Herds were visited to ascertain information on the management system, possibility of contact with other species of domestic animals, introduction of new animal into the herd and to collect serum samples from feedlot and matched milk and serum samples from dairy herds. Commercial indirect ELISA-kit for the detection of specific antibodies to BVDV was used. According to this study, the apparent individual and herd seroprevalence of BVDV was 35.9 % and 70.9 %, respectively. The individual and herd seropositivity difference among the two production systems was not significant ($p>0.05$). There was a significant difference; however, in the prevalence of the different age groups ($p<0.05$) of the studied animals. Management was found to significantly ($p>0.05$) affect the antibody prevalence where 88.9% of the extensive and 75.5 % of the semi intensive herds had at least one seroreactor. Although a significant difference ($p<0.05$) in herd positivity was observed with respect to herd size, introduction of new animal to the herd and contact with other species of animals in the univariate analysis, only herd size ($p<0.01$) was found to significantly affect herd positivity in the multivariate analysis. The overall individual milk prevalence was 17.1%. The reliability of using individual milk ELISA results as a diagnostic tool for BVDV prevalence was evaluated. The correlation between milk and serum ELISA was moderate ($r=0.473$, $p=0.000$) and the test agreement between milk ELISA and serum ELISA also indicated a moderate level of agreement (kappa=0.409, $p=0.000$). In conclusion, exposure to BVDV was widely distributed in feedlot and dairy farms in and around Adama. , the overall situation of BVDV and its economic impact in Ethiopia should be well studied and documented. Further studies on the identification of Persistently Infected (PI) animals and validation of milk ELISA tests were recommended.



**Key words**: BVDV, Dairy, ELISA, Feedlot, Milk, Prevalence, Serum

## 1. INTRODUCTION

Ethiopia is believed to have the largest livestock population in Africa. This livestock sector has been contributing considerable portion to the economy of the country, and still promising to rally round the economic development of the country. It is eminent that livestock products and by-products in the form of meat, milk, honey, eggs, cheese, and butter supply the needed animal protein that contribute to the improvement of the nutritional status of the people. Livestock also plays an important role in providing export commodities, such as live animals, hides, and skins to earn foreign exchanges to the country. The total cattle population for the country is estimated to be 47.57 million. (CSA, 2008)

Despite the above fact, livestock diseases coupled with poor supply of veterinary services, will have numerous negative impacts on productivity of herds i.e death of animals, loss of weights, slow down growth, poor fertility performance, decrease in physical power and the likes (CSA, 2004).

Among such livestock health problems, the bovine viral diarrhea(BVD), a disease of viral origin, causes several different diseases including the benign infection of bovine virus diarrhea which is usually subclinical, mucosal disease which is usually fatal and occurs in animals which are persistently viremic and specifically immunotolerant as a result of a congenital infection acquired in early fetal life; and congenital abnormalities in calves as a result of infection of the fetus in mid gestation. The virus may also cause reproductive failure and be immunosuppressive (Radostitis *et al.,* 1994).

BVDV was first recorded in the United States and has since been recognized in most countries. Serological surveys have highlighted the widespread infection rate which occurs in many cattle populations in spite of a low incidence of clinical disease (Radostitis *et al.,* 1994). From 1946 to 1960, Bovine viral diarrhea and Mocosal Disease were regarded as distinct entities with many clinical features in common but with epidemiological differences. Bovine viral diarrhea virus (BVDV) is a small, enveloped, single stranded, positive sense RNA virus of the *Pestivirus* genus within the family Flaviviridae. Two types of BVDV; namely BVDV-1 and BVDV-2, synonym *Pestivirus* 1 and *Pestivirus* 2, can be distinguished from one another and from the two other

principal *Pestivirus* types, namely Ovine Border Disease Virus (BDV or *Pestivirus* 3) and classical swine fever virus (CSFV or *Pestivirus* 4) (Paton *et al.,* 1999).

Most cattle which are exposed to the virus seroconvert and have antibody present for the remainder of their life, being virus free. Calves born to cows which are seroconverted gain antibody from colostrum which will be found at blood-test but which wanes in the first six months of life (Synge *et al.,* 1999). Several differences in cattle population structure, housing systems and management can account for the variation in prevalence of infection (Houe, 1999)

Although variability amongst isolates of BVDV has been demonstrated by a variety of means, the extent of genetic variability amongst isolates of BVDV in most countries remains unknown. Two genetically distinct forms have been identified, namely, BVDV type I and type II. BVDV type II appears to be a newly emerging variant that has become increasingly common in North America since the 1980s, but has been only rarely described in Europe. Whereas Molecular epizootiology has been used to study the relationship between outbreaks of classical swine fever, little has been done to relate patterns of BVDV similarity and spread. However, it has been shown that BVDV isolates from the same herd are often indistinguishable genetically (Vilcleka *et al.*, 1999).

The implementation of a programme to control the infection must be based on first, the identification of animals and herds either free of infection or presence of active infection, secondly, the clearance of virus shedder (s) from the infected herds and thirdly, control measures to prevent the transmission of the virus within and between herds (Garoussi *et al.*, 2008).

Bovine viral diarrhea virus (BVDV) infection is an important risk factor for development of shipping fever pneumonia in feedlot cattle, and infects but does not cause morphologic evidence of damage to airway epithelial cells (Haddawi *et al.,* 2007).

While Serological studies were conducted and recorded in neighboring countries as in Kenya, where 19 % of adult cattle and 49% of grade cattle are serologically positive and in Zimbabwe where 64% of cattle were positive (Radostitis *et al.,* 1994), no or little recorded study on the prevalence of BVDV has been conducted in Ethiopia.

Based on the above indicated gaps, objectives of this study are

- To determine the seroprevalence of Bovine viral diarrhea Virus (BVDV) in feedlot and dairy animals
- Identify the possible risk factors for Bovine viral diarrhea Virus (BVDV)
- To compare milk with serum ELISA for detection of antibodies to Bovine viral diarrhea Virus

## 2. LITERATURE REVIEW

### 2.1. Definition

In 1946, Olafson and associates described gastroenteritis with severe diarrhea in dairy herds in New York State. Some affected animals developed ulcers in the nasal and oral mucosa. Abortions were also seen. Although morbidity rates seemed high, mortality rates were low. Bacteria were not found in blood or tissues that produced the same clinical symptoms in healthy cattle. Through transmission and immunity studies, the ailment was differentiated from rinderpest, which has similar symptoms. The new disease was termed Bovine viral diarrhea (Niza-Ribeiro *et al.*, 2005).

### 2.2. Etiology

Bovine viral diarrhea virus is classified in the genus *Pestivirus*, which also comprises classical swine fever virus (CSF) and border disease virus (BDV). Together with the genera *Flavivirus* and *Hepacivirus*, it forms the family Flaviviridae (Gennip *et al.*, 2008). From 1946 to 1960, bovine viral diarrhea and mucosal disease were regarded as distinct entities with many clinical features in common but with epidemiological differences (Paton *et al.*, 1999).

#### 2.2.1. Virus Morphology

Bovine viral diarrhea virus (BVDV) is a small, enveloped, single stranded, positive sense RNA virus of the *Pestivirus* genus within the family Flaviviridae. Two types of BVDV (BVDV-1 and BVDV-2; synonym *Pestivirus* 1 and *Pestivirus* 2) can be distinguished from one another and from the two other principal *Pestivirus* types, namely ovine border disease virus (BDV or *Pestivirus* 3) and classical swine fever virus (CSFV or *Pestivirus* 4). All of the *Pestiviruses* are serologically related and many can infect a variety of artiodactyl host. Recent data on genomic organization, virion composition and genomic RNA structure showed significant differences with the members of the Flaviviridae family (Pellerin *et al.*, 1994).

#### 2.2.2. Genomic organization

The genome of BVDV consists of a single strand of positive sense RNA which is about 12,300 nucleotides long. A single open reading frame exists which is approximately 3900 codons long

(Gennip *et al.*, 2008). RT-PCR has been proved to be a rapid and sensitive method to detect viral nucleic acids and this technique has been used by several investigators for the detection of *Pestiviruses* using oligonucleotide primers located in conserved regions of the viral genome.

Most of the PCR primers that were selected in the 5'UTR recognized the greatest number of *Pestivirus* isolates but failed to differentiate BVDV from other *Pestivirus*es. Based on the sequence comparison of 5'UTR, the BVDV isolates were subdivided in two genotypes (Letellier *et al.*, 1999). The open reading frame is flanked by 5' and 3' untranslated regions which are important for the initiation of translation and RNA stability. The open reading frame is translated into a single polyprotein which is then processed by both viral and cellular enzymes into mature viral proteins (Gennip *et al.*, 2008)

The BVDV genome encodes for both structural and nonstructural proteins. The structural proteins include the capsid protein C (p14) and three glycoproteins Erns, E1, and E2 (gp48, gp25, and gp53). The capsid protein functions to package the genomic RNA and to provide structure for the formation of the virion envelope. The three glycoproteins are associated with the lipid envelope. The E2 protein contains the major recognition sites for the production of neutralizing antibodies against BVDV by infected or vaccinated cattle. The genetic sequence which codes for part of the E2 protein is extremely variable between strains of BVDV which probably reflects selective pressure against these neutralizing sites by the immune system (John *et al.*, 2008)

Six nonstructural proteins are encoded by the noncytopathic BVDV genome. Npro (p20) is the first protein produced from the open reading frame. It has papain-like protease activities. The next nonstructural protein produced is NS23 (p125). This protein has several unique characteristics which suggest its involvement in multiple functions. These characteristics include a very hydrophobic domain, a zinc-finger, a protease, and a helicase. Cytopathic BVDV strains have changes within the coding region for p125 which result in the production of the protein NS3 (p80). This protein is unique to the cytopathic BVDV biotype. The NS3 protein of cytopathic BVDV contains the protease and helicase activity of the NS23 protein. Other nonstructural proteins include NS4A (p10), NS4B (p32), and NS5A (p58). Knowledge of these proteins is limited. However they appear to be important in viral replication. The final protein produced is

NS5B (p75) which is thought to be the RNA-dependent-RNA polymerase needed to replicate the viral genome (Gennip *et al.*, 2008).

Replication of BVDV begins with receptor mediated endocytosis into a cell. The E2 glycoprotein appears to mediate this step. Once in the cell, the viral RNA is released into the cytosol, RNA translation then begins with the 5' untranslated region serving as an internal ribosomal entry site. Viral proteins can be detected as early as three hours after cell infection (Lindberg *et al.*, 2002)

Following gene translation, the large polyprotein product is processed by both cellular and viral enzymes into mature proteins. Once the RNA-dependent RNA polymerase is produced, new genomic RNA is produced to be packaged into virus packages. Viral packaging occurs in either the golgi apparatus or endoplasmic reticulum where they acquire their lipid envelope through budding into the vesicle lumen. Mature virus packages are then released from the cell exocytosis. New virus can be released as early as 10 hours post cell infection (Yamane *et al.*, 2008)

2.2.3. Genetic variation

*Pestivirus*es have been typed using different genomic regions. For most studies a fragment of the 5 untranslated region (5'-UTR) was utilized, but also the regions coding for $N^{pro}$ and E2. Comparison of the phylogentic analyses showed that BVDVs form defined clusters (Vilcek *et al.*, 2003)

BVD virus is genetically variable; containing a single positive-stranded RNA genome with a size of approximately 12.3 kb having a single large open reading frame (ORF) flanked by 5'and 3'non-coding regions (NCR). The ORF encodes a polyprotein during viral replication, which is then processed by cellular protease and viral autoprotease into mature viral proteins arranged in the following order (from the N terminus to the C terminus): $N^{pro}$, C, $E^{rns}$, E1, E2, p7, NS23, NS4A, NS4B, NS5A and NS5B (Xingran *et al.*, 2006).

The nucleotide regions 5_-NCR, Npro and E2 are generally used in phylogenetic analyses of *Pestivirus*es. BVDV- 1 is one of the most important species in *Pestivirus*es and has been further divided into at least 11 subgroups from A to K on the basis of either 5_-NCR or Npro gene (Xingran *et al.*, 2006).

2.2.4. Antigenic variation

BVD viruses are considered to constitute one group, although serological differences do occur. These differences are evident when BVD strains escape neutralization by antibodies formed after vaccination (Bolin *et al.*, 1991). Dekker *et al.* (1995) could characterize different clusters *Pestivirus*, using a limited set of monoclonal antibodies, into four different antigenic groups namely BVDV, BDV, CSFV and 5250.

Antigenic analysis of BVDV isolates with a panel of 10 monoclonal antibodies revealed marked antigenic differences in the major envelope glycoprotein, gp53/E2, compared to standard laboratory and vaccine BVDV strains (Floresa *et al.*,2000).

2.2.5. Subtypes and biotypes

The genetic and antigenic diversity between different strains of BVDV is quite varied. However, it has become clear that two major groups of virus exist which differ significantly with respect to both their genetic and antigenic makeup. These two groups are referred to type I BVDV and type II BVDV. Although differences exist at the molecular level, the major aspects of the pathogenesis of disease caused by type I and type II BVDV appear to be the same. The major difference is that acute infections with type II BVDV have been associated with more severe clinical syndromes. The reasons for this increase in severity are unknown (Dekker *et al.,* 1995)

Moreover, classification of type I and type II BVDV are based on significant differences in the genetic sequence of the 5' untranslated region of the genome. Correlated with this grouping are differences in the antigenic makeup of the virus and the pathology manifested by the virus (Vilcek *et al.*, 2003). While infections of immuno competent cattle with BVDV-1 are generally subclinical, infections with highly virulent BVDV-2 strains can lead to severe haemorrhagic syndrome with high mortality (Carman *et al.*, 1998).

Likewise, a characteristic of BVDV which applies to how the virus interacts with cells in an artificial cell culture system is one criterion for biotype categorization. In cell culture, noncytopathic BVDV does not cause visible cell damage and death. However, cytopathic BVDV

causes characteristic visible cell damage and death. Cytopathic BVDV arises from genetic changes in noncytopathic virus (Ridpath *et al.,* 2006).

In nature, noncytopathic BVDV is the most common biotype isolated. It causes acute infections with various clinical outcomes, intrauterine infections of fetuses, and persistent infections. Isolation of cytopathic BVDV in association with acute infections or intrauterine infections is rare. Cytopathic BVDV has not been shown to cause persistent infections. Cytopathic virus is most important in the pathogenesis of mucosal disease (OIE, 2004).

Biotype does not correlate to virulence in acute infections as BVDV strains associated with severe acute BVD outbreaks are all noncytopathic based on their growth characteristics in cultured epithelial cells. The practical significance of biotype is that, invivo, noncytopathogenic viruses may establish persistent infections following in utero infection but cytopathogenic viruses do not. Noncytopathogenic viruses predominate in nature. Cytopathogenic viruses are relatively rare and usually found in association with outbreaks of mucosal disease, a relatively infrequent but highly fatal form of BVDV infection (Ridpath *et al.,* 2006).

**2. 3. Epidemiology**

Bovine viral diarrhea was first described in New York, United State of America, in 1946 (Radostitis *et al.,* 1994; Afaleq *et al.*, 2007). A significant consequence of BVDV infection, from the epidemiologic standpoint, is the birth of calves persistently infected (PI) with BVDV as a result of transplacental infection of the fetus from pregnant cows that become infected with NCP strains during the first trimester of gestation. PI animals shed large amounts of BVDV into the environment throughout their life and thus, represent a mechanism by which the virus persists and spreads among cattle populations. The detection and elimination of PI cattle, along with vaccination, are necessary for control of BVD in cattle herds (Saliki *et al.*, 1997)

2.3.1. Geographical distribution

Diseases caused by BVDV have been recorded in most countries where cattle are reared. The infection is worldwide in distribution and is regarded in some countries as the single most

important virus infection of cattle. The prevalence of infection is high but the incidence of the disease is low (Radostitis *et al.,* 1994).

2.3.2. Host range

The classification of virus types was made according to the host-species that was infected. *Pestivirus*es are, however, able to cross the species barrier. BVDV can cross-infect cattle, sheep, goats and pigs. BVD is a bovine pathogen that occasionally infects pigs. Differentiation between CSFV and other *Pestivirus*es can be accomplished by the use of CSFV-specific monoclonal antibodies (Mabs) but the search for ruminant *Pestivirus*-specific Mabs has failed due to a great antigenic diversity (Letellier *et al.*, 1999).

BVDV also infects pigs, alpacas, sheep, goats, and wild deer. Of these species, BVDV infection of sheep and goats could be of most importance in transmission to cattle because of the frequent pasturing of these species in common. Several outbreaks of BVDV infection in sheep have been reported, where the most common manifestation of the disease is reproductive losses (Radostitis *et al.,* 1994).

2.3.3. The Role of carrier animals

Persistently infected (PI) animals have an important role in the perpetuation of BVDV infections. These animals multiply BVDV at a high rate for months or years and have therefore, been described as BVDV producing factories. Although it might be thought that this considerable virus multiplication would generate diversity, limited studies have, on the contrary, failed to detect antigenic or genetic changes in viruses isolated at different times from persistently infected (PI) animals. These animals may have reduced viability both in utero and after birth, but a proportion of PI animals survive, acting as an important reservoir of infection for other cattle This is consistent with the hypothesis of immunologically unimpeded multiplication of the resident virus and the immune elimination of emerging variants (Paton *et al.*, 1999).

This contrasts with acute infections which would favour variant BVDV able to escape the immune response. If such selection really occurs, resulting effects should be particularly visible on the 53 kd glycoprotein (E2 gene), known to play a role in protective immunity (Hamersa *et*

*al.*, 1998). Animals with persistent BVDV infection are the major source of the virus to infect non immune animals (Radostitis *et al.,* 1994).

Transplacental infection of the fetus with a noncytopathic BVDV strain during the first trimester of gestation may lead to persistently infected, immunotolerant calves. When these animals are superinfected with an antigenically similar cytopathic strain, they may suffer of the fatal mucosal disease (Letellier *et al.*, 1999)

2.3.4. Mode of transmission

The virus is transmitted by direct contact between animals, and by transplacental transmission of the fetus. The virus can be isolated from nasal discharge, saliva, semen, feaces, urine, tears and milk, each of which would allow wide dissemination of the virus. Discharge from the reproductive tracts of an infected cow, either persistently infected or systematically immune, including aborted fetuses, can be sources of the virus (Radostitis *et al.*, 1994). Since BVDV is immunosuppressive, it causes increased susceptibility to other infectious agents (De Melo., 2004).

**2. 4. Pathogenesis**

Acute infection is characterized by a period of systemic viral infection and virus shedding, which is followed by recovery and development of long lasting immunity. Although acute infection is frequently unnoticed, it can cause or contribute to a variety of problems, including diseases of the reproductive, respiratory, and enteric systems (Paton *et al.*, 1999). The clinical cases affected young animals (8-18 months old) and were characterized by diarrhea, respiratory signs, extensive oral and digestive tract erosions, conjunctival and vulvar congestion, occasional digestive bleeding and vulvar and heart petechial hemorrhages (Floresa *et al.*, 2000).

In cases of so-called virulent BVDV, there may be haemorrhagic disease and significant mortality in both young and adult animals. BVDV readily crosses the placenta and can cause infertility and embryonic or fetal death. Fetuses infected before the onset of immunocompetence can become persistently infected. (Paton *et al.*, 1999)

The interaction of BVDV with secondary pathogens is thought to be one of the contributing factors in Bovine respiratory disease complex (Ridpath *et al.,* 2007).

**2.5. Immune Response**

Most cattle which are exposed to the virus seroconvert and have antibody present for the remainder of their life, being virus free. Calves born to cows which are seroconverted gain antibody from colostrum which will be found at blood-test but which wanes in the first six months of life. (Paton *et al.*, 1999).

The two host factors associated with BVDV infecition are the immune status of the heifer entering into pregnancy and the physiologic immune suppression which occurs during pregnancy (Evermann, 2002).

Neutralizing antibody to BVDV has been widely used as an important parameter to evaluate the immune responses for both vaccination and natural infection. T cell assays for antigen-specific cell-mediated immune (CMI) responses have more recently become available and can provide valuable information about another important aspect of the immune response. Studies indicated successful development of CMI responses without active antibody responses in calves exposed to virulent BVDV in the face of high-titered, colostrum-derived maternal immunity. The seronegative calves with an active CMI response were protected from challenge with virulent BVDV after their maternal antibody titers became undetectable (Platt *et al.*, 2008).

Recognition of BVDV antigen by T- cells was determined by evaluating the proliferation of either or purified $CD_4$ or $CD_8$ T cells under standard condition (5 days at 37 $^0$C in a $CO_2$ gassed followed by pulsing with H-methyl thymidine) for 18 hours and evaluation of incorporated radioisotopes. In these assays, virus-specific lymphoproliferation was detected earlier in animals that had been infected with cythopatic than in those infected with noncythopatic virus. $CD_4$ and $CD_8$ T cells from recovered animals were found to recognize virus infected cells whereas non infectious viral antigen was recognized only by $CD_4$ T cells. These responses were strain cross reactive and MHC restricted (Collen *et al.*, 2002).

## 2.6. Clinical Signs

### 2.6.1. Acute infections

Acute infections of cattle occur particularly in young animals, and may be clinically inapparent or associated with diarrhoea. Affected animals may be predisposed to concurrent infections, for example respiratory disease, perhaps due to an immunosuppressive effect of the virus. Bulls may suffer a temporary depression of fertility and can show transient shedding of virus in the semen. Cows may also suffer from infertility, likely associated with changes in ovarian function and secretions in gonadotrophin and progesterone. During acute infections, a brief viraemia may be detectable and nasal shedding of virus may occur. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals (Letellier *et al.*, 1999; OIE, 2004).

A serological response is the most certain means of diagnosing a previous infection. The clinical picture is generally one of high morbidity and low mortality, though more severe disease is sometimes seen. In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries and infection with Type 2 viruses in particular has been demonstrated to cause altered platelet function. Other acute outbreaks may show fever, pneumonia, diarrhoea and sudden death in any age group, with haemorrhagic signs (Letellier *et al.*, 1999; OIE, 2004). Mucosal disease is characterized by severe erosive to ulcerative lesions at the mucosal surfaces and depletion of lymphoid follicles in lymphoid tissues (Liebler *et al.*, 1995).

### 2.6.2. Congenital infection

If non cytopathogenic virus infects the bovine fetus, this may result in abortion, stillbirth, teratogenic effects or a congenital infection that persists in the neonatal calf. Confirmation that an abortion is caused by BVDV is often difficult to establish, but virus may be isolated from the fetal tissue in some cases, or viral antigen or genome may be demonstrated. An attempt should also be made to detect specific antibody in samples of fetal fluids or serum, or in the supernatant fluid from a tissue suspension. Stillbirths or teratogenic effects may be associated with an active fetal immune response to the virus during mid-to-late gestation. The dam will often have high

antibody titers (>1/2000) to BVDV, which is suggestive of fetal infection and is probably due to the fetus providing the dam an extended challenge of virus (OIE, 2004)

Although congenital infection with BVDV often leads to abortion, it is not always recognized in the field. Infection during the first third of the gestation period can result in the abortion of a conceptus that is small and goes unnoticed by the farmer. The cow would return to service and the failure to maintain pregnancy would be classified as an example of early embryonic death. Another possible outcome of infection is the death and subsequent resorption of fluids from the fetus that results in mummification. It is frequently observed that aborted fetuses have subcutaneous oedema and copious pleural and peritoneal effusions. There may also be congenital abnormalities that result in growth retardation and in selective central nervous system (CNS) defects, such as cerebellar hypoplasia and dysmyelination, and eye defects, such as cataracts. Sometimes there are skeletal defects, the most advanced of which is arthrogryposis (OIE, 2004). The congenital infection is usually caused by non-cytopahogenic strains of BVD virus. In the case of congenital infection, and before the fetus becomes immuno-competent, the virus attacks the pregnant cow causing death and abortion of the foetus, mummification, stillbirths and teratogenesis (Al-Afaleq *et al.*, 2007)

Stillborn calves are a common sequel to congenital infection. The calves usually appear to be fully developed at parturition, but fail to survive. After day 150 of pregnancy, the immune system of the fetus will be developed and infection of the fetus will usually result in an antibody response and the birth of a normal calf. (OIE, 2004; Munoz-Zanzi *et al.*, 2003)

2.6.3. Persistent viremia

PI claves result when a pregnant, susceptible cow is exposed to the virus between 40 and 125 days of gestation. If a cow is exposed before that time she will return to heat, and after that time she will either abort or have a weak calf. During the 40 to 125 day range, however, the fetus is inventorying its tissues for the development of the immune system and it categorizes the virus as self tissue. Because of this, it will never be able to respond immunologically to the virus, and when born becomes a super shedder of the virus. Normal calves that become infected usually get sick and can shed from 1 thousand to 10 thousand virus particles per day in the nasal secretions.

PI calves, may appear perfectly normal, but they will be shedding from 1 million to 10 million virus particles per day for their entire life (Radostitis *et al.*, 1994).

When infections of the fetus occur before approximately 110 days of gestation and before immunocompetence, the calf may be born with a persistent viraemia. Identification of these animals is readily made by detection of noncytopathogenic BVDV in blood. The virus can also be identified in the skin by immunohistochemistry. Furthermore, animals with a persistent viraemia will also lack specific antibody, but diagnosis in the young calf, up to approximately 3 months of age, may be confused by the presence of maternal antibody to BVDV. Maternal antibody may also interfere with virus isolation. In older animals with persistent viraemia, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) 'heterologous' (antigenically different) from the persisting virus. To confirm a diagnosis of persistent viraemia, animals should be retested after an interval of at least 3 weeks. (OIE, 2004)

Observations in the field as well as results from recently performed experimental studies indicate that transiently infected animals are very inefficient in transmitting the virus. The main route of transmission with in an infected herd seems to be one-way: from PI animals to susceptible animals (Lindberg and Alenius, 1999, Houe, 1999)

**2. 7. Diagnosis**

Since most of the ruminant *Pestivirus*es share the basic properties essential in the pathogenesis and epidemiology of BVD, diagnostic methods should be designed to recognize the whole range of viruses isolated from cattle. Together with the better understanding of the disease complex induced by BVDV, the currently available diagnostic tests have made disease control projects based on detection and removal of viremic animals from unvaccinated populations as realistic options to the more conservative vaccination and non-intervention strategies for control of BVD (Sandvik, 1999).

Currently there are a variety of diagnostic tests used to detect PI animals, including virus isolation from the blood or tissues, immunohistochemical staining of the skin samples, reverse transcriptase–polymerase chain reaction (RT-PCR), microtiter virus isolation, Enzyme-Linked

Immunosorbent Assay (MTVI-ELISA) and Antigen Capture Enzyme Linked Immunosorbent Assay (AC-ELISA) (OIE, 2004; Susan *et al*., 2006)

2.7.1. Virus isolation

BVDV is not among the most fragile viruses, but still, reliable results from virological testing can only be obtained with suitable sample material. If clinical cases are to be investigated for BVDV, the choice and handling of samples may influence the success of assays for BVDV (Sandvik., 1999).

In principle, three different methods for detection of BVDV can be distinguished. The virus may be cultivated for one or more passages in cell cultures, which subsequently are fixed and stained with virus-specific antibodies for visualization of ncp BVDV. Alternatively, BVDV antigens can be detected with specific antibodies directly in organ samples without propagation of the virus. Using molecular biological methods, viral RNA can also be detected in a variety of biological samples (Sandvik., 1999).

2.7.1.1. Virus isolation in cell cultures

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). Growth of both biotypes is usually satisfactory. Noncytopathogenic BVDV is a common contaminant of fresh bovine tissue, and cell cultures must be checked for freedom from adventitious virus by regular testing. Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked before routine use. Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free (OIE, 2004).

The fetal bovine serum that is selected for use in cell culture must also be free not only from virus but also from BVDV neutralising antibody. Heat treatment (56°C for 30-45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation at 25 kilo Grays is more certain. Commercial batches of fetal bovine serum mostly test positive by PCR even after the virus has been inactivated by irradiation. Where appropriate, horse serum can be substituted

for bovine fetal serum, although it is often found to have poorer cell-growth-promoting characteristics (OIE, 2004).

2.7.1.2. Detection of viral antigens

The basic principle of this diagnostic method is identical to the cell culture propagation /immunostaining procedure referred to above, but instead of cultured cells for production of antigen, preformed antigen is either detected inside or extracted from cells taken from the host animal (Sandvik, 1999).

Immunohitochmistry

By morphological recognition of cytoplasmatically located BVDV antigens in infected cells, immunohistochemical examination of tissue sections provides a more specific diagnosis than detection of extracted antigens in a solution. Test results from IHC examination of skin biopsies has correlated well to findings from blood testing, but tissue samples are more difficult to obtain from live animals than blood. Immunohistochemical staining of tissue sections from brain cortex have also given reliable test results for calves PI with BVDV. These methods are most promising for postmortem diagnosis of persistent infection with BVDV, but should be first evaluated on samples from acutely infected cattle (Sandvik., 1999).

Enzyme-Linked Immunosorbent Assay for antigen detection

Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published 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. The test is suitable for detection of persistently infected animals, and usually measures BVD antigen in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISA is able to detect BVD antigen in the blood as well at in plasma or serum samples. The best of the methods gives sensitivity similar to virus isolation, and may be preferred in those rare cases where persistent viraemia is combined with seropositivity. The ELISA can have low sensitivity in the presence of BVDV cloistral antibodies. In the presence of antibodies,

reverse transcription PCR (RT-PCR) should be used as it is the most sensitive detection method for this circumstance. The antigen ELISA appears to be less useful for virus detection in acute BVD infections. (OIE, 2004)

2.7.1.3. Nucleic acid detection

The RT-PCR method can be adapted to the detection of BVD viral RNA for diagnostic purposes. This may have a special value where low-level virus contamination is suspected, for example in screening batches of fetal calf serum, or biological products such as vaccines. Caution is needed in the interpretation of results, as the detection of viral RNA does not imply that infective virus is present. 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. Due to the more conserved nucleotide sequences in the 5' untranslated region and the NS3 gene of BVDV, RT-PCR assays using primers specific for these regions have offered the best epidemiological sensitivity (Sandvik., 1999; OIE, 2004).

Newer methodologies incorporate the use of probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate among *Pestivirus*es. Testing for virus after inoculation of cell cultures using PCR should be avoided as it may give positive results if commercial bovine fetal serum has been used in the growth medium. Primers should be selected in conserved regions of the genome, such as the 5'-noncoding region, or the NS3 (p80 gene). Molecular tests can be prone to contamination in unskilled hands. Stringent precautions should therefore, be taken to avoid DNA contamination in the test system, and rigorous controls must be mounted (OIE, 2004).

2.7.2. Detection of Immune Responses against BVDV

Immune responses against an infectious agent are classified as cellular or humoral, and immunity may be acquired actively by infection or passively by transfer of colostral antibodies. Cellular immunity, measured as proliferation of peripheral blood mononuclear cells after *invitro* exposure to BVDV, has been demonstrated for seropositive cows, but not for a calf believed to be passively immunized with colostral antibodies .However, most studies on detection of immune responses against BVDV have concentrated on demonstration of antibodies to the virus, which

can be detected in serum from 3 weeks, and thereafter for years after acute infection of immunocompetent animals (Sandvik., 1999).

BVDV specific antibodies can be classified in two functional groups. Antibodies to viral glycoproteins (primarily E2 or gp 53) may block the infectivity of or neutralize the virus. These antibodies cross react with other strains of BVDV, but if sera raised against one virus strain are tested against other challenge viruses, different antibody titres may be obtained (Brock, 1995). Conversely, the highly immunogenic non-structural viral protein NS2-3 (p125), which is essential for the intracellular replication of the virus, is antigenically conserved between all *Pestivirus*es. Antibodies to NS2-3 do not neutralize BVDV, but since they can be readily detected by other serological tests, this antigen is important in BVDV serology. If effective recombinant sub-unit vaccines against BVDV become available, simultaneous vaccination and anti-NS2-3 serology for monitoring of natural infection with BVDV should be possible (Sandvik., 1999).

2.7.2.1. Virus neutralization test

Virus neutralization (VN) assays are sensitive and specific assays for detection of antibodies to BVDV, and have long been recognized as the reference test for BVDV serology. Today, the test is usually performed in 96 well microtitre plates, where serially diluted test sera are incubated with a cytopathogenic challenge virus before susceptible bovine cells are added to and incubated with the neutralized virus for 4 days (Edwards, 1987).

Because of the high specificity of the test, it is essential to use a challenge virus antigenically similar to the field viruses in the population to be tested. The VN assay requires substantial investments in selection and monitoring of cell cultures and media to give satisfactory test results, and is therefore, not suitable for examination of few or sporadic samples.

2.7.2.2. Enzyme immunoassays

Both indirect and blocking types of test can be used. A number of commercial kits are available. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for

harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents (OIE, 2004).

Two principally different protocols are possible, activity amplification or activity modulation systems. Among commonly used activity amplification systems are indirect ELISAs, where antigen is immobilized and used to trap specific antibodies, which subsequently are detected by enzyme-conjugated species-specific antiglobulins and a chromogenic substrate. In activity modulation systems, virus-specific antibodies in the test sample compete with or block the binding of conjugated virus-specific antibodies, resulting in a lowered signal for positive samples (Paton *et al.*, 1991; Sandvik, 1999).

Among problems observed with activity amplification ELISAs for BVDV serology are high and variable background signals caused by contaminations in the test antigen, or cross reactivity of the enzyme-conjugated anti globulins resulting in low signal-to-noise ratios. Activity modulation ELISAs based on blocking of enzyme-conjugated Mabs can be designed to have a high analytical specificity, but it is essential that the employed Mabs recognize all virus strains in order to ensure a sufficient epidemiological sensitivity (Sandvik, 1999).

2.7.2.3. Other serological tests

Many serological tests have been adopted for BVDV serology, including immuno- diffusionin agar gel, complement fixation, indirect immuno-fluorescence, and western blotting (Sandvik, 1999).

## 2. 8. Prevention and Control

Recently, some European countries have started eradicating BVDV infection either in single herds or on a nation-wide basis. In Norway, BVD became a notifiable disease in 1989. The Norwegian BVDV control programme includes identification of positive herds, and culling of the PI animals. Sweden started a control and eradication program on a voluntary basis in 1993, which includes monitoring of herds by annual bulk milk surveys (Grom and Barlic-Maganja, 1999)

The decision to vaccinate will usually be based on a risk-cost analysis. If the risk that a herd will get infected is high, and consequently the expected economic loss is also high, the recommendation will be to vaccinate. Considering the high prevalence of BVDV that causes high economic losses, BVDV vaccination of cattle herds is certainly indicated, provided efficacious and safe vaccines are available (Oirschot Van *et al.*, 1999).

While variation in virulence among BVDV2 strains has been extensively reported, much less information is available on variation in virulence among BVDV1 strains. In the United States, the BVDV1 strainNY-1 has been made available by the USDA as a challenge strain for evaluating efficacy of vaccine protection against BVDV1. However, this strain is also present in at least one commercial vaccine (Ridpath *et al.*, 2007)

The ability of modified live BVDV I and II components of a commercially available modified live virus (MLV) vaccine is to prevent fetal infection and abortion, and therefore, the birth of persistently infected animals. Heifers immunized with vaccine 4–8 weeks before insemination showed no adverse effects. All vaccinated animals had seroconverted to BVDV 4 weeks after immunization (Kovacs *et al.,* 2003). Modified live virus vaccine should not be administered to pregnant cattle (or to their suckling calves) due to the risk of transplacental infection (OIE, 2004).

**2. 9. Economic Importance of Bovine viral diarrhea**

BVD infection in a dairy herd can result in large economic losses, primarily due to reproductive problems in infected cattle, decreased overall animal health, and decreased milk production (Houe, 1999; Heuer *et al.,* 2007). BVD causes two types of bovine viral diarrhea virus (BVDV) infection is an important risk factor for development of shipping fever pneumonia in feedlot cattle, and infects but does not cause morphologic evidence of damage to airway epithelial cells (Al-Haddawi *et al.*, 2007). Bovine viral diarrhea (BVD) is considered an economically important disease and attempts at costing BVD have been made using empirical data from case studies of dairy herds in a number of areas (Gunn *et al.*, 2004).

Acute clinical BVD can cause a significant drop in milk production during the course of the illness. In an outbreak of acute clinical BVD in a 273 cow herd in the UK, around 40% of the

adult cows were affected and the average daily total yield of milk dropped from 7,500 litres to 5,724 litres (23%) by a fortnight after the onset of the disease (David *et al.*, 1994).

Although some outbreaks can be devastating for the individual producer, calculations of the national losses are more relevant as a basis for deciding between control and eradication within a particular country. However, due to the variation among losses in individual herds, these calculations can only be approximated. The losses at the population level have been estimated in the range of USD 10-40 million per million calvings. In Denmark, with an estimated annual incidence of acute infections of 34%, the total annual national losses in 1992 were estimated to be USD 20 million (Houe and Palfi, 1993).

### 2.10. Situation of BVDV in Ethiopia

The overall disease situation coupled with the impact of BVD infection in dairy and feedlot cattle in Ethiopia is not yet studied. However, the silent nature of the disease, in adult cattle, could be deceptive to field veterinarians, who can only see frequent affection on the fetuses. Such affections as abortion, stillbirth, malformations and birth of weak calves that die within few days, can be manifestations for other diseases such as blue tongue and akabane. Hence, it is pertinent that, the actual situation of BVD should be investigated in dairy as well as feedlot farms in the country (Al-Afaleq *et al.*, 2007).

# 3. MATERIALS AND METHODS

## 3.1. Description of the Study Area

The research was conducted in one district, namely Adama woreda, of the East Shoa Zone of Oromia Regional State. It is located about 94 from East of Addis Ababa in an altitude of about 1850 m.a.s.l. The agroecological conditions including the soil and the climate are similar to many highland areas in Ethiopia. The main rainy season occurs between June and September with an average rainfall of 800 mm. There is also a short rainy season from March to May. The annual average temperature ranges from 12.3 $^{0}$Cto 27.7 $^{0}$C with an average of 18.7 $^{0}$C. Highest temperatures are in May.

According to the study made by the Central Statistical Agency, the livestock populations in the East Shoa Zone is approximately 1,204,478 million cattle, 702,544 sheep and 733,537 goats (CSA, 2008).

## 3. 2. The Study Population, Study Type and Sampling Technique

### 3.2.1. The Study Population

In Adama, feedlot and dairy animal keeping is a widely practiced. Cattle kept for fattening purpose are mainly for export purposes. The main breed of these animals is Boran breed whose average body weight and age range between 200 to 500 and 3 to 7 years, respectively.

In Adama, there are two dairy cooperatives namely Awash Dairy Cooperatives with a membership of 120 and Yakla Micro Dairy cooperatives with 105 members. These cooperatives supply raw milk to Holland and Mama dairy processing industries in Debre Zeit and Sebeta, respectively. Each dairy farmer has two or more lactating Holstein Friesian crosses or local breeds of cows. The estimated average milk yield is 7.5 liters/ day/ cow. The number of lactating cows ranges from 1 to 10, with an average of 5 dairy animals per farm. The dairy farmers deliver the milk to the nearest milk collection centers of the aforementioned dairy cooperatives or sell locally. They may also use it for home consumption.

3.2.2. Study Type

The study was a cross-sectional study to determine the seroprevalence of BVDV virus antibodies using the indirect Enzyme Linked Immuosorbet Assay (ELISA) on milk and sera samples collected from feedlot and dairy cattle in and around Adama. The risk factors associated with the occurrence of BVD virus antibodies were collected using a standard questionnaire.

The sampling method for the dairy herds was the one stage cluster sampling. Samples of clusters (dairy herds) were selected first and all lactating cows in each herd were sampled. A list of dairy farms and cattle were acquired from the offices of the two cooperatives.

In feedlot, however, the sampling method employed was the two stage cluster sampling where a sample of clusters (feedlot herds) were selected first and random sampling of cattle was then employed. A list of feedlot herds were obtained from the Adama Animal quarantine office of the Ministry of Agriculture and Rural Development (MoARD).

3.2.3. Sample Size

The sample size determination was made considering the 225 and 170 dairy and feedlot herds in the study area, respectively. 95% confidence interval, 5% expected error and 50% expected prevalence were taken under consideration. The sample size was determined using the Win Episcope 2.0 version software. The adjusted sample size became 110 herds. Through proportional allocation to maximize precision, 40 herds from the feedlots and 70 herds from the dairy herds were selected.

**3. 3. Sample Collection**

During the study, a total of 110 herds (70 from dairy and 40 from feedlot production systems) were selected and sampled between November 2008 and January 2009. 402 and 372 serum samples from feedlot and dairy animals as well as 415 milk samples of which 372 were matched samples from the dairy herds were collected.

3. 3. 1. Serum samples

Whole blood samples were collected from the jugular veins of selected lactating by venopuncture using a 10 ml sterile plain vacutainer tubes. The latter were then stored overnight at room temperature for serum collection. Each tube was labeled with identification number. Data including owners name, herd size, species, age, sex, housing, body condition, vial number and other relevant information were recoded.

The blood was allowed to clot for at least four hours in a shed. The serum was then transferred into a sterile cryovial bearing the identification number, site, age and sex and transported in an icebox to the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta for analysis. In the laboratory, the serum was stored at – $20^0$c until laboratory analysis as recommended by the OIE. (2004).

3. 3. 2. Milk samples

Milk sample was collected from individual lactating cows in a sterile tube. The milk was transported in an ice-box to the NAHDIC for laboratory analysis. The milk samples were centrifuged at 1000 × *g* for 10 min. Skimmed milk from beneath the cream layer was obtained from the milk samples and stored at −20°C until analyzed. At analysis, thawed skimmed milk aliquots of milk samples were assayed for the presence of antibodies to BVDV, using a commercially available indirect ELISA.

**3. 4. Laboratory Diagnosis**

3. 4. 1. Enzyme-Linked Immunosorbent Assay for Antibody detection

Enzyme-linked immunosorbent assays (ELISAs) are versatile diagnostic methods which can be designed to detect immunoreactive molecules. For prevalence studies, ELISA are applied to samples of serum or milk. For BVDV serology they have become popular for several reasons. They are independent of cell cultures, can easily be applied for mass screening, test results are read in few hours (Sandvik, 1999).

The test was conducted using the commercial ELISA kit following the procedure supplied by the manufacturer, Svanova Biotech AB, Uppsala, Sweden.

The ELISA steps were sequentially as follows;

Serum Samples

-100µl of PBC tween-buffer was added to each well that were used for serum samples and serum controls

-4µ of positive control serum and 4µl of negative control serum were added, respectively to selected wells coated with BVD antigen

-4µl of serum samples were added to a selected well coated with BVDV antigen

Milk Samples

-100µl of positive control milk and 100µl of negative control milk respectively to selected wells coated with BVDV antigen

-100µl of skim milk sample was added to selected wells coated with BVDV antigen

-The plates were shaken thoroughly, sealed and incubated at 37 $^{o}$ C for 1 hour.

-The plates were rinsed three times with PBS Tween Buffer. The wells were filled up at each rinse, emptied the plate and tap hard to remove all remains of fluid.

-100µl of HRP conjugate to each well and incubate at 37 $^{o}$ C for 1 hour.

-The plates were again rinsed three times with PBS Tween Buffer. The wells were filled up at each rinse, emptied the plate and tap hard to remove all remains of fluid.

-100µl of substrate solution was added to each well and incubated for 10 minutes at room temperature. The timing began after the first well was filled.

-The reaction was stopped by adding 50µl of stop solution to each well and mixed thoroughly.

-The optical density (OD) of the controls and the samples was measured at 450 nm in a microplate photometer. The OD was measured within 15 minutes after the addition of stop solution to prevent fluctuation in OD values.

Result interpretation

Serum

Calculation of cut – off

A=OD value of negative control X 2.0

If A ≥0.2; A was used as a cut-off

If A≤o.2; 0.2 was used as a cut-off

M ilk

Calculation of cut – off

A=OD value of negative control X 2.0

If A ≥ 0.1; A was used as a cut-off

If A≤ 0.1; 0.1 was used as a cut-off

### 3.5. Data analysis and management

The data was entered and managed in MS Excel and SPSS software programs. The SPSS version 15 was applied for the data analysis. Milk and serum prevalence estimation of BVD in cattle was done. Its association with a number of potential risk factors were determined by Chi-square ($\chi^2$) tests and Odds Ratio (OR) obtained using the logistic regression were undertaken between the milk and serum. Descriptive statistics such as percentages and frequency distributions were used to describe the characteristics of the data. A Pearson's correlation coefficient and Kappa statistics

were calculated to determine the correlation and the agreement between individual milk and serum prevalence results. The $\alpha$=0.05 was used as the statistical significant level.

## 4. RESULTS

Cattle sera and milk were stratified according to herd size, age and sex for statistical analyses. Overall, 70.9 % of the herds tested positive for BVDV virus antibodies to the milk or the serum ELISA tests. While, individual seropositivity and milk positivity were 35.9 %and 17.1 %, respectively.

Both production systems namely feedlot and dairy, were found seropositive (with at least one reactor) to BVDV antibodies. The individual seroprevalence for each production system was 38.4% in dairy and 33.6% in feedlot farms. Likewise, 70% and 72.5% of dairy and feedlot farms were positive (at least one sero or milk positive animal is present in the herd). However, the difference in the seropositivity of individual animals among the two production systems and the difference in herd positivity was not significant ($p>0.05$) (Table 1).

Table 1. Summary of seroprevalence of bovine viral diarrhea virus antibodies in individual cattle of the Adama area on production system category

| | Production system | Number of animals tested | Seroprevalence (%) | 95% Confidence Interval | P-value |
|---|---|---|---|---|---|
| Individual | Dairy | 372 | 38.4 | 33.4 - 43.34 | 0.091 |
| | Feedlot | 402 | 33.6 | 28.9 – 38.2 | |
| Herd | Dairy | 70 | 72.5 | 62.03 – 82.96 | 0.480 |
| | Feedlot | 40 | 70 | 55.8 – 84.2 | |

With regard to the herd size, irrespective of physiological status, a herd with at most 5 animals was considered a small herd and medium ones with up to 15 while a herd with more than 15 animals was considered as a large one. Considering at least one positive reactor in a herd, the overall herd positivity for BVDV antibodies were 70.9% (Table 2). The positivity was found to increase with an increase in the sized herds ranging from 14.1% and 33.3% in the small and medium sized herds, respectively, and 52.6 % in large herds. The prevalence difference was significant ($\chi^2=15.377$, df= 2, $p=0.000$) among the different herd size categories.(Table 2)

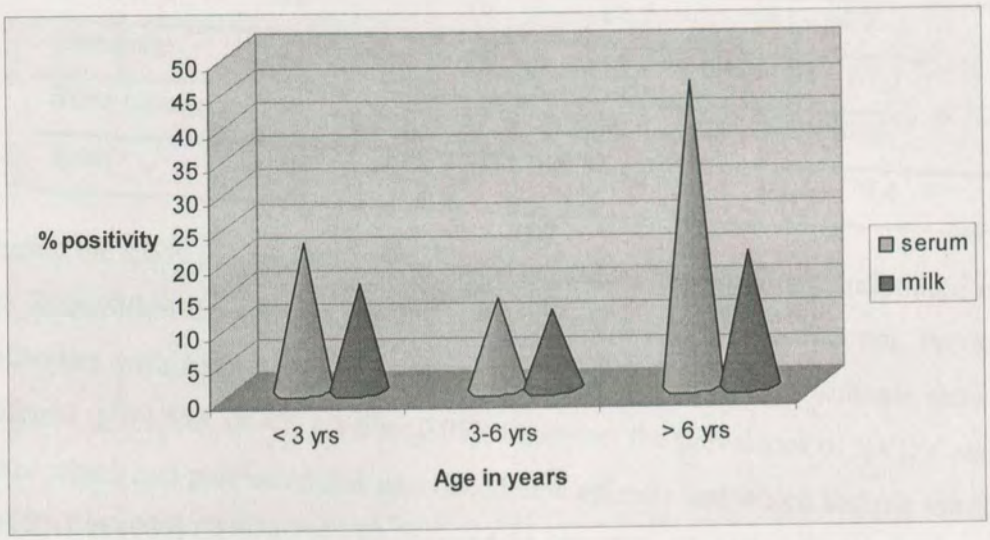
The multivariate analysis of risk factors also indicated that large and medium size herds had a 9.3 (95% CI = 2.44 - 35.47) and 7.09 (95% CI = 2.16 – 23.3) more likely chance of being positive than the small one as indicate in Table 6.

Table 2. Summary of prevalence of BVD virus antibodies in cattle of the Adama area on herd size basis

| Herd size | No of herds tested | No positives | Prevalence (%) | 95% CI | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|
| Small | 21 | 11 | 14.1 | 7.59 – 20.6 | 15.377 | 0.000 |
| Medium | 44 | 26 | 33.3 | 24.4 – 42.1 | | |
| Large | 45 | 41 | 52.6 | 43.2 – 61.9 | | |
| Total | 110 | 78 | 70.9 | | | |

The animals were categorized into three age groups; less than 3 years (calves and heifers), 3 to 6 years (young), and more than 6 years (adult). Age specific seroprevalence increased from 21.7% (95% CI= 18.7% - 24.6%) in heifers and calves to 45.2 % (95% CI= 41.5%-48.5%) in adult animals with more than 6 years of age. The seroprevalence difference was significant ($\chi^2$= 68.36, df= 2, $p<0.001$) among age categories. In the mean time, milk prevalence also increased from 15.8 % (n=415) in younger cows to 20.2 % (n=415) in older cows with more than 6 years old. However, the difference in the milk prevalence among the different age categories was not significant ($p>0.05$).

Figure 1. Summary of milk and seroprevaleces of BVDV antibodies in cattle of Adama area on age category




Logistic regression analysis for age and seropositivity provided an Odds Ratio (OR) value of 5.3(95 % CI= 3.46-8.218) for age group of >6 years compared to animals less than 3 years of age. The association of age as a risk factor for seropositivity was significant ($p<0.05$).

The differences of the herd positivity among the three management systems namely extensive (animals graze communally outside their houses and return back at nights), intensive (animals are managed indoors with little or no chance of leaving their stalls) and semi intensive (animals are managed housed but occasionally left freely to graze and water outside their shelters) was significant ($\chi^2$ =6.723, df=2, $p<0.05$). In the mean time, while cattle managed under the extensive system were found to have a higher positivity than the intensiveelly managed ones in the univariate analysis, the multivariate analysis indicated that there was no difference in positivity among the three management systems as indicated in Table 6.

Table 3. Summary of prevalence of BVDV antibodies on management basis in cattle of the Adama area.

| Management system | No of herds tested | No positives | Prevalence (%) | 95% CI | $\chi^2$ | P value |
|---|---|---|---|---|---|---|
| Extensive | 18 | 16 | 88.9 | 83 - 94.7 | 6.723 | 0.038 |
| Intensive | 43 | 25 | 58.1 | 48.8 – 67.3 | | |
| Semi intensive | 49 | 37 | 75.5 | 67.4 -83.5 | | |
| *Total* | 110 | 78 | 70.9 | 62.4 – 79.4 | | |

During the study period, 28 (25.5%) of the herds had purchased and introduced new animals into the farm within the past six months while 82 (74.5 %) of them had not. Prevalence of BVDV antibodies with respect to the purchase and introduction of new animals showed significantly different ($\chi^2$=3.553, df =1, p value<0.05). Likewise, the prevalence of BVDV antibodies in those farms which had purchased and introduced new animals and which had not introduced new ones was 85.7 % (95% CI =79.2% - 92.2%) and 65.9% (95% CI=57% - 74.7%), respectively.

With regard to the possibility of contact with other species of animals, 97 (88.2 %) and 13 (11.8 %) of the herds had a high and very low possibility of contact with other species of animals, respectively. Herd positivity was higher in herds which had a higher possibility of contact with other species of animals, 74.2 % (95% CI = 66% - 82.3%) compared to those which had very low possibility, 46.2 % (95% CI =36.8% - 55.5%). The difference in herd positivity was statistically significant ($\chi^2$= 4.38, df= 1, p value= 0.043).

Table 4. Summary of herd positivity of cattle in Adama area with respect to introduction of new animal and possibility of contact with other species of animal

| | | Number of herds tested | Number positive | Prevalence (%) | 95% CI | $\chi^2$ | P value |
|---|---|---|---|---|---|---|---|
| New animal introduction | Yes | 28 | 24 | 85.7 | 79.2 – 92 | 3.553 | 0.035 |
| | No | 82 | 54 | 65.9 | 57 – 74.7 | | |
| Possibility of contact with other species of animal | High | 97 | 72 | 74.2 | 66 – 82.3 | 4.38 | 0.043 |
| | Less | 13 | 6 | 46.2 | 36.8 – 55.5 | | |

Though new animal introduction to the herd and possibility of contact with other species of animal were significant in the univariate analysis, did not show any significant association in the final model.

History of abortion of both the farm and individual animal was assessed. Positivity of the herd increases with the presence of at least one previous abortion in the herd. The herd positiviy was 88.9 % (95% CI= 83% - 94.7%) and 67.2% (95% CI= 58.4% - 75.9%) the history of abortion and in herds without the history of abortion, respectively. In the mean time, milk and serum positivity, 20% (n= 415) and 42.3% (n=372), respectively, increases in cows with the previous history of abortion than in cows with no history of abortion, 16.9 % and 38.2%. Though there is an increase both in individual milk and seropositivity and herd positivity with abortion history, the difference observed was not statistically significant ($p > 0.05$).

Frequency of veterinary supervision of the herd was categorized into four groups. 8 (8 %) of the study herds never had any veterinary advice while 69 (69 %) rarely had veterinary advise specifically during purchase of new animals, 17(17%) and 6 (6 %) herds have veterinary advise and interventions often (for the treatment of clinical cases) and almost always (frequent

veterinary supervision of animas during entry to the farm, feeding, clinical case treatment etc), respectively.

Overall, herd prevalence decreases with the increase in the frequency of veterinary consultation from a 78.3 % (n=110) herd prevalence in farms with a rare contact with a veterinarian to 50% (n=110) herd prevalence in farms with an up to date veterinary intervention. However, the herd prevalence is not shown to be statistically significant ($p>0.05$).

Likewise, the difference in the seroprevalences between the sex groups was not significant ($p>0.05$) though there is a slight increase in the seroprevalence of female, 38.5 (n=774) as opposed to male, 33.5 (n=774).

The milk and seroprevalence of local breed cattle 24.1% (95 % CI= 21% -27.1%) and 35.7 % (95 % CI= 32.3% – 39%) were higher than that of cross breed ones 16% (95 % CI=13.4% – 18.6%) and 36.2 (95 % CI=32.8% – 39.6%), respectively. However, the milk and seroprevalence of individual animal were not statistically significant ($p> 0.05$).

Table 5. Summary of milk and seroprevalences of BVDV antibodies on sex and breed basis

| | | | Number of tested | Number positive | Prevalence (%) | 95% CI | $\chi^2$ | **P value** |
|---|---|---|---|---|---|---|---|---|
| Sex | Serum | Female | 371 | 143 | 38.5 | 35 – 41.9 | 2.13 | 0.083 |
| | | Male | 403 | 135 | 33.5 | 30.1 – 36.8 | | |
| | Serum | Local | 456 | 163 | 35.7 | 32.3 – 39 | 0.014 | 0.482 |
| | | Cross | 318 | 115 | 36.2 | 32.8 – 39.6 | | |
| Breed | Milk | Local | 58 | 14 | 24.1 | 21 -27.1 | 2.349 | 0.092 |
| | | Cross | 357 | 57 | 16 | 13.4 – 18.6 | | |

With regard to parity number of dairy cows, both milk and seroprevalences were found to increase with an increase in parity number. The seroprevalence ranges from 22.2 % (95 % CI= 17.9% – 26.4 %) in heifers to 64 % (95 % CI = 59 % - 68.9%) in dairy cows with a parity number of at least five. Likewise, the milk prevalence of dairy cows increased from 10.6 % (95 % CI= 7.6 % - 11.25%) in younger cows with 1 or 2 parity number to 25.7 (95 % CI= 21.5 %– 29.9 %)

in older cows with a parity number of at least five. Both the milk and seroprevlences were to be significantly different ($p<0.05$).

The body condition of individual sampled animal recorded during sampling of both the serum and milk was categorized into three; namely poor, moderate and good. As the body conditions of individual sampled animals were getting better, both the milk and seroprevalences tend to decrease from 41.3 % (95 % CI= 37.8 % – 44.8%) seroprevalence and 17.4 % (95 % CI= 13.7% - 21 %) milk prevalence in animals with a poor body condition to 31.0% (95 % CI= 27.7% – 34.26%) seroprevalence and 14.3 % (95 % CI= 10.9% – 17.7 %) milk prevalence in animals with a good body condition. However, the difference in both milk and seroprevalences of individual animal were not statistically significant ($p> 0.05$).

Table 6. Summary results of multivariate analysis of association between BVDV herd status and potential risk factors

| Variable | Level | Odds Ratio | P value | 95% Confidence Interval |
|---|---|---|---|---|
| Herd size | Small | - | | |
| | Medium | 7.09 | 0.001* | 2.16 – 23.3 |
| | Large | 9.3 | 0.001* | 2.44 - 35.47 |
| Management | Extensive | - | | |
| | Intensive | 0.28 | 0.15 | 0.051 – 1.587 |
| | semi intensive | 3.23 | 0.28 | 1.133 – 9.21 |
| Age | Less than 3 years | - | | |
| | More than 6 years | 5.332 | 0.000* | 3.460 – 8.218 |

*Significant ($p<0.05$)

The difference in the serum and milk prevalence of individual cows was statistically significant (p= 0.000). The Pearson's correlation coefficient between matched samples of individual serum and milk samples was 0.473 (n = 368) indicating a moderately positive association. Likewise, the level of agreement between the milk and serum ELISA test results was assessed using a kappa statistic which also indicated a moderate level of agreement (kappa value= 0.409, p= 0.000) (Table 7).

Table 7. Summary of correlation and Test Agreement between milk and serum prevalences of the ELISA test.

| | *Number* | *% positivity* | *Correlation coefficient* | *Kappa value* | *P value* |
|---|---|---|---|---|---|
| Both positive | 57 | 39.9 | | | |
| Serum positive, milk negative | 86 | 60.1 | 0.473 | 0.409 | 0.000 |
| Serum negative, milk positive | 7 | 3.1 | | | |
| Both negative | 218 | 96.9 | | | |
| Total | 368 | 100 | | | |

## 5. DISCUSSION

Niskanen (1993) classified a herd level exposure to BVDV from the bulk tank milk using BVDV antibody ELISA into four categories. A herd was classified as actively infected if more than 60 % of the cows were immune, moderately exposed to the infection if 30-60 % of the cows were immune, low exposure if 10-30 % of the cows were immune and naive if less than 10 % were immune. Different investigations based on the detection of BVDV antibodies, either in individual animals or in bulk milk, have shown that the prevalence of BVDV within herds, in a number of countries, mostly ranges from 70% to 100% (Garoussi *et al.*, 2008).

The overall herd prevalence of BVDV in this study area was 70.9 % which showed high exposure of dairy and feedlot animals to bovine viral diarrhea Virus. The herd prevalence studies undertaken by different researchers have reported 57.7% in England and 95% in Wales (Vilceck *et al.*, 1999). Serum and milk prevalences of BVDV in the study area were 35.9 % 17.1%, respectively. Similar studies in different countries obtained 60 % seroprevalence in Switzerland (Rufenacht *et al.*, 2000), 46% and 64 % in Kenya and Zimbabwe, respectively (Radostitis *et al.*, 1994).

However, an antibody positive herd test does not necessarily mean that a PI animal is still present, since anti-BVDV antibodies can persist for many years after removal of all PI cattle. Nor does herd negative indicate absence of a PI animal, since it can have been recently introduced (Drew *et al.*, 1999).

Previous work in Scandinavia and Britain established close agreement between serum BVDV ELISA antibodies with milk ELISA although the absorbance levels in milk were low (Niskanen *et al.*, 1989). Another study in the USA showed that matched serum and milk samples obtained a high overall correlation r=0.93. In this study, however, the agreement level between the milk and serum ELISA values had a kappa value of 0.409 and a correlation coefficient of 0.473 which indicated a moderate level of agreement between the two tests.

The results of this study have confirmed the prevalence of BVDV in dairy farms can hardly be estimated in the study animals using milk samples. This could be attributed to the low

sensensitivity of the ELISA when testing of individual milk samples (Garoussi *et al.*, 2008) compared to using serum samples. This can be explained by the immunoglobulin levels in the milk which was 20 to 40 times lower than in serum (Kramps *et al.*, 1999). Management practices of lactating dairy cows which included poor milking, handling, preservation as indicated by Francisco *et al.* (2008) coupled with the very low level of antibodies in the milk compared to serum could be the reasons for the moderate level of agreement and correlation between serum and milk ELISA values demonstrated in this study in comparison to the well managed animals which resulted in high levels of agreement in Europe and the United States (Francisco *et al.*, 2008).

As herd size increased, the herd seroprevalence of BVDV increased from 14.1 % in small size herds to 52.6 % in large ones. This could be attributed to the high presence of Persistently Infected (PI) cattle in large sized herds than in the small ones (Houe *et al.,* 1995; Niza-Ribeiro *et al.*, 2005). This finding is consistent with reports from other investigations that large herds often have higher infection rates than small herds (Houe *et al*, 1995).

Niza-Ribeiro *et al.* (2005) studied age-specific seroprevalence of BVDV and indicated that seropositivity increased from 16 % in young animals to 41 % in cows. Moreover, Rufenacht *et al.* (2000) indicated that antibody-prevalence was 70% in cows older than 25 months than the overall prevalence of 57.7 % which showed that antibody prevalence increased with increases in age of an animal.

The present study showed that older animals were 3.45 more times of becoming BVDV seropositive than the young stock. This finding is in agreement with the observation of Niza-Ribeiro *et al.* (2005). Comparatively higher seropositivity in calves than young cows could be due to the maternal colostral antibodies which are present for up to six months of age in calves born from immune cows (Radotitis *et al.*, 1996). Meanwhile, the older the animals are, the higher the chance of being in contact with PI animal. The immune response developed, once a susceptible animal is in contact with PI, is life long (Platt *et al.*, 2008). Thus, older animals have higher chance of becoming seropositive than the younger ones.

Bovine viral diarrhea virus is mostly introduced into a susceptible herd by introduction of PI animals or pregnant animals carrying a PI fetus (Houe, 1999). Houe (1999) studied the risk of

introduction of BVDV in the farm through the purchase and introduction of new animals into a herd provided PI prevalence is known. Thus, if the prevalence of PI animals is 2% (including animals carrying a PI fetus), the risk (P) of introducing PI animals when buying from random sources without testing is 1-(probability of buying a non-PI animal) $^{n}$, where n is the number of animals purchased i.e. P= 1-(0.98) $^{n}$ .Hence, introducing 20 animals, the risk would be 1-$(0.98)^{20}$=33%.

In the univariate logistic analysis of risk factors for BVD, herds which had purchased and introduced new animals had higher positivity than those which had not. This result is in consistent with Houe (1999) and Taylor (1997).

Possibility of contacts of a herd with other species of animal was assessed using both the univariable and multivariable logistic analyses. Though there was no significant association between the possibilities of contact with other species of animals, the present study indicated an increase in seropositivity in herds with higher possibilities of contacts with other species of animals than those herds without such possibilities. This finding is in agreement with Broaddus *et al.* (2007) and Carlsson (1991).

Broaddus *et al.* (2007) demonstrated that pasturing goats with cattle increases the risk of BVDV infection in goats. The potential for BVDV transmission from cattle to goats and the generation of PI goats would have signifiant implications, such as abortions due to BVDV infection in goats and propagating PI goats that can serve as sources of BVDV infection for cattle and also other non PI goats. BVDV also infects sheep. Both BVDV and boarder disease virus (BDV) may be isolated either from cases of boarder disease (BD) in sheep or goats (Carlsson, 1991). On the contrary, the results of the present study were in disagreement with Valle *et al.* (1999) which concluded that contacts with sheep was not a major risk factor for BVD in the Mreland Romsdal County, Norway.

With regard to the management of herds, 44.5 % of the herds were managed under semi-intensive management system in which cattle in a herd were housed but could move and get mixed with other herds if necessary especially during watering and pasturing. The present study found out that herd positivity was significantly associated with the type of management practices. The result showed that intensification of a herd increases herd level seropositivity. This finding is

similar to the findings by Houe *et al.* (1995), Taylor *et al.*, (1997) and Grom and Barlic-Maganja, (1999). In herds where animals were housed and pastured in segregated groups, the transmission from PI animals may be prevented until the PI animals come into closer contacts with susceptible animals (Houe *et al.*, 1995, Taylor *et al.*, 1997). Moreover, one hour of direct nose-to-nose contact is sufficient for transmission to occur (Traven *et al.*, 1991). In this context, Grom and Barlic-Maganja, (1999) reported that the seroprevalence was much higher in exensivelly managed animals than the intensive, where animals were strictly housed, and pasturing was not practiced.

## 6. CONCLUSIONS AND RECOMMENDATIONS

The finings of this study suggested that exposure to BVDV is widely distributed in feedlots and dairy farms in and around Adama. It is also conceivable from the results that the population of studied animals had acquired a certain level of immunity. This was confirmed by the overall high seroprevalence observed in both at an individual and a herd level. Most of the herds had at least one serum or milk antibody positive. In addition, therefore, there is a high probability of having PI animal (s) in some herds. Intrinsic factors including ages and parity numbers and extrinsic factors like herd sizes, management practices, introduction of new animals into herds and contacts with other species of animals are the predisposing factors for BVDV seropositivity. The study also indicated the moderately low level of correlation between the serum and milk ELISA results. Further investigation using both bulk tank and individual milk samples with matched serum samples are imperative for validation of milk ELISA for routine diagnosis of BVDV.

In view of the above points, the following recommendations are forwarded:

- Due to its significant economic impact in dairy farms evidenced in studies in developed countries, the overall situation of BVDV and its economic impact in Ethiopia should be well studied and documented.
- Further studies on the identification of Persistently Infected (PI) animals with subsequent isolation and characterization of the circulating viral antigen is mandatory
- Further research on the use of bulk tank milk (BTM) or individual milk samples for routine diagnosis of BVDV should be undertaken.
- National and regional disease control schemes should give due consideration to diseases which have immunosuppressive effects and which predisposes animals to further infections.

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## 8. ANNEXES

Annex 1. Sample Collection Format

Owner's name---------- Keble---------- Date of collection----------

Production system---------- Age the animal ----------

Sample type serum ☐ Lab code---------

Milk ☐ Lab code---------

Breed Local ☐ Cross ☐

Sex M ☐ F ☐

Parity number----------

Body condition of the anima

Poor ☐ moderate ☐ good ☐

History of abortion of the animal----------

Remarks_____

_____

_____

_____

Annex 2. Herd assessment of Risk factors to BVDV

Production system----------

Name of the cooperative----------

Herd size----------

Presence of other species of domestic animal in the herd

Herd Management ☐ Extensive (animals share a common pasture)

☐ Intensive (a herd is housed strictly with less not in contact with any animal)

☐ Semi intensive (herds may share watering or pasturing points)

Purchase and Introduction of new animals in to the herd

Yes ☐ No☐

Possibility of contact with other species of animal

Yes☐ No☐

History of abortion in the farm

Yes ☐ No ☐

Purchase of farming Home consumption of milk ☐

Supply of milk to the dairy processing plants ☐

Processing of milk for commercial purposes ☐

Local supply of fattened cattle ☐

Export of fattened cattle ☐

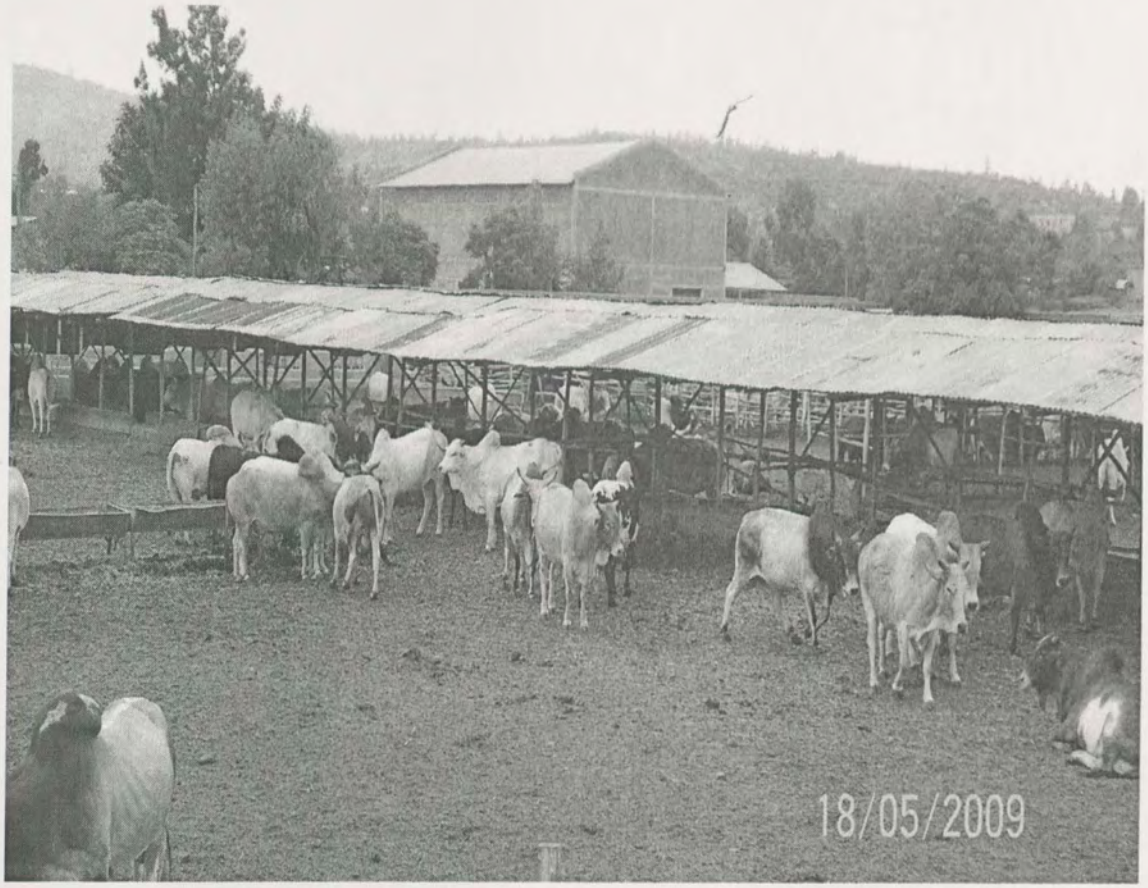
If Intensive and Semi Intensive, presence of other species of small ruminants in the herd

Yes ☐ No ☐

Frequency of veterinary supervision of the farm

None ☐ Often ☐ Rarely ☐ Almost always ☐

Annex 3. A typical large sized Feedlot farm in the Study area



Annex 4. A typical large sized dairy farm in the study area

## 9. CURRICULUM VITAE

### Personal Data

| | | |
|---|---|---|
| **Name** | | Kumilachew Belay Bogale |
| **Date of Birth** | : | September 17, 1981 |
| **Place Of Birth** | | Addis Ababa |
| **Sex** | : | Male |
| **Marital Status** | : | Single |
| **Nationality** | : | Ethiopian |

### Summary of Qualification

Doctor of Veterinary medicine (DVM) Degree in Veterinary Medicine from the Addis Ababa University, Faculty of Veterinary Medicine.

Master of Science (MSc) Degree in Tropical Veterinary Microbiology from the Addis Ababa University, Faculty of Veterinary Medicine.(Under Study)

### Education

| | |
|---|---|
| 1988-1995 | Elementary School |
| | Yekatit 23 Primary School, Addis Ababa |
| 1995-1998 | High School |
| | Kolfe Comprehensive Secondary School |
| 1998-1999 | Pre- Veterinary Studies |
| | Addis Ababa University, Faculty of Science, Addis Ababa |
| 1999-2004 | Doctor of Veterinary Medicine |
| | Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit |
| 2007-2009 | Master of Science |
| | Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit |

## Professional Experience

| | |
|---|---|
| 9/2004 - 3/2005 | **Field Veterinary Officer, Boreda Woreda Agricultural and Rural Development Office, Gamo Gofa Zone, SNNPR**<br>**Organization Type:** *Government* |
| 5/2005 - 1/2008 | **Livestock and Veterinary Section Head, Shag Import and Export Enterprise, Adama**<br>**Organization Type:** *Non-Government, Plc* |
| 5/2008 till Date | **Livestock Opertions Manager, Tewodros Teshome Import and Export, Adama**<br>**Organization Type:** *Non-Government, Plc* |

## Languages

| | Oral Level | Written Level |
|---|---|---|
| ▪ Amharic | Advance(Fluent) | Advance(Fluent) |
| ▪ English | Advance(Fluent) | Advance(Fluent) |

## Membership

**Type** : Ethiopian Veterinary Association
**Member Since** : July, 2004

## Papers

| | |
|---|---|
| **2003** | Trans Boundary Disease Control Policy in Ethiopia, Senior Seminar, FVM, AAU, Debre zeit |
| **2004** | A Studu on Ethnoveterinary Medical Practices and th Anthelminthic effect of Witharia Somnifera in East Shoa, Oromiya, DVM Thesis, FVM, AAU, Debre Zeit |
| **2008** | Implications on the Applications of Sanitary and Phytosanitary (SPS) Measures in Ethiopia, MSc Seminar, FVM, AAU, Debre Zeit |
| **2009** | A Study on the Prevalence of Bovine viral diarrhea Virus (BVDV) |

Antibodies in Selected Feedlot and Dairy Farms in and around Adama, Oromiya, MSc Thesis, FVM, AAU, Debre Zeit

## Workshops Attended

| | |
|---|---|
| **2005** | The Effect of FMD on Livestock Export Trade in Ethiopia, SPS-LMM, Adama, Ethiopia |
| **2007** | Standardization of Agricultural Products in Ethiopia, EQSA, Addis Ababa, Ethiopia, |
| **2008** | The Management of Feedlots, SPS-LMM and EMDTI, Debre Zeit, Ethiopia |

## References

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## 10. SIGNED DECLARATION SHEET

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any university and that all sources of material used for the thesis have been duly acknowledged.

Name .....

Signature.....

Date of submission.....

This thesis has been submitted for examination with my approval as an academic advisor,

Dr. Moses N. Kyule .....

Prof. Mahendra Pal .....