SEROPREVALENCE AND THE ASSOCIATED RISK FACTORS OF INFECTIOUS BURSAL DISEASE IN CHICKENS IN JIMMA TOWN AND BONGA DISTRICT OF SOUTH WEST ETHIOPIA

MSc. THESIS

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Seroprevalence and the Associated Risk Factors of Infectious Bursal Disease in Chickens in Jimma Town and Bonga District of South West Ethiopia

MSc. Thesis

By

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DEDICATION

To my wife S/r Tigist Tadesse Kebamo

&

My child, Maereg Debebe, for their unlimited passion and moral support throughout my MSc education life.

STATEMENT OF THE AUTHER

I declare that the thesis hereby submitted for the MSc degree at Jimma University College of Agriculture and Veterinary Medicine is my own work and has not been previously submitted to any other University or Institution for the award of any degree. I concede copyright of the thesis in favor of the Jimma University, College of Agriculture and Veterinary Medicine.

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BIOGRAPHICAL SKETCH

The author was born in Baenara kebelle of soro woreda in Hadiya Zone, Southern Nations Nationalities and People's Regional State, Ethiopia in September, 1986 G.C. He completed his elementary and junior secondary school education at Jajura St. Peter and Paul Catholic Missionary of elementary school, and secondary school of Jajura respectively; and high school and preparatory education was from Wachemo comprehensive high school. He joined Addis Ababa University in October 2006 and graduated with Doctor of Veterinary Medicine in the department of Veterinary Medicine in July, 2010.

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immuno-deffusion
BF	Bursa of fabricius
CSA	Central Statistical Agency
I- ELISA	Indirect Enzyme-Linked Immunosorbent Assay
IBDV	Infectious bursal disease Virus
OIE	Office International Des Epizooties
RE	Restriction endonucleases
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcriptase- polymerase chain reaction
SPF	Specific-pathogen-free
USAID	United state Agency for International Development
VN	Virus neutralization
VP	Viral protein
VP2	Viral protein 2
vvIBD	Very virulent Infectious bursal disease
vvVP	Very virulent viral protein

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ABSTRACT

Infectious bursal disease is recognized as a worldwide concern for the poultry industry, resulting in a great economic loss in different type of poultry production system including Ethiopia. A cross-sectional study was conducted to determine seroprevalence of IBD and assess associated risk factors in non-vaccinated, apparently healthy chickens in Jimma town and Bonga district of south west Ethiopia: between October, 2015 to August, 2016. A Multistage sampling technique was used to pick out chickens for serum sample collection. A total of 422 chickens serum samples were examined using Indirect ELISA test, 407 (96.4%) of them to be found positive. Univariate and multivariate logistic regression analysis was carried out by SPSS version 20 to determine independent association between variables and occurrence of the infectious bursal disease. Multivariate analysis revealed that the odd of infecting with the disease in adult aged chickens was lower than young aged groups (P=0.002, OR=0.07, CI=0.009-0.547), where as females are more likely affected than that of male chickens (P=0.004, OR=3.6, CI=1.197-10.548)and the occurrence of the disease was lower in Bonga district than in chickens reared in Jimma Town (P=0.016, OR= 0.3, CI=1.190-9.83) in this investigation area. Finally, the result of this research shows Infectious bursal disease is very prevalent and widely distributed in chickens reared in the study areas. Therefore, creating awareness to poultry owners, and vaccination, especially at early age was recommended to prevent and control the disease and to reduce further losses.

Key words: Chickens, I-ELISA, Infectious Bursal Disease, Risk Factors, Seroprevalence, South West Ethiopia

1. INTRODUCTION

The poultry sector is one of the segments of livestock sector in Ethiopia which can be characterized into three major production systems: large commercial, small scale commercial and village or backyard poultry production system. These production systems have their own specific chicken breeds, inputs and production properties. Each can sustainably coexist and contribute to solve the socio-economic problems of different target societies (Tadelle *et al*., 2003c). Among the total of 183.04 million livestock population, 56.87 millions are chicken (CSA, 2014/15). They play a role by providing the needed animal protein that contributes to the improvement of the nutritional status of the people (USAID, 2013).

Ethiopian poultry production has a long traditional practice which is characterized by low input and low output (Mulugeta and Tebkew, 2013). Attempts are underway to enhance chicken productivity and their contribution via importing and distributing improved breeds to farmers living in different parts of Ethiopia (Zeleke *et al.,* 2005; Mulugeta and Tebkew, 2013). Accompanying intensification of poultry farming, there is occurrence of epidemics of newly introduced diseases and/or epidemics of endemic diseases. Among those diseases, Infectious bursal disease is the one that become a serious threat to cause frequent outbreaks and a challenge to the young growing poultry farms (Zeleke *et al.,* 2005; Solomon and Abebe, 2007).

Infectious bursal disease is an acute, highly contagious, immunosuppressive and economically important young (mostly 3-6 weeks old) poultry disease caused by *Infectious bursal disease virus*. The virus is a member of the genus *Avibirnavirus* and family *Birnaviridae* RNA virus (Islam, 2005; Okwor *et al.,* 2011). It has a bi-segmented, double-stranded RNA genome which contains a single-shelled, non-enveloped, icosahedral capsid structured and having a diameter of 58 nm -60 nm. This relatively simple structure renders the virus very resistant to the outside environment (Jacqueline, 2010). There are two known serotypes (Serotype 1 and 2). Only serotype I viruses are naturally pathogenic to chickens whereas serotype 2 virus apathogenic for chicks (Van den Berg *et al*., 2004; Jackwood and Sommer, 2005). *Infectious bursal disease virus* has five proteins recognized as VP1 to 5. Among these proteins, the VP2 protein is the one major protective antigen of IBDV that contains specific epitopes responsible

for inducing neutralizing antibody responses (Muller *et al.,* 2003; Coulibaly *et al*., 2005; Jacqueline, 2010).

Infectious bursal disease is a severe acute disease of 3–6-week-old birds which is associated with high morbidity and mortality, but a less acute or subclinical disease is common in 0–3-week-old birds resulting in immunosuppression and increase susceptibility of poultry to opportunistic secondary infections (Khan *et al*., 2011; Mahgoub, 2012). The virus preferentially affects actively proliferating and differentiating B lymphocytes in the bursa of fabricius, which leads to an age-dependent immunosuppression. The immunosuppressive effects of IBDV infections also frequently interfere with effective immune responses to vaccination (Van den Berg, 2000; Jacqueline, 2010). The disease is characterized with a typical clinical signs of those an acute immunodepression, with depression, prostration of the affected birds, diarrhoea, during the first weeks of life. It is transmitted through orally via contaminated feed and water (Sharma *et al*., 2000).

In the report of 63rd General Session of the Office International des Epizooties, it was estimated that IBD has considerable socio economic importance at the international level as the disease is present in >95% of the member countries, and the occurrence of acute clinical cases (vvIBDV) was reported in 80% of the country (Jinda *et al*., 2004). The recent ‘re-emergence' of the infectious bursal disease virus (IBDV) in the form of antigenic variants and hyper virulent strains has been the cause of significant losses; because of IBD virus remains infectious for a very long period of time and has resistance to commonly used disinfectants (OIE, 2012).

The Infectious bursal disease was first reported in the country by Zeleke *et al.* (2003) in 2002 at privately owned commercial poultry farm in Debre Zeit in which 45-50% mortality and 90.30% of overall seroprevalence of IBD antibody in different farms was documented. Prior to February 2006, the health measures at the government owned poultry multiplication and distribution centers with the exception of Bonga and Bedelle, all the multiplication centers were devastated by the outbreak of Infectious bursal disease i.e. Gumboro disease (Yilma, 2007); and a commercial broiler farm (Chanie *et al.,* 2009**).** On the other hand most of the researchers (Hailu *et al*., 2009; Hailu *et al.*, 2010, Shiferaw *et al*., 2012; Sindu *et al*., 2015) in

Ethiopia performed serological surveys in different parts of the country and documented results indicates that IBD is a threat on both backyard chickens and commercial chickens. Currently, IBD is the most important threat to poultry production in the country and widely distributed in all regions in the backyard chickens, commercial farms and poultry multiplication centers. The disease has since spread to all investigated commercial farms and multiplication centers occurring at an average outbreak rate of 3-4 farms per year (AHY, 2011).

Likewise, different researchers reported various risk factors for the occurrence of infectious bursal disease in different production systems. Age, sex, breed, production type, immune status of the chickens, seasonal variation, agro-ecology, biosecurity measure, social awareness, a previous history of infectious bursal disease etc… are factors that contributes for the occurrence of the disease in the poultry flocks (Hailu *et al.,* 2009; Hailu *et al*, 2010; Tesfaheywet and Getnet, 2012; Natnael, 2015). A cross sectional study conducted by Natnael (2015) revealed that the incidence of the IBD affected by study area, farm systems, age of chickens, and breeds and reported seroprevalence (67.11%) of Koekoek which is more prone to IBD infection followed by Bovans brown (57.69%), local breeds (48.31%), and Bovans white (40.28%) and crossbreed (38.71%). Tesfaheywet and Getnet (2012) reported that the prevalence of IBD in local chickens with no history of previous vaccination against infection was from field exposure to the virus.

Since, the presence of the infectious bursal disease in many parts of the Ethiopia and its occurrence of the disease is increasing at alarming rate in all over the country where commercial poultry production is intensified and even in the backyard chickens, and also a serious problem for the poultry industry (Shiferaw *et al*., 2012; Aregitu, 2015). This disease has been causing considerable economic loss to the country and posed a challenge especially for the success of vaccines used at this time (Wit and Baxendale, 2004). In addition, the information on the presence of the disease in chickens reared in different type of production system in the study area: Jimma town and Bonga district of South West Ethiopia is very limited. Similarly, the status of diagnostic coverage of poultry diseases including Infectious bursal disease particularly in Jimma town and Bonga district is limited to the extent that, even from commercial farms, and remains undiagnosed and dead chickens are simply discarded.

Only a few cases may be reported to diagnostic investigation centers. Therefore, considering the significant economic losses associated with IBDV and study gaps, to realize the importance of the infectious bursal disease among different types of production and relevant control and prevention programs could then be promoted through determining prevalence of the disease and the associated risk factor available in this study area so that this disease can be controlled among chicken production thereby increasing their productivity.

It is for this reason that this study was carried out with the following general and specific objectives:

General objective:

- To investigate the occurrence of infectious bursal disease and to assess the associated risk factors among unvaccinated, apparently health chickens in different level of production system in Jimma town and Bonga district of south west Ethiopia.

Specific objectives:

- To determine seroprevalence of infectious bursal disease in Jimma town and Bonga district of south west Ethiopia.
- To assess potential risk factors in relation to infectious bursal disease occurrence in the study areas.

2. LITERATURE REVIEW

2.1. The Disease

Infectious bursal disease is also known as Gumboro Disease which is a highly contagious disease of young chicken (*Gallus gallus domesticus*) caused by *infectious bursal disease virus* (IBDV*)* (Caston *et al.*, 2008*)* which belongs to a genus *Avibirnavirus* (Fauquet *et al*., 2005), of family *Birnaviridae* (Delmas *et al.,* 2004) that causes disease and mortality in young chickens mainly 3–6-week-old in the worldwide distribution (Van den Berg *et al.,* 2000; Lukert and Saif, 2003). The causal agent of the disease was first isolated in Gumboro, Delaware in United States of America (USA), and the disease was originally known as Gumboro disease. It is a viral infection, affecting the immune system of poultry (OIE, 2008). The disease is characterized by sudden of short course and extensive destruction of lymphocyte particularly in the bursa of fabricius, where B lymphocytes mature and differentiate (Quinn *et al.*, 2002; OIE, 2008); however, IBD viral replication also occurs in other lymphoid structures including the spleen, thymus, harderian gland, and cecal tonsils (Vegand, 2008). It is considered as AIDS of chicken since it severely affected chicken immune system (Kaufer and Weissi, 2005).

Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. The main clinical signs include watery diarrhea, depression, ruffled feathers, anorexia, trembling, prostration and death after two to three days of clinical signs onset (OIE, 2012). The major post-mortem lesions may include dehydration of the muscles with numerous ecchymotic hemorrhages, swelling and discoloration of the kidneys, with urates in the tubules, inflammation, edema and bursal hemorrhages or atrophy (Chansiripornchai and Sasipreeyajan, 2009). Disease severity depends on the age and breed of the affected birds, the degree of passive immunity and the virulence of the strain of virus and secondary infections associated with the immunosuppressive effects of the disease (Van den Berg *et al.,* 2000; Aregitu, 2015).

2.2. Etiology

2.2.1. Classification

Infectious bursal disease virus (IBDV) is classified as a member of the *Birnaviridae* family. The family includes 3 genera: *Aquabirnavirus* whose type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks, and crustaceans; *Avibirnavirus* whose type species is *infectious bursal disease virus* (IBDV), which infects birds; and *Entomobirnavirus* whose type species is *Drosophila* X virus (DXV), which infects insects (Delmas *et al.,* 2004). Viruses in this family possess bi-segmented, double-stranded RNA (dsRNA) genomes, which are packaged into single-shelled, non-enveloped virions (Delmas *et al.*, 2004).

IBDV genome consists of two segments, A and B, being 3.4 and 2.9 kb, respectively which are enclosed within a non enveloped icosahedral capsid (Jacqueline, 2010; Tahiri *et al.*, 2013). They are known to show clinical disease in chickens younger than 10 weeks (OIE, 2008). Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. There two types of serotypes. Serotype 1 is responsible for clinical cases of Gumboro to which commercial vaccines against Gumboro disease were mainly produced (OIE, 2008), which can be further classified as attenuated (vaccine strains), classical (standard), antigenic variant, and very virulent (also known as hypervirulent) strains based on their phenotypic traits (such as antigenicity and pathogenicity) and by the genetic traits that is, VP2 amino acid sequence differences (Van den Berg *et al*., 2000; Lukert and Saif, 2003). Of the 4 phenotypic traits of serotype 1 that exists in the field, the hyper or very virulent IBD virus is capable of infecting chickens in the presence of maternally derived or higher levels of vaccinal antibodies causing very high mortalities which exceeds 50% in susceptible chickens, and bursal damage with severe economic losses (Dobos *et al*., 2001). Variant IBDVs do not cause mortality, whereas the classical strains cause up to 20% mortality (Muller *et al*. 2003; Musa *et al.,* 2012). Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus and are thus apathogenic for chicks (Jackwood and Sommer, 2005).

In the reports of Coulibaly *et al*., 2005 and Durairaj *et al*., 2011 the virus has five proteins recognized as VP1 to 5. The small segment B of the genome encodes for VP1 and the large segment A encodes for VP2, 3, 4, and 5. The VP2 and VP3 (which has Endopeptidase activity) are the major proteins constituting 51 and 40% respectively; whereas VP1 (3%) and VP4 (6%) are minor proteins of the total proteins. VP1 is the viral RNA polymerase, and VP4 is a viral protease. The function of VP5 is not clearly established, but it was suggested that it might have a regulatory function playing a role in virus release and dissemination. While the VP2 has the serotype specific epitope protein contains important neutralizing antigenic sites and elicits protective immune response and most of the amino acid (AA) changes between antigenically different IBDVs are clustered in the hypervariable region of VP2 **(**Coulibaly *et al*., 2005; Durairaj *et al*., 2011). Thus, this hypervariable region of VP2 is the obvious target for the molecular techniques applied for IBDV detection and strain variation studies (Muller *et al.,* 2003; Caston *et al.,* 2008).

2.3. Epidemiology

2.3.1. Distribution

Infectious bursal disease (IBD) is an acute, highly contagious viral infection of young chickens that has lymphoid tissue as its primary target with a special predilection for the bursa of fabricius. It was first recognized as a specific disease entity by Cosgrove in 1962 and was referred to as “avian nephrosis” because of the extreme kidney damage found in birds that succumbed to infection. Since the first outbreaks occurred in the area of Gumboro, Delaware, “Gumboro disease” was a synonym for this disease and is still frequently used. The first outbreak of infectious bursal disease (IBD) that had occurred in 1957 in a broiler farm near Gumboro, the Delaware area in the USA, was caused by the classical serotype 1 IBDV (Cosgrove, 1962). Infections with serotype 1 IBDV are of worldwide distribution, occurring in all major poultry producing areas. The incidence of infection in the areas where there is serotype 1 is high; essentially, all flocks are exposed to the virus during the early stages of life, either by natural exposure or vaccination.

Between 1960 and 1964, the disease affected most regions of the USA and reached Europe in the years 1962 to 1971 (Fragher, 2001). The variant IBDV strains then emerged in the 1980's in IBDV-vaccinated farms in the Delmarva area and were antigenetically different from the classical strain. Since 1986, Europe has experienced the emergency of vv strain of IBDV, which are characterized by a per acute onset of severe clinical disease and high mortality, which can cause up to 70% flock mortality in laying pullet (Van Den Berge *et al*., 2004). Although these new serotype 1 viruses demonstrate increased virulence in their ability to break through the existing level of maternal immunity; they are antigenically similar to the classic strains of IBDV (Van den Berg *et al*., 2004). Strains of vvIBDV have rapidly disseminated to every poultry-producing country such as Middle East, Asia, and Africa, South and Central America in 1999, and in the USA in 2009 were detected (Animal Health Australia [AHA], 2009; Jackwood *et al.,* 2009), but there was no any report that shows the existence of Infectious bursal disease in Canada, Mexico, Australia, and New Zealand (Aregitu, 2015). In addition a survey conducted by World organization for Animal Health (OIE) in 1995, 95% of the 65 countries was responded to declare the case of the infection (Entradossi, 2000).

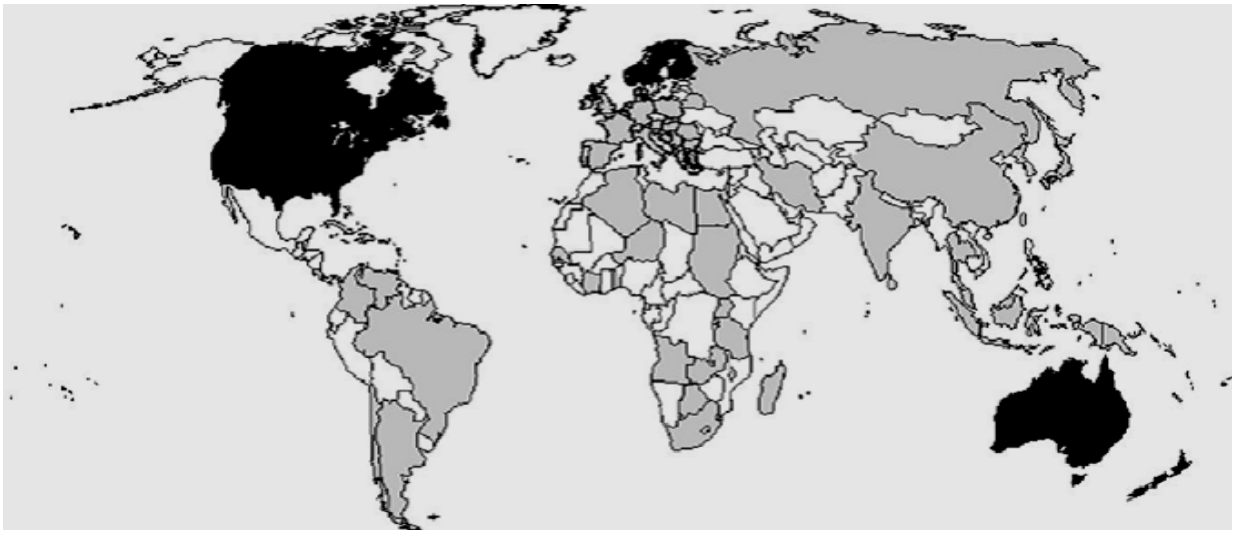

Fig. 1 Worldwide geographical distribution of the acute forms of IBDV

In gray, countries where acute forms have been reported. In black, countries where no acute forms have been reported. In white, countries with no report (updated from Eterradossi, 1995) as cited by Aregitu, (2015).

2.3.2. Host range

Infectious bursal disease virus (IBDV) is host specific. Although serologic evidence of natural infection with the virus has been reported in turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens (OIE, 2008). It is strongly believed that the serotype IBDV 1 is highly host specific to chickens which develop IBD after infection by serotype 1 viruses. Reports have shown that serotype 2 of IBDV is more prevalent in many species of wild birds, with the natural host considered to be turkeys (Okoyo and Uzoukwu, 2005). Infectious bursal disease virus has recently been isolated from a sparrow in China suggesting that wild birds could act as carriers (Wang *et al*., 2007). The duck can also be an asymptomatic carrier of serotype 1 viruses (Van den berg *et al*., 2004). There is no evidence that IBD virus can infect other animals, including humans.

2.3.3. Physicochemical nature of the virus

The most interesting feature of IBDV is its ability to remain infectious for a very long period of time and its resistance to commonly used disinfectants (Jackwood, 2014). The virus has been shown to remain infectious for 122 days in a chicken house, and for 52 days in feed, water and faeces in the chicken houses; and for up to 4 weeks in the bone marrow of infected chickens (Elankumaran *et al.,* 2002). It is susceptible to mutation, highly stable and resistant to variety of chemical and disinfectant like phenolic derivative and a quaternary ammonium compound, but iodine complex has a toxic effect on virus. It is also resistant for treatment with chloroform and ether, remains viable from pH 2-12 and is inactivated only in 70^0C for 30 minutes. The virus is unaffected by exposure for 1 hour at 0.5% to 30% phenol but Virus infectivity was markedly reduced when exposed to 0.5% formalin for 6 hours. IBDV also heat stable, viable after treatment at 56 °C for 5 hours (Dwight *et al*., 2005; Eterradossi and Saif, 2008).

2.3.4. Transmission of IBD Virus

Chickens are the only known avian species to develop clinical disease and distinct lesions when exposed to IBDV. The IBD transmit with horizontal way only, with healthy subjects being infected by the oral or respiratory pathway. The most common mode of infection is

through the oral route. Conjunctival and respiratory routes may also be involved (Sharma *et al.*, 2000). The high persistence of the virus and its resistance to several disinfections and virucidal procedures may contribute to the rapid distribution of the virus (Van den berg *et al.*, 2000; Garriga *et al*., 2006). IBDV may spread through contaminated equipment (Flensburg *et al*., 2002; Jackwood and Sommer-Wagner, 2010). Infected subjects excrete the virus in faces as early as 48 hours after infection, and may transmit the disease by contact over a sixteen-day period. The possibility of persistent infection in recovered animals has not been researched. The disease is highly contagious, can also spread through the movement of poultry products, equipment, feed bags, vehicles and people and to a lesser extent, through aerosols of dust (Sharma *et al*., 2000; Elankumaran *et al*., 2002).

There is no evidence to suggest that IBDV is spread via transovarial transmission (Eterradossi and Saif, 2008). No specific vectors or reservoirs of IBDV have been established, but the virus has been isolated from mosquitos (*Aedes vexans*), rats, and lesser mealworms (*Alphitobius diaperinus*) (Eterradossi and Saif, 2008). Viable vvIBD virus was recovered after 2 days from the faeces of a dog that had been fed tissues from experimentally infected chickens, indicating that dogs may act as mechanical vectors for the virus (Pages-Mante *et al*., 2004). There is no data that suggest IBDV is transmitted by wild birds, however direct or indirect transmission of the virus between wild birds and domestic chickens probably occurs (Motohiko *et al*., 2005). In the absence of effective cleaning, disinfection and insect control; can increases the possibilities for transmission when they are scavenging of dead chickens, ingestion of contaminated water, or exposure of respiratory or conjunctiva membranes to contaminated poultry dust (Okoyo and Uzoukwu, 2005).

2.4. Incubation Period and Clinical Signs

The incubation period of Infectious bursal disease virus has very short, and clinical signs of the disease are seen within 2-3 days after exposure. One of the earliest signs of infection in a flock is the tendency for some birds to pick at their own vents. In the report of Tsegaye and Mersha in 2014 infectious bursal disease follows one of two courses such as subclinical, and or clinical IBD, depending on the age at which chickens are infected. The subclinical form of the disease occurs in chickens less than 3 weeks of age. Chickens present no clinical signs of

disease, but experience permanent and severe in immunosuppression. The reason young chickens exhibit no clinical signs of disease are not known. However, immune- suppression occurs due to damage to the bursa of fabricius (Jordan *et al*., 2002). The disease is clinically seen only in chickens older than 3 weeks. While the clinical symptom is described as acute on set of depression, trembling, white and watery diarrhea, anorexia, prostration, ruffled feather, and vent feather solids with urates; in severe cases, affected birds became dehydrated, and in terminal stages of the disease, had a subnormal temperature and death (Zeleke *et al*., 2005; Ahmed *et al*., 2009).

Mortality commences on the third day of infection, reaches a peak by day four, then drops rapidly and the surviving chickens recover a state of apparent health after five to seven days. Disease severity depends on the age and breed sensitivity of the infected birds, the virulence of the strain and the degree of passive immunity. If the virus persists on the farm and is transmitted to the next flocks, the clinical forms of the disease appear earlier and are gradually replaced by subclinical forms. Moreover, a primary infection may also be unapparent when the viral strain is of low pathogenicity or if maternal antibodies are present (Van den berg *et al*., 2004).

2.5. Pathogenesis

Pathogenesis is defined as the method used by the virus to cause injury to the host with mortality, disease or immunosuppression as a consequence. The injuries can be evaluated at the level of host, the organ and the cell bursal disease virus usually infects young chickens between 3-6 weeks of age and causes a clinical disease, while sub-clinically infecting older birds. The outcome of IBDV infection is dependent on the strain and amount of the infecting virus, the age and breed of the birds, route of inoculation and presence or absence of neutralizing antibodies (Moody *et al*., 2000)

The most common route of infection is oral, but conjunctiva and respiratory route may also be important. Four to five hours after oral infection virus can be detected in macrophages and lymphoid cell in the cecum, duodenum, jejunum and kuppfer cell of the liver. The bursa is infected via the blood stream and by 11 hour many cell in this organ contain antigen. A

viremia follow when the virus infect other organ including spleen, the harderian gland and the thymus lymphocyte and their precursor appear to viral antigen can be found in the bursa up to 14 days post infection (Jordan *et al*.,2002).

In some birds the kidneys appear swollen and may contain urate deposit and cell debris which is probably result of blockage of ureters by severely swollen bursa. The cause of muscle hemorrhage is unknown. Bursa depletion as the result of virulent IBD virus infection in early life can result in impaired immune responses to antigen and the response to IBD virus itself. Although there are reports indicating that infection as late as 4weeks of age results poor response to certain antigen. This not all the cases and the severity of infection and whether or not maternal derived antibody (MDA) modified the disease could be important. The consequence of immunosuppression is lowered resistance to disease and suboptimal response to vaccine given during this time (Minalu *et al*., 2015).

2.6. Diagnosis

Diagnosis of IBD involves consideration of the flocks' history, and of the clinical signs and lesions. Obviously, chickens less than 3 weeks of age present no clinical signs of disease, while chickens greater than 3 weeks of age present clinical signs as described. The severity of the clinical signs will depend upon the factors described. while the clinical diagnosis of the acute forms of IBD is based on disease evolution of a mortality peak followed by recovery in five to seven days and relies on the observation of the symptoms and post-mortem examination of the pathognomonic lesions, in particular of the bursa of fabricius (Rajaonarison *et al*., 2006).

2.6.1. Symptomatology and Gross lesions

Hyper virulent IBDV infections are characterized by severe clinical signs and high mortality. Indeed, the vvIBDVs produce disease signs similar to conventional type 1 infection, with the same incubation period (4 days), but the acute phase is exacerbated and more generalized in the affected flock. Severe outbreaks are characterized by sudden onset of depression in susceptible flocks (OIE, 2008). Animals in the acute phase of the disease are prostrate and reluctant to move, with ruffled feathers and frequently watery or white diarrhoea. The age

susceptibility is extended, covering the entire growing period in broilers, and the peaks of mortality show a sharp death curve followed by rapid recovery. Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most cases lasting for 5-7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2-3 days. Usually between5% and 10% of birds die, but morbidity can reach 30-40% (Van den Berg *et al*., 2000).

On post mortem examination of birds that died during the acute phase of vvIBD, the bursa of fabricius is the principal diagnostic organ: it is turgid, eodematous, and sometimes haemorrhagic and turns atrophic within 7 to 10 days. This atrophy might be more rapid, even 3 to 4 days after inoculation (Tsukamoto *et al*., 1992). In addition, dehydration and nephrosis with swollen kidneys are common, and ecchymotic haemorrhages in the muscle and the mucosa of the proventriculus are observed in the majority of the affected birds. Severe depletion of lymphoid cells is observed not only in the bursa of fabricius, but also in the non-bursal lymphoid tissues. Pathogenicity of IBDV has been associated with virus distribution in non-bursal lymphopoietic and haematopoietic organs. Indeed, using various immunostaining methods, a higher frequency of antigen-positive cells could be demonstrated after infection of birds with vvIBDV compared with other strains, in the thymus (Sharma *et al*., 2000), the spleen and the bone marrow. In particular, atrophy of the thymus has been associated with the acute phase of the disease and might be indicative of the virulence of the isolate, although it is not associated with extensive viral replication in thymic cells. An increased number of macrophages are found in various organs (Tanimura *et al*., 1997). Thrombocytes also represent a target for IBDV, and acute disease is characterized by disseminated haemorrhages probably related to an impairment of the clotting mechanism (OIE, 2008).

Confirmation of a diagnosis of clinical IBD can be made at necropsy by examining the BF during the early stages of disease for characteristic gross lesions. During later stages of disease it is difficult to confirm a diagnosis of IBD by examining only shrunken, atrophied BF, as other diseases (for example, Marek's disease, mycotoxicosis) produce similar changes. In birds less than 3 weeks of age or in young chickens with maternal antibodies, IBD virus infections are usually subclinical. Thus, typical clinical signs are not present, and diagnosis

should be supported by histopathologic study of suspect bursa of fabricius, serologic studies, or by virus isolation (Rajaonarison *et al*., 2006; Gary and Richard, 2015).

2.6.2. Isolation and Characterization of the virus

Confirmation of diagnosis should be accomplished by virus isolation or detection of viral antigens in tissues (BF) from suspect cases. Isolation is commonly done in embryonating chicken eggs inoculated via the chorioallantoic membrane route. A variety of primary and established cell lines have been used for isolation and propagation of the virus. Once the virus is isolated, it could be identified by reacting it with a known anti-IBDV serum using any of a number of antigen antibody tests such as virus neutralization (VN), fluorescence antibody (FA) test, ELISA, or agar gel precipitation (AGP) (Lukert and Saif, 2003; OIE, 2008).

Therefore, diagnosis depends on the isolation and characterization of the virus and its differentiation from serotype 1 viruses; it can be made through following methods:

a. Serological Diagnosis

Serological tests such as AGID, ELISA, and VNT are carried out in serum sample to detect antibodies used for monitoring vaccine responses and might be additional information for diagnosis of infection of unvaccinated flocks (OIE, 2012). The enzyme linked immuno sorbent assay (ELISA) is the most commonly used test for the detection and quantification of IBDV antibodies to check response to vaccination, natural field exposure and decay of maternal antibody titer (Lukert and Saif, 2003). It is economical, simple, and quick tests a large number of samples at the same time and is adaptive to automation to computer software (Lukert and Saif, 2003). It is likewise essential to confirm the disease-free status of flocks. Viral antigens in the bursa of fabricius can be demonstrated based on plates coated with IBDV specific antibodies by the antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) or by agar-gel precipitin assay or (Islam *et al*., 2001a). The agar gel Immuno-diffussion (AGID) test can be used to detect viral antigen in the bursa of fabricius. A portion of the bursa is removed, homogenized and used as antigen in a test against known positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response (OIE, 2008). The VN titers accurately correlate with

protection of chickens against IBDV (Knoblich *et al*., 2000). Differentiation of classic and variant strains has been made by using ELISA and monoclonal antibodies (Sapats *et al*., 2005). However, these methods may not be as rapid and sensitive as molecular methods (Jackwood, 2004).

b. Virological Diagnosis

Infectious bursal disease virus may be detected in the bursa of fabricius of chicks in the acute phase of infection, ideally within the first three days following the appearance of clinical signs. Isolation: A filtered homogenate of the bursa of fabricius is inoculated in nine to eleven-day-old embryonated eggs originating from hens free of anti BDV antibodies. The most sensitive route of inoculation is the chorioallantoic membrane (CAM); the yolk sac route is also practicable, and the intra-allantoic route is the least sensitive (OIE, 2008).

The specificity of the lesions observed must be demonstrated by neutralizing the effect of the virus with a monospecific anti-IBDV serum. Isolation in embryonated eggs does not require adaptation of the virus by serial passages, and is suitable for vvIBDVs. In the absence of lesions, the embryos from the first passage should be homogenized in sterile conditions and clarified, and two additional serial passages should be performed (Lukert and Saif, 2003).

c. Molecular Identification

Molecular diagnostic assays are most often used to identify IBDV in diagnostic samples. They use reverse-transcriptase PCR to identify the viral genome in bursa tissue. Sequence analysis of the VP2 coding region has been used to further characterize the viruses (Jackwood, 2014). Most efforts at molecular identification have focused on the characterization of the larger segment of IBDV (segment A) and especially of the vvVP2 (very virulent viral protein) encoding region.

Several protocols have been published on characterization using restriction endonucleases of RT-PCR products. These approaches are known as RTPCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (Zierenberg *et al.*, 2001). The usefulness of the information

they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified, however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis, provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture-adapted strains, which exhibit amino acid pairs 279 N–284 T (Lim *et al*., 1999). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (Eterradossi *et al.*, 1999). However, it is not yet known whether these amino acids play a role in virulence or if they are just an indication of the clonal origin of most vvIBDV isolates. Several recent studies indicate that although VP2 is an important virulence determinant, it may not be the only one. It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments), however some potentially reassortant viruses have been identified (Le nouen *et al*., 2006).

d. Histological Diagnosis

Histological diagnosis is based on the detection of modifications occurring in the bursa. The ability to cause histological lesions in the non-bursal lymphoid organs, such as the thymus spleen or bone marrow (Inoue *et al*., 1999) has been reported as a potential characteristic of hypervirulent IBDV strains. The histological diagnostic method has the advantage of allowing for diagnosis of both the acute and chronic or subclinical forms of the disease.

Detection of viral antigens: thin sections of the bursa of fabricius prepared to detect viral antigens specific to IBDV done by direct and indirect immunofluorescence or by immunoperoxidase staining in the bursal follicles of infected chickens between the fourth and sixth day after inoculation. No viral antigen is detectable from the tenth day. However, the virus can be isolated from bursa sampled from the second to the tenth day, with a maximum infectious titer after four days (OIE, 2008).

2.7. Treatment, Control and Preventions

2.7.1. Treatment

There is no specific therapy for the disease. Facilitate the access to water to prevent dehydration. As with every disease optimize climate and reduce stress to a minimum. Use of antibiotics can sometimes be advisable to limit the impact of secondary infections (Austic and Nesheim, 2000).

2.7.2. Control and Prevention

Control of IBD has been further complicated by the recognition of variant strains of the IBD virus. Variant viruses induce damage in the BF in chickens, even when high and uniform antibody titers are present. Variant strains do not cause obvious clinical disease, but immune-suppression. In chicken affected by classical IBDV the bursa of fabricius undergo rapid atrophy (lymphocyte depletion) without inflammatory changes observed early in the infection. These variants are not from a different serotype, but are antigenically different enough to cause immunosuppression problems (Jackwood and Sommer, 2005). An additional important feature of IBD is its immunosuppressive action that may interfere with the efficiency of vaccination programs, e.g. those for Newcastle disease (ND).

Often IBD is a serious problem in integration, and losses occur persistent efforts at reducing field virus's exposure through a biosecurity program, maintenance of adequate and uniform maternal titers and an effective vaccination program with a suitable vaccine and at a proper age. In this case, consideration should be given to vaccinating breeders with inactivated vaccines containing standard and variant strains of the IBD virus occurs (Jackwood and Sommer, 2005). Immunization of breeders is an important part of the IBD control program. Antibodies produced by the hen are passed through the egg to the broiler chick. These maternal antibodies, if present in adequate levels, protect the chicks against subclinical IBD.

Live vaccines are administered to achieve active immunity but interference of maternally derived antibody (MDA) is the crucial problem in determining a successful live IBDV vaccination schedule. Vaccinating chickens in the presence of high levels of maternally

derived antibodies results in vaccine virus neutralization and no immunity (Moraes *et al*., 2005). Currently as reported by (Hagazi *et al*., 2015) in Mekelle, Tigray, Ethiopia, determining the proper time for administration of live intermediate IBD vaccine important than giving IBD vaccine to chickens whose parents that have taken IBD vaccine without determining maternally derived antibodies (MDA) titer and age for vaccination. And therefore, in order to have chickens protected from IBDV field challenge, it is crucial to determine the optimal timing for IBD vaccine delivery. The optimal timing is often predicted based on serological data following detection of IBDV MDA by an ELISA system during the first week post hatch (De Wit, 2001).

The dramatic impact of a very virulent IBD virus can be reduced by proper clean-up and disinfection between flocks, and that traffic (people, equipment and vehicles) onto the farm be controlled. The development and enforcement of a comprehensive biosecurity program is the most important factor in limiting losses due to IBD because the IBD virus is very resistant and can survive for more than 100 days in a contaminated area. Phenolic and formaldehyde compounds have been shown to be effective for disinfection of contaminated premises (Gary and Richard, 2015). Since the virus is very stable for months. It is largely excreted through feces hence contaminated litter, feed and water have to be burnt or buried deep under the lime cover. Besides this other measures are; lower stocking densities, increasing intervals between flocks and complete removal of organic waste between batches. In areas where management practices to reduce virus concentration are used, the disease trends to occur at a later age, and immunosuppressive form of infection is reduced (Stwart-Brown and Grieve, 1992). Administration of inactivated vaccines to breeder hens induces long-standing and high levels of antibodies in the hatched chicks. In some areas where the producers do not adopt inactivated vaccination very virulent IBD virus has caused significant loss. Intensive live virus vaccination program is used in the hatched chicks from the unvaccinated breeder hens. Such chicks escape the strong risk of immunosuppressive form of the disease (Chuahan and Roy, 1998).

2.8. Current Status of Infectious Bursal Disease in Ethiopia

2.8.1. Prevalence of Infectious bursal disease

Various studies have been conducted with the main objective of determining the prevalence, and the associated risk factors of infectious bursal disease in different poultry production system of Ethiopia (Zeleke *et al.,* 2005; Solomon and Abebe, 2007; Hailu *et al.,* 2009; Tesfaheywet and Getnet, 2012; Natnael, 2015).

Gumboro disease was first reported in 2002 in Ethiopia at privately owned commercial poultry farm in which 45-50% mortality rate was documented (Zeleke *et al*., 2003); and diagnosed first in commercial poultry and thereafter in a government-owned poultry multiplication center (Solomon and Abebe,, 2007) and a commercial broiler farm (Chanie *et al.*, 2009) with serological tests. Infectious Bursal Disease is a newly emerging disease of chicken in Ethiopia, as described by Zeleke *et al.*, (2005) the disease has been considered to be introduced concurrent with the increased number of commercial state and private poultry farms flourishing in the country. Different researchers reported from various regions of the country indicated that viral diseases are posing a growing threat to the young poultry industry (Hailu *et al.*, 2009, AHY, 2011; Shiferaw et al., 2012, Natnael, 2015). Therefore, infectious diseases like IBD are becoming real threats to chicken under different production system (Aregitu, 2015).

In study conducted by Sindu *et al*., 2015, an overall seroprevalence of 45.05%(173/384) of Infectious bursal disease (IBD) in chicken reared under backyard poultry production systems around Mekele town; Out of 552 serum samples tested 458 (83%) in backyard chickens at selected woredas of Eastern Ethiopia by Tadesse and Jenbere, (2014); and 27.8% of with a case fatality rates of 98.56% and 77.73% the incidence of IBD in chickens owned by 775 households in Amhara region of Bahir Dar and Farta district respectively by Hailu *et al*., (2009) were indicated. Agar gel Immuno-deffusion test revealed the presence of antibodies against IBD in the serum of most recovered birds from IBD. Thus, it is of paramount importance to design cost effective control methods against IBD in order to improve the

productivity and welfare of village chickens and also to conserve the indigenous chicken genetic resource (Hailu *et al*. 2009).

Hailu *et al*., (2010), also reveals overall seroprevalence of 76.64% of IBD on local chicken (269/351) on study which was conducted in three selected districts of south and west Showa zones of Oromia region namely: Waliso, Welemera and Ambo; that has a prevalence of 89.78% in Waliso that had significant difference with seroprevalence in Ambo (70.69%) and in Welemera (40.81%). They reported an overall seroprevalence of 76.64% (269/351). There was also a significant difference on the seroprevalence based on different age groups (87.26% in 3-12 weeks, 74.4% in 13-24 weeks and 55.38% in 25-36 weeks old), hygienic condition also of birds kept in poor hygienic (83.33%) condition was very high as compared to those kept in good hygienic condition, and in chickens sharing the same house with the owners than those kept in separate shed.

An investigation reported by Shiferaw *et al*., 2012 in eight different districts of Ethiopia within objective of determining the seroprevalence and associated risk factors of infectious bursal disease among the total of 2,597 chicken serum samples, 83.1 % (2158/2597) positive for IBD examined using ELISA. Among the predisposing factors location, age, breed significantly associated with occurrence IBD of from those associated risk factors there were statistically significant ($p < 0.05$) in seroprevalence between districts (highest at Mekele (90.3 %) while the lowest at Gondar district (69.8 %), breed in crossbreed of chicken (91.4 %) while the lowest was recorded in indigenous breed of chicken (81.4 %),). Production system can as well influence the occurrence of IBD; i.e. seroprevalence recorded in intensive production system (85.9 %) higher than in extensive production system (81.6 %).

In addition, molecular characterization of the Ethiopian IBD virus isolates was done for the first time in 2005 from the samples collected from Kombolcha Poultry Multiplication Center, and in commercial and breeding poultry farms in Ethiopia between 2009 and 2011 (Shiferaw *et al.,* 2012). In both cases the samples were processed at the National Veterinary Institute, Ethiopia, for virus isolation using chicken fibroblast cell culture, and the positive isolates were submitted to OIE IBD Reference Laboratory, France, for further antigenic and genomic characterization, and were identified as virulent classical viruses and very virulent IBD virus.

In all cases the situation of the disease at small scale commercial flocks, and back yard poultry farms indicate the disease is widely distributed in the country. The importance of the disease reflected by the high mortality, reduced productivity amongst infected chicks and increased susceptibility to other infections accordingly, chickens also develop a poor immune response to vaccination against other pathogens (Zeleke *et al*., 2005). More over chicken traders also suffer from huge financial losses due to IBDV mortality in chicken, particularly those who buy young aged chicken and rear them for several weeks after purchase (Zeleke *et al*., 2005).

Table 1: Reported prevalence of IBD in Ethiopia

Study area	Prevalence	Authors
Mekele region	90.3%	Shiferaw *et al*., 2012
Southwest showa of Ethiopia	76.64%	Hailu *et al.,* 2010
Mekelle town	45.05%	Sindu *et al*.,2015
Debre-Zeit	82.2%	Tesfaheywet and Getnet, 2012
Andassa poultry farm	100%	Solomon and Abebe, 2007
Eastern Ethiopia	83%	Tadesse and Jenbere, 2014
Debre Brehan	94.7%	AHY, 2011

3. MATERIALS AND METHODS

3.1. Study Areas and Period

The study was carried out between October 2015 to August 2016 in selected parts of South West Ethiopia particularly in Jimma town and Bonga district. Jimma town is one of district in Jimma zone of Oromia Regional State, South West Ethiopia which contains 11 Kebeles (i.e. is the lowest administrative unit within the district). It is located at 355 km from South-West of Addis Ababa, the Ethiopian capital, at 7^0 41'N latitude and $36^0$50' E longitudes and has an altitude of 1704 meters above sea level. The area is characterized by a humid tropical climate of heavy annual rainfall that ranges from 1200-2000 mm per annual. The mean annual maximum and minimum temperature ranges from 6^0C and 31^0C respectively, with an overall average of 18.7^0C. Mixed crop-livestock production system is the main form of agriculture in the area. Although the area is mainly known for its coffee production but crop and livestock production is important agricultural activities as well (Tolosa *et al*., 2013).

The zone is the one containing largest livestock population in Ethiopia with an estimated population of 2,200, 106 of cattle, 824, 208 of sheep and 411, 180 of goats, 92, 093 of horses, 20, 011 of mules, 71,880 of donkeys, 1, 818,613 of poultry and 570,241of beehive. Among the 64.5 million of total livestock population that is found in regional national state of Oromia is 18.8 million are poultry population. While, Jimma zone contain the largest part of poultry population as compare to among the 17 regional zones with an estimated total population of 1, 818,613 of poultry. Among the total population of the zone chickens, 125,174 chickens population are found in Jimma town. The area is well practiced and dominant with different type of poultry production system (CSA, 2014/2015).

Bonga district is the administrative center of the Kaffa zone which contains a total of three Kebeles with a specific name called Bonga Zuria. It is located at a distance of 449 Km. that situated between 7^0 00' – 7^0 25' N and 35^0 55' – 36^0 37' E, South West of Addis Ababa. Topographically it lies at an altitude of 1650 meters above sea level and has a Woyena dega type of climate; and the mean maximum and minimum temperatures are 27.1^0C and 11.8^0C, respectively (FARM Africa, 2002a). Its average annual rainfall is 1750 millimeters.

Agriculture is the principal source of livelihood for most of the population. It is characterized by a subsistence mixed farming system, where rain-fed crop farming and livestock production coexist. Cattle, sheep and goats, with a total population of 931, 307; 420, 782; and 55, 210 respectively; are the main livestock types raised in the area. The zone is potential in poultry production at about total population of 1,924,450 (CSA, 2014/15). Among a total, 57,608 poultry population is found in Bonga district. The district is well practiced by intensive, semi-intensive and extensive farming system.

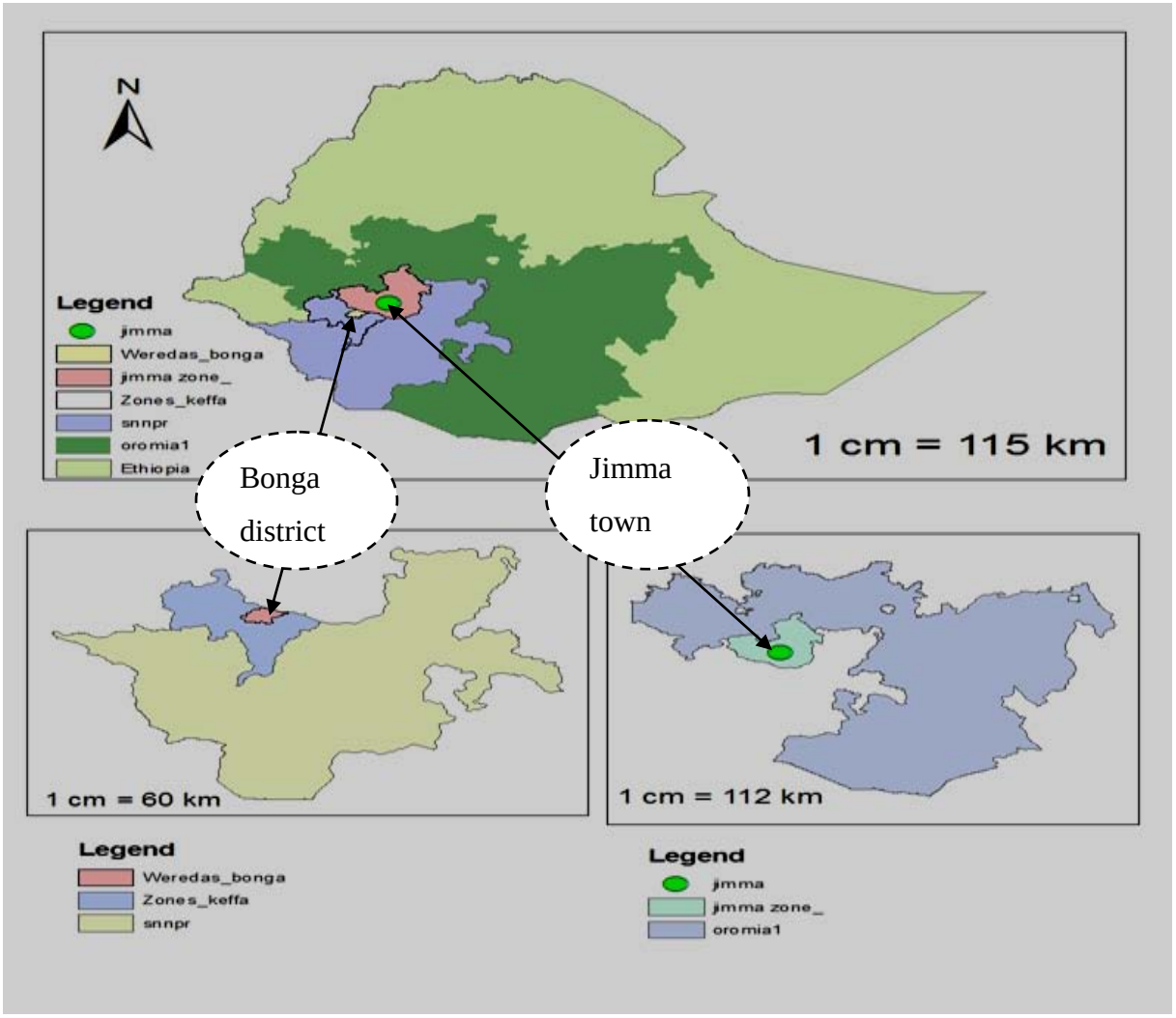

Fig.2 Map of study area

3.2. Study Animals

The study animals were apparently healthy and unvaccinated chickens (referring those chickens seemingly normal based on clinical observation and symptoms and function, normally assessed by routine physical evaluation and those which was not vaccinated before by IBDV vaccine during study) reared under different level of management systems such as extensive, semi-intensive and intensive production system at different Peasant Association of Jimma town and Bonga district of southwestern Ethiopia.

3.3. Study Design

The type of study design was cross-sectional study type which was conducted from October 2015 to October 2016 to establish serological prevalence of IBD and to assess the associated risk factors in the investigation areas.

3.4. Sample Size Determination

For this particular study the sample size was determined for a define precision and level of confidence, and calculated according the formula by Thrustfield (2005). Since there was no previous estimated prevalence of infectious bursal disease in the area of current study were carried out; 50% prevalence was expected with desired absolute precision 5% and 95% confidence interval were used to determine the minimum sample size.

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

n= 384

Where,

n = sample size of the study population
d = Absolute desired precision
p = previous/expected prevalence in the study area
CI = confidence interval
1.96^2= Z- value for the confidence levels.

However, according to Tedesse and Jember, (2014) the correction for multi-stage sampling design effect was considered and multiplied of 10%, the final sample size become a total of 422.

3.5. Sampling Method

A Multi- stage sampling technique was used to select the sampling units. The woreda and Kebeles were considered as primary unit, the flock owned by the house hold or farm as secondary unit and individual chickens as tertiary unit (Tesfaheywet and Getnet, 2012). During study, these study areas were selected purposively; since areas are well practiced by different poultry production type. About 50% of the Kebeles in each of the districts were considered representative to the districts and included in the study on the basis of feasibility and affordability or cost. Hence, eight kebeles such as 6 Kebeles namely Kito, Ginjo, Jiren, Bore, Hermata, and Kochi were from Jimma town; and two Kebeles namely Shata Kentery and Meskel Adebabay from Bonga district were selected based on total chicken population presented within Kebeles which was documented in livestock statistics data of Livestock and Fishery development office of Jimma town and Bonga district respectively in 2008 E.C. Chickens which were presented under extensive management system, semi intensive and intensive farms have been considered.

Table 2: Number of sample size within production type in the study area

Study area	Sample number per production type			Total
	Intensive	Semi- intensive	Extensive	
Jimma Town	43	6	240	289
Bonga district	37	24	72	133
Total	80	30	312	422

An average of 39 (312/ 8 kebeles= 39) extensively managed chickens within kebeles of both districts while the rest of sampled population were obtained from semi- intensive and or intensive farms presented with in selected kebeles. About 20 households were randomly selected within kebeles who have moderately high number of back yard chickens and an average of two chickens were to be sampled per house hold; and 5% per semi intensive and

or intensive farms presented within selected kebeles. The number of households was calculated by dividing an average of 39 chickens to be sampled per kebeles by the average of two chickens to be sampled per household (Sule *et al*., 2013). Following, blood sample were drawn from randomly selected chickens. The chickens were categorized based on their sexual maturity, chickens between the ages ranges of ≤8 weeks old were considered Young while those >8 weeks were considered Adults chickens (Shiferaw *et al*., 2012). Similarly, production type, sex, breed and location of chickens were recorded. The total sample size calculated was comparatively allocated between the two districts based on total population owned in the districts. Among the total sample size, 68.5% of sample i.e. 289 of sera sample was collected from Jimma town whereas 31.5% of i.e. 133 of sera sample were collected from Bonga district.

3.6. Blood Sampling and Storage

Approximately 3 lm of blood was collected from the humeral region of the wing vein with a 5-ml syringe. The syringe was laid nearly horizontally until the blood clotted. After clotting, the syringe was returned to a vertical inverted position to permit the serum to ooze out. The sample was then kept at 37^0C for several hours or left overnight before the serum was removed. The separated serum was transferred to plastic cryovials, labeled for identification, and stored at $–20^0$C until the Indirect enzyme-linked Immunosorbent assay (I-ELISA) was performed to detect antibodies against the IBD virus (Singh *et al.,* 2010).

3.7. Serum Analysis

The ELISA test was done following procedures outlined by indirect ELISA diagnostic kit which designed to detect antibodies directed against the infectious bursal disease virus, also known as Gumboro Disease (OIE, 2004; De Herdt *et al.,* 2005).

3.7.1. Sample processing

The serum samples test were carried out at OIE diagnostic laboratory center of National Veterinary Institute (NVI) in Debrezeit, Ethiopia based on the procedures employed for Indirect Enzyme-Linked Immunosorbent Assay (IELISA) using commercially available

ProFLOK® IBDV antibody test kit. The ProFLOK® IBD ELISA kit is a rapid and specific serologic test for the detection of IBD antibody in chicken serum samples.

3.7.2. Sample preparation

Blood samples were collected from non-vaccinated chickens. Five ml sterile disposable syringes were used to collect blood samples aseptically directly from the wing vein. Soon after the collection of blood, the syringes with blood were kept at room temperature overnight for clotting of blood in one side of the syringes. Then sera were decanted into cryotubes bearing identification. Finally, the clarified sera were stored at -20^0c until tested. This serum was used as a test sample for the detection of IBDV specific antibody level in the chicken using I-ELISA. Finally, application of ELISA for IBD was carried out depending on procedure indicated below (Appendix 2).

Calculation of antibody level using equation provided in ELISA Kit: The presence or absence of antibody to IBDV was determined by the color reactions quantified by measuring the optical density of each well at 450 nm. The positive control has been standardized and represents the significant antibody level to IBDV in chicken serum. The relative level of antibody in the unknown can be determined by calculating the sample to positive (S/P) ratio.

ELISA validity test**:** In a valid IBD ELISA result, the mean Optical Density (OD) value of positive control serum (OD) is greater than 0.25 ($OD_{PC} > 0.250$) and the ratio of the mean values of the Positive and Negative Controls (OD_{PC} and OD_{NC}) is greater than 3. But the OD value is out of these ranges, IBD ELISA result was considered invalid.

Interpretation of ELISA test: Serum sample positive control ratio was calculated for test interpretation. Accordingly, the following equation was applied:

S/P ratio

$$S/P = \frac{OD_{Sample} - OD_{NC}}{\mathrm{OD_{PC}} - OD_{NC}}$$

For each sample, the S/P (sample to positive ratio) value is >0.3 the IBD immune status was considered to be positive but $\leq$ 0.3 was taken as negative.

3.8. Data Management and Analysis

Microsoft excel spread sheet program was used to store all the data, filtered, coded and recorded in window 7 Microsoft excel before transferred and analyzed by using Statistical Package for Social Science (SPSS) version 20. Descriptive statistics was used to summarize the data in the tables. The prevalence was calculated by dividing the number of chickens that were test positive by the total number of chickens examined. Logistic regression was used to determine degree of association between risk factors and IBD. Any variables with $P \leq 0.2$ after univariate analysis were included in multivariate logistic regression analysis to examine the independence effects of each risk factor in SPSS 20. The degree of independence within variables was determined by odd ration obtained from the analysis. A backward stepwise variable-selection strategy was used to make a final model with a significance level of $P < 0.05$ at 95%confidence level.

4. RESULT

A total of 422 chickens' sera were collected from different breeds, mixed age groups, sex and management system with no previous history of vaccination against infectious bursal disease from the Jimma town and Bonga district of southwestern Ethiopia. Of total the sera presented to examine by the test of Indirect Enzyme Linked Immunosorbent Assay (I-ELISA), 407 sera were tested positive with an overall seroprevalence of 96.4% (94.62- 98.18) 95% confidence interval.

4.1. Univariate analysis of different risk factors in relation to Seroprevalence of IBD

4.1.1. District and Kebeles

Among a total of 289 sera samples collected in Jimma district, 283 (97.9%) were tested positive while of 133 sera sample collected from Bonga district, 124 (93.2 %) of chickens sera were tested positive. This was statistically significant ($P<0.05$) association. Although there was no statistical significance observed ($p>0.05$) with in kebeles in the analysis, the highest prevalence rate was recorded in Kito (98.5%) and followed by Ginjo (98.3), Bore (97.7%), Hermata and Kochi (97.5%), Jiren (97.4%), Meskel Adebabay (96.4%) and Shata Kentery (91.4%) (Table 3).

Table 3: Seroprevalence of IBD in relation to district and kebele

Variables	Tested	Positive	Seroprevalence (%)	P value	OR	95% CI
District						
Jimma town	289	283	97.9	-	-	-
Bonga	133	124	93.2	0.016	0.29	0.102-0.838
Kebeles						
Kito	67	66	98.5	-	-	-
Ginjo	60	59	98.3	0.091	0.16	0.019-1.336
Bore	44	43	97.7	0.433	0.38	0.033-4.296
Hermata	40	39	97.5	0.739	0.62	0.038-10.21
Jiren	38	37	97.4	0.685	0.56	0.034-9.23
Kochi	40	39	97.5	0.713	0.59	0.036-9.717
Meskel Adebabay	52	50	96.2	0.764	0.65	0.040-10.696
Shata Kentery	81	74	91.4	0.918	0.86	0.053-14.122

4.1.2. Sex, Breed and Age

Seroprevalence of infectious bursal disease was compared in both sexes in different production system, age group and breeds in the study areas. Of 294 total number of sera collected from females and tested, 288 (98%) were found positive for IBD antibodies. The total sera collected from males was 128 of which 119 (92.9%) were tested positive. The occurrence of infectious bursal disease is 3.6 times more likely prevalent in females than male's chickens. This result was significantly associated ($P<0.05$) as shown in table 4.

Even though there was no statistically significance association ($P>0.05$) in between breeds, the highest seroprevalence was recorded in exotic breed followed by local and cross breed. Of 126 total number of sera collected from exotic chickens and tested by I-ELISA test, 120 (96.8%) were found positive for IBD antibodies. The total number of sera collected from local chickens were 253; of which 244 (96.4%) were positive followed by cross of 45 sera collected

from chickens 43 (95.8%) were positive tested by I-ELISA also. Detailed results according to breeds were shown on table 4.

There was higher prevalence recorded under young age groups (≤8 weeks old) chickens. Of 195 total number of sera collected and tested by I-ELISA test, 194 (99.5%) were found positive for IBD antibodies. The total number of 227 sera collected from adult group of >8 weeks old chickens, 213 (93.8%) were positive tested by I-ELISA. This association was statistically significant (P<0.05) shown on table 4.

Table 4: Seroprevalence of IBD in relation to sex, breed and age groups

Variables	Tested	Positive	Seroprevalence (%)	P value	OR	95% CI
Sex						
Male	128	119	92.9	-	-	-
Female	294	288	97.9	0.017	3.5	1.235-10.179
Age in weeks						
Young(≤ 8)	195	194	99.5	-	-	-
Adult (>8)	227	213	93.8	0.014	0.08	0.011-0.645
Breed						
Exotic	124	120	96.8	-	-	-
Cross	45	43	95.8	0.504	0.64	0.087-3.327
Local	253	244	96.4	0.459	0.51	0.165-2.258

4.1.3. Production System

Though, the result recorded among chicken reared under different level of production system was not indicted a statistically significant association (P>0.05), relatively highest prevalence was recorded in intensive production system where as the lowest was recorded under extensive management system. Detail result was shown in the table 5.

Table 5: Seroprevalence of Infectious bursal disease on different production system

Variables	Tested	Positive	Seroprevalence (%)	P value	OR	95% CI
Production type						
Extensive	312	300	96.2	0.272	0.32	0.041-2.471
Semi-intensive	30	29	96.7	0.164	0.18	0.015-2.031
Intensive	80	78	97.5	-	-	-

4.2. Risk Factors Analysis in Relation to Occurrence of IBD Using Multiple Logistic Regression

Multiple logistic regression analysis was carried out to examine the independence of effects of each risk factor in relation to prevalence of the disease. Any variables with $P \leq 0.2$ after univariate analysis were considered in the multivariable analysis and a backward stepwise variable-selection strategy was used to make a final model with a significance level of $P \leq 0.05$ at 95%confidence level. Followed, variables such as age, sex and variation in the district were significantly (P<0.05) associated and revealed as a major risk factors for the highest prevalence of infectious bursal disease in the study area. The statistical analysis indicated, for one week of age increase in chicken, the log odds of seroprevalence of the IBD decreases by 3 times. The variation in the sex influences significantly and for one female's chicken increase in number, the log odds of the disease prevalence increases by 1.3 times whereas when increasing in sampled chickens number from Bonga district of negatively influences the log odds of disease by 1.2 times than from Jimma district.

Table 6: Multivariate logistic regression analysis of risk factors

Risk factors	B	P value	OR	95% of CL
Adult to young	-2.659	0.011	0.070	0.009-0.547
Female to males	1.268	0.022	3.554	1.197-10.548
Bonga to Jimma town	-1.172	0.035	0.32	0.104-0.920

B: coefficient; CI: Confidence interval; OR: Odd ratio

5. DISCUSSION

Although it has been more than 50 years ago, in 1962 in Gumboro, Delaware, USA, since Gumboro Disease joined the list of poultry diseases and vaccines have been used, the most striking fact regarding this condition is that it is still widely present, and still ranks among the top infectious in almost all countries (Van den Berg, 2000; Jinda *et al*., 2004; Fantay *et al*., 2015). This can be explained by the extremely high resistance of the Gumboro virus, allowing it to survive in the poultry house in the absence of chickens during down periods, despite cleaning and disinfection, as well as by its capacity to escape post infection and/or post vaccination passive and active immunities, by selection of antigenic mutants (Van den Berg, 2000; Zanella, 2007; OIE, 2012). Previous serological surveys in Ethiopia also showed that the infectious bursal disease is prevalent and widely distributed in different chicken rearing areas of the country (Zeleke *et al*., 2005, AHY, 2011; and Shiferaw *et al*., 2012). Hence, a cross-sectional study was conducted in two selected woredas: Jimma town and Bonga district of south western Ethiopia to determine seroprevalence of infectious bursal disease and its associated risk factors in unvaccinated and apparently healthy chickens of the area.

In the present study, an overall seroprevalence of 96.4% was reported from the total of 422 chickens serum examined through I-ELISA test, 407 were tested positive for IBD. The highest seroprevalence of IBD in this study is clearly showed that IBDV is widely distributed and is a problem of all types of production systems and breeds of chickens in the investigation areas. It is suggested that although there was no any clearly seen clinical sign of the disease observed during sample collection in chickens reared in the area, there might be due to the presence of field exposure of chickens to the virus and remained sub clinically infected during early of the age. Since the presence of IBD antibody in the sera of chickens tested during diagnosis is an indication of previous exposure of chicken of natural infection in the field (Hailu *et al*., 2010; Mahasin *et al*., 2016). Similarly, Shrestha *et al*. (2003) reported in non vaccinated chickens the level of antibodies against IBDV might remain detected zero maternal immunity after a few days or a week and this might increase the chance of 100% susceptibility to IBD. It might be also distribution of improved breed of chickens from infected poultry breeding and multiplication centers to the other centers is suspected of disseminating diseases and enhanced prevalence of disease to chickens reared in different

production systems. This is in agreement with the report of Alemu (1995) and Zeleke *et al.* (2005).

The overall seroprevalence (96.4%) obtained from this study further supports the previous findings of Ethiopian animal health year book (2011) which reported 94.7% in Debre Bran; Zeleke *et al*. (2005) who reported 93.3 % of overall seroprevalence in non vaccinated chicken of Debre Zeit; Solomon and Abebe (2007) on the case-report study from Andasa poultry farm of which indicated a 100% seroprevalence in non vaccinated flocks; and study conducted by Hermandez-Divers *et al*. (2006) showed a 100% seroprevalence in northwest Ecuador. In addition, a comparable reports of seroprevalence 83.1 % (2158/2597) in eight selected districts of Ethiopia by Shiferaw *et al*., (2012); 83% in backyard chickens at selected districts of eastern Ethiopia by Tadesse and Jenbere, (2014); and 82.2% in chickens managed under backyard production system in Central Oromia Tesfaheywet and Getnet, (2012) by documented by using commercialized ELISA kit.

In contrast to current result, studies conducted by Hailu *et al.* (2009) who reported an overall seroprevalence (38.9%) in village chickens; and Tesfaye (2008) reported of 29% in non-vaccinated backyard local chickens of East Showa zone, Akaki, Debrezeit and Adama. Similarly, Ndanyi (2004) from Kenya reported a seroprevalence rate of 49.3%; and Tsai and Lu (1993) and Singh *et al*. (1994) also indicated a seroprevalence rate of 45 and 46.2%, respectively by using agar gel immune diffusion (AGID) test as diagnostic tool. The reason underlying variations in the result might be attributed due to sample collection followed outbreaks of infectious bursal disease. This is in line with the result of Shiferaw *et al*. (2012) who reported 90.3% of highest seroprevalence in sample collected followed an outbreak season in Mekele compared to other study areas. The difference in sensitivity and specificity of the test used by different authors also influences the prevalence of the disease. This is in line with the Manual of Office International des Epizooties (OIE, 2004), that described ELISA as the most ideal, sensitive and specific diagnostic tool used for serological diagnosis of viral antibodies. In addition, the difference in the result, might be also the difference in agro-ecological zone of the study conducted, number of sample size, age of chicken sampled, the breed difference, immune status of the host, availability of veterinary services and

awareness in the public services towards the disease control and prevention in the study area influences the seroprevalence of the disease (Hailu *et al*., 2010; Sindu *et al.*, 2015).

Regarding results of districts, there is variation within district statistically significantly (P=0.035, OR=0.32, CI=0.104-0.920) association at 95% of CI as indicated in table 6. The IBD infection in chickens reared in Bonga were 0.3 times less likely prevalence than that of Jimma district i.e. the disease was more likely prevalent in Jimma town than that of Bonga district. The result agrees with findings of Hailu *et al*. (2010) and Sindu *et al*. (2015) reported differences within agro-ecology statistically influences occurrence of IBD. For instance, Swaia, *et al.* (2011) reported statistically significant IBD prevalence which varies from 37.5 % to 91 % between districts and from 75 to 90 % between regions in northern Tanzania free ranging chickens.

Higher seroprevalence obtained among chickens serum collected from Jimma town as compared to Bonga district revealed that there is high virus activities in the area. The resoan might be suggested that there might be higher access of importing young chickens from differet multiplication centers and redistributing to surrounding farmers and private farmes as well as organized youth without immunizing chickens against IBDV. While, reportes indicated that all of government owned poultry multiplication and distribution centers with the exception of Bonga and Bedelle were devastated by the outbreak of Infectious Bursal Disease i.e. Gumboro disease (Yilma, 2007; Chanie *et al.,* 2009). The occurrence of antibodies to infectious bursal virus of local chickens is suggestive of a high viral activity that may have a significant implication of the prevalence of the disease in exotic chickens reaered mixed to village chickens (Sule *et al*. (2014). Since village chickens are considered to be relatively resistant to most poultry diseases including IBD, they can serve as carriers of the virus and play a vital role in the transmission of the disease to more vulnerable exotic breed chickens and vice-versa (Mahasin *et al*., 2016) in the environment. The absence of programmed vaccination against IBDV in exotic breeds and as well as local or cross type of chickens in the study area might also resulted in high seroprevalence of IBD. This findings is ingreement with finding of Natnael (2015) vaccination influences occurrence of disease in the specific area. In addition, this result may also supports previous finding of Hailu *et al.* (2010), Shiferaw *et al*. (2012) and Sindu *et al*., (2015) who reported the difference in

prevalence's of IBD within districts as well as kebeles can be associated with factors like differences in geographic and climatic condition of the area, age, immunity status of chickens, husbandry and the hygiene condition with in area.

Furthermore, although there was no statistically significance difference among the kebeles, the highest prevalence obtained within chickens sampled in Kito (98.5%) and followed by Ginjo (98.3), Bore (97.7%), Hermata and Kochi (97.5%), Jiren (97.4%), were indicative of a high virus activity. On the other hand, relatively low prevalence in Meskel Adebabay (96.4%) and Shata Kentery (91.4%) as shown on table 3 were indicative of low virus activities in the area as compare to others. This result might be due most of Kebeles of Jimma town are more crowded and densely populated settlement and most of chickens are getting their food through scavenging; therefore, there is high chance of contact with virus contaminated sources. Since infectious bursal disease can be transmitted through contact exposure to excreting subject or by indirect contact with any an animate objects it is probable that the high prevalence within kebeles of Jimma district was due to horizontal transmission through direct contact with contamination sources and products (Adene *et al.*, 1985; Okoye *et al.*, 1999) that occurred around the many garbage's generated by densely populated settlements. The low prevalence obtained within Meskel Adebabay and Shata Kentery may be associated with the dispersed nature of their settlements. This result agrees with studies conducted by Tesfaheywet and Getnet (2012) on seroprevalence of infectious bursal disease in chickens managed under backyard production system in Central Oromia, Ethiopia; and Sule *et al*. (2013) on serological survey for infectious bursal disease virus antibodies among village chickens in Yobe State Nigeria concluded that settlement nature of the district and or kebeles influences prevalence of IBD.

Relatively higher seroprevalence of infectious bursal disease was recorded in females 288 (98%) compared to males of which 119 (93%) in both sexes in different production system. This result was statistically significant (P=0.022, 3.6 (1.197-10.548)) as shown in table 6. The infectious bursal disease in females is 4 times more likely prevalent than in males in the investigation area. This difference might be due to physiological and immunological differences between both sexes. Moreover according to report Bettridge *et al*. (2014) of the reproductive demands placed in females may increases the risk of infection as compare to

males. It is also possible that there are some other unmeasured risk factors in common, such as different male and female behaviours, which increases exposure to pathogens. This is supported by Sindu *et al*. (2015) on study conducted in non vaccinated chickens in Mekele of Northern Ethiopia. In contrast to current result, Shiferaw *et al*. (2012); Tesfaheywet and Getnet (2012); and Tadesse and Jenbere, (2014) reported there was no statistically significant (P>0.05) variation between sexes in relation to infectious bursal disease since they have equal probability to exposure of infection by this contagious virus.

Even though there was no significant association (P>0.05) observed statistically with in breeds in relation to susceptibility of chickens to IBDV, slightly highest seroprevalence was recorded in exotic (96.8%) breed followed by local (96.4%) and cross breed (95.8%) as indicated above on table 4. This result showed that seroprevalence was slightly higher in the exotic group indicating that they might be more susceptible to infection by the virus (Mahasin *et al*., 2016). Similar studies were conducted with seroprevalence rates exceeding 90% among exotics compared to that recorded in local breeds (Zeleke *et al*, 2005). Similarly higher prevalence is seen in locals followed by crossbreed chickens. Besides, the relatively considerable seropositivity among locals compared to crossbreed chickens in the study might be because farmers prefer rearing local chickens with low cost of production compared to improved breeds in the study area. This might explain the higher number of local breed was examined compared to that of the crossbreed. Even if there were a slight difference in between breed prevalence, the result might indicate that the local breed or crossbreed of chickens was also exposed to IBDV. The reason was the detection of this considerable antibody levels was done in chickens that has no history of previous vaccination and in apparently healthy flocks. The result supports findings of Tesfaheywet and Getnet, (2012) who reported that all chickens were susceptible to IBDV infection and might show subclinical infection and immunosuppression due to field exposure especially at early ages. However, this result is in contrast to previous findings of Shieferaw *et al* (2012) who reported the highest seroprevalence in crossbreed of chicken (91.4 %) followed by exotics while the lowest in indigenous breed of chicken (81.4 %) in which there was a statistically significant differences within seroprevalence of IBD among the three breeds of chickens. The different

might due to the fact that indigenous breeds have better resistance to most diseases compared to exotic breeds.

Concerning the result of the age group, the higher seroprevalence (99.5%) was recorded on young age groups (≤8 weeks) chickens than chicken on age above 8 weeks old (93.8%). As the chickens got older and older, the seroprevalence decreases significantly. This indicated age is one of potential factors which statistically influences (P=0.011, OR= 0.070, CI= 0.009-0.547) the seroprevalence of IBD in the investigation area (Table 6). Infectious bursal disease among adult age groups (>8 weeks old) is 0.07 times less likely occurred than among young aged groups of chickens. The reason of variation in age group is might be within this age, naturaly IBD is the disease of chickens and which bursa of fabricius (BF) attains it maximum growth and development with sufficient B lymphocyte for IBDV multiplication. This finding is more likely supported by the result of Saif *et al.* (2000), Tesfaheywet and Getnet, (2012); and Natnael (2015) who reported that chickens of age 3 to 6 weeks were more susceptible to IBD because the bursa, the site for IBDV multiplication, is matured and maximum in size at this age. Similarly, in the report of Shiferaw *et al*. (2012) as the age increases the BF begins to atrophy starting from seven weeks old and the number of B lymphocyte decrease gradually. The maximum susceptibility is observed between 2 and 7 weeks of age (Hittchner, 1978). An investigation conducted by Sigh and Dhawedkar (1992) reported a high seroprevalence of IBD (61.82%) in chicken between 7 and 11 weeks and lowest in those above 22 weeks of age which was closely related to the findings of present study. As result of this, IBDV infectivity decrease as B lymphocyte decrease with age of the chickens (Rashid *et al.*, 2013).

Management system influences the occurrence of infectious bursal disease viruses. Though, the result recorded among chicken reared under different level of production system was not indicates a statistically significant association (P>0.05), relatively highest prevalence was recorded in intensive production system where as the lowest was recorded under extensive management system (Table 8). This might be due to intensification being able to easily share infection horizontally through the flock. Similarly as reported by Negash (2013) and Natnael (2015) the susceptibility for IBDV varies according to management level. In the report of Shiferaw *et al*., (2012) production type can as well significantly (P<0.05) influence the occurrence of IBD i.e. seroprevalence recorded in intensive production system (85.9 %)

higher than in extensive production system (81.6 %). In addition in the report of Hailu *et al*. (2009) and Hailu *et al*. (2010) seroprevalence of IBD of chickens kept in different production system influenced by improper cleaning, keeping used litter, poor ventilation and overcrowding. The occurrence of IBD in semi-intensive chicken production system is a relatively higher than that of extensive chicken flock; lower than intensive production system (Natnael, 2015). So this is may be due to that intensification of production system can act as a means of protective factor against IBDV.

6. CONCLUSION AND RECOMMENDATIONS

This research documented a high seroprevalence of infectious bursal disease in unvaccinated chickens reared in Jimma town and Bonga district of south western Ethiopia. The result of this study is clearly showed that IBDV is widely distributed in almost all parts of study areas, resulting in considerable economic impact through mortality and secondary losses manifested in subclinical infection among all types of production systems and breeds of chickens. This may lead to immune suppression which adversely affects response to vaccination to other pathogens.

Chickens within young age group, sex in females and variation in districts were only major variables significantly associated with the disease and were found to be predominant that could predispose chickens and favors the increased incidence of the disease. This high seroprevalence of IBD reduces the income of producer from chicken in the investigation areas. Finally depending on above conclusion the following recommendations are forwarded:-

- Attention should be given in administration of scheduled vaccination program at early age of chickens and proper biosecurity measures in intensive and semi-intensive farms to reduce masses of infectious bursal disease virus infection and to enable a suitable control program in the study area.
- Responsible stock holders should have to plan vaccination schedules in chickens before distribution of young chickens to different production system.
- Farmers should be encouraged to adapt management system and vaccinate chickens in different level of production regularly to reduce further loss.
- Investigation should be carried out at molecular diagnostic level to isolate all the currently circulating strains of IBDV for developing vaccines that match to wild virus in the study area.
- Further, regular surveillance in the vaccination response and characterization of field strains would help in checking out and re-evaluating control strategies.

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8. APPENDICES

Appendix 1: Serological sample data collection sheet

Region……Zone……District……PA……

Date of sample collection…….....…/……../2008E.C

Laboratory code……..Geo reference

N^0……E^0……Altitude……..

Purpose of Sample collection ……

N^0	Owner name	Sample N^0	Breed	Age in week	Sex	Vaccination status	Clinical status	Production system	Test result

Appendix 2: I-ELISA testing procedure

Allow all reagents to be come to room temperature (21^0c $\pm$ 5^0c) before use homogenize all reagents by inversion or Vortex.

1. Samples are pre-diluted 1:500 in Dilution Buffer 14. In a pre-dilution plate, add:

- 245 micro liter of Dilution Buffer 14 to each well.
- 5 micro liter of the Negative Control to wells A1 and B1.
- 5 micro liter of the Positive Control to wells C1 and D1.
- 5 micro liter of each sample to be tested in the remaining wells.

2. In the ELIZA microplate, add

- 90 microliter of Dilution Buffer 14.
- 10 microliter of pre-diluted samples mentioned above.

3. Cover the plate and incubate 30 min $\pm$3 min at 21^0c ($\pm$ 5^0c).

4. Prepare the Conjugate 1x by diluting the Concentrate 10x to 1:10 in Dilution Buffer3.

5. Empty the wells. Wash each well 3 times with approximately 300 microliter of the Wash solution 1x. Avoid drying of the wells between washings.

6. Add 100 microliter of the Conjugate 1x to each well.

7. Cover the plate and incubate 30 min $\pm$ 3 min at 21^0c ($\pm$ 5^0c).

8. Empty the wells. Wash each well 3 times with approximately 300 microliter of the Wash solution 1x. Avoid drying of the wells between washings.

9. Add 100microliter of the Substrate Solution to each well.

10. Incubate 15 min $\pm$ 2 min at 21^0c ($\pm$ 5^0c) in the dark.

11. Add 100 microliter of the Stop Solution to each well in order to stop the reaction. The Stop Solution should be added in the same order as in step Nº9.

12. Read and record the O.D. at 450 nm.

Appendix 3: IBD IELISA Plate Layout Form

Region…….Zone……District……PA……

Plate…….Date……Code…….

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												