# EVALUATION OF SOME COAGULATION FACTORS IN COCKERELS EXPERIMENTALLY INFECTED WITH VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS (IBDV37/KADUNA NG/2009)

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**DEPARTMENT OF VETERINARY PATHOLOGY, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

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## DECLARATION

I declare that the work in this Thesis entitled **"Evaluation of some coagulation factors in cockerels experimentally infected with very virulent infectious bursal disease virus (IBDV37/ KD NG/2009)"** has been carried out by me in the Department of Veterinary Pathology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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| Lukman Muftahu OYEDEJI | SIGNATURE | DATE |

## CERTIFICATION

This thesis entitled **"EVALUATION OF SOME COAGULATION FACTORS IN COCKERELS EXPERIMENTALLY INFECTED WITH VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS (IBDV37/KADUNA NG/2009)"** by Lukman Muftahu OYEDEJI meets the regulations governing the award of the degree of Master of Science in Veterinary Pathology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

DR. K. A. MAJIYAGBE

Chairman, Supervisory Committee _____ Signature _____ Date

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Dean, School of Postgraduate Studies _____ Signature _____ Date

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## DEDICATION

This work is dedicated to my brother, Bashir Muftahu, my sister, Hajiya Shakirah Muftahu and to anyone who fell victim of Gumboro disease.

## ABSTRACT

Young chickens in Nigeria are known for their high susceptibility to very virulent infectious bursal disease virus (vvIBDV). Almost all clinical cases of infectious bursal disease (IBD) have been associated with haemorrhage. Coagulopathies and thrombocytopaenia have been associated with haemorrhage. This study aimed at evaluating the coagulation defects in IBD. One hundred Near Black cockerels were purchased at day old. They were acclimatized for the period of 28 days before being randomly divided into two groups of 50 chickens each and kept separately. One group of fifty cockerels were innoculated with vvIBDV strain IBDV37\Kaduna NG\2009 (GenBank accession number JX 424062) intraocularly at the age of 35 days. Four and half milliliter of blood was collected to determine the haemogram and coagulation factors at 6, 12, 24, 36, 48, 60, 72, 84, 96, 120 and 168 hours post inoculation (hpi). Necropsy was carried out during sampling and tissue sections of the bursa of Fabricius, spleen, thymus, liver, kidney, keel and thigh muscles were taken for histopathology. The clinical signs observed beginning at 48 hours pi included depression, anorexia, ruffled feathers, and greenish yellow diarrhea. The thrombocyte count reduced from $7.86 \pm 0.79 \times 10^3/\mu l$ at 6 hpi to $2.55 \pm 0.14 \times 10^3/\mu l$ at 84 hpi ($p < 0.05$) in the IBDV innoculated group. Activated partial thromboplastin time revealed prolonged clotting time between 60 hpi and 120 hpi. The major lesions observed at necropsy include bursal oedema and haemorrhage beginning at 72 hpi and later atrophy, petechial and ecchymotic haemorrhages in thigh and keel muscles and at the mucosal junction between proventriculus and gizzard with higher frequencies being observed in dead birds than sacrificed ones. Histopathological examination of bursal sections affirmed that the predominant lesion was lymphoid cell necrosis. The spleen and thymus were much less severely affected and

possess greater regeneration potential. The results showed that vvIBDV of Nigerian field isolate caused severe lymphoid cell necrosis and later bursal atrophy and. Haemorrhage is associated with mortality in IBD. The marked thrombocytopaenia observed which could be the cause of the delayed coagulation time contributes significantly to the haemorrhages.

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# CHAPTER ONE

## INTRODUCTION

For many years, infectious bursal disease (IBD) has constituted a serious problem to the poultry industry, and the recent re-emergence of infectious bursal disease virus (IBDV) in the form of antigenic variants and hyper virulent or very virulent strain has been the cause of significant losses in the poultry sub-sector (van den Berg *et al*.,2000).

Infectious bursal disease was first recognized as a specific disease entity by Cosgrove in 1957 (Cosgrove, 1962) and was referred to as "avian nephrosis" because of the kidney damage found in birds that succumb to the infection. Because the first outbreak occurred in area of Gumboro, Delaware (USA), "Gumboro disease" become a synonym for this disease and is still frequently used (Lukert and Saif, 2003).

Gumboro disease is a highly contagious viral disease of young chickens, characterized by destruction of the lymphoid cells in the Bursa of Fabricius; other lymphoid organs are also affected but to a lesser degree (Cheville, 1967; Kibenge *et al.*, 1988). The disease is caused by IBDV, a virus that is a member of the genus *Avibirnavirus* of the family *Birnaviridae* (Saif, 1998; van den Berg *et al*., 2000, Lukert and Saif, 2003; Okoye, 2005; OIE, 2008; Mac Lachlan and Dubovi, 2011).

Infectious bursal disease virus is a non-enveloped, icosahedral, bisegmented double stranded RNA virus with a diameter of about 55-60nm (Isma'il and Saif, 1990; Mbuko *et al*., 2010; Mac Lachlan and Dubovi, 2011). Two distinct serotypes of IBDV are known to exist (van den Berg *et al.*, 2000; OIE, 2008; Mac Lachlan and Dubovi, 2011) namely: serotype 1 which is pathogenic for chicken and serotype 2 which is apathogenic and has been isolated from

chickens and turkeys (van den Berg *et al*., 2000; Mahgoub, 2012). The two serotypes are differentiated *in vitro* by the absence of cross neutralization, and *in vivo* by the absence of cross protection (Jackwood *et al.*, 1982; Jackwood *et al*., 1984, Isma'il *et al*., 1988 and Betcht *et al*., 1988; van den Berg, 2000).

Development of antigenic drift among serotype 1 viruses has been reported in the last few decades (Rosenberger and Cloud, 1986; van den Berg, 2000). These new viruses or strains are known as IBDV antigenic variants or subtypes. They lack complete antigenic similarities or homogeneity when compared with the classical or standard strain of IBDV by virus neutralization test. The practical implication of this is that when the variant strains attack the flocks that have been vaccinated with the classical strains, the later will not offer complete and satisfactory protection against clinical IBD (Okoye, 2005). Another development was the report of the emergence of very virulent strains of infectious bursal disease virus (vvIBDV) in Europe in 1987 (Chettle *et al.*, 1989; van den Berg 2000). These viruses cause mortalities at least twice as high as those of classical IBDV strains (van den Berg *et al.*, 1991 and Eterrodossi *et al*., 1992). Mortalities of up to 100% in specific pathogen free (SPF) chickens, 60% in layers and 30% in broilers have been reported (van den Berg *et al*., 1991; Nonaya *et al.*, 1992, van den Berg *et al*., 2000; Okoye, 2005).

Although turkeys, ducks, guinea fowls and ostriches may be infected, clinical disease occurs solely in chickens (Lukert and Saif, 2003; OIE, 2008). Clinical IBD occurs mainly in young chicken (van den Berg *et al.*, 2000) 3 to 6 weeks old when bursa is normally at its maximum development (Lukert and Saif, 2003). A sub-clinical disease is common in day-old to 3 week-old birds. This can cause immunosuppression due to the lymphoid depletion of the bursa of Fabricius caused by IBDV. If this occurs in the first 2 weeks of life, significant

depression of the humoral antibody response may result (OIE, 2008). Chicks at day-old to 2 weeks of age have a high level of maternally derived antibody (MDA) and hence resistance to IBD (Mahgoub, 2012). However, the MDA wanes with age (Abdu, 1997) depending on the level of passive antibodies obtained from the parent stock. The bursa of Fabricius, which is the target organ for the virus reaches its maximum development between 3-6 weeks after hatch rendering the chicken highly susceptible to the IBDV (Muller *et al*., 2003, Mbuko *et al*., 2010). Incubation period in susceptible birds is between 2 to 3 days (van den Berg *et al*., 2000; Lukert and Saif, 2003). The clinical signs are not specific (Saif, 1998) and may include: exhaustion, prostration, watery yellowish to greenish diarrhoea, pasty vent, drooping wings, ruffled feathers and even death (Saif, 1998; van den berg *et al.*, 2000; Lukert and Saif., 2003; Okoye, 2005). A variable degree of petecchial and ecchymotic haemorrhages in the bursa of Fabricius, thigh and pectoral muscles as well as at the mucosal junction between proventriculus and gizzard are typical gross lesions in acute IBD (Hair-Bejo *et al.*, 2004; Roosevien *et al*., 2006; Zeryehun *et al*., 2009; Zeryehun *et al*., 2012). Meanwhile, the severity of the disease depends on the age, breed, the virulence of the strain, the level of passive immunity (van den Berg *et al.*, 2000), amount of virus, route of inoculation, secondary pathogens as well as environmental and managemental factors (Muller *et al*., 2003; Mbuko *et al*., 2010).

Infectious bursal disease is recognized as an important disease of young chicken world-wide (Oladele *et al*., 2005). From the mid 1970s, IBD assumed a worldwide distribution and has been a major threat to the poultry industry (Okoye, 2005). Ojo *et al.* (1973) reported an IBD-like disease in western Nigeria. Onunkwo (1975) described the first confirmed outbreak of IBD in Nigeria (Okoye, 2005). Although the disease was first reported from the South

Western Nigeria, it has now become nation-wide in distribution (Nawathe and Lamorde, 1982).

While vaccination is the most practical procedure for prevention of IBD (Saif, 1998), it has not been very successful in many parts of the world including Nigeria (Okoye, 2005). Outbreaks have been frequent and devastating in many vaccinated and unvaccinated flocks (Okoye, 1984b; Abdu, 1986a,b; Onunkwo and Okoye, 1991; Hatmono,1999 and Hassan *et al.,* 2002) in Nigeria.

The resistance of IBDV to physical and chemical agents (Benton, *et al*., 1967) accounts for persistence of the virus in the environment, particularly on contaminated farms despite disinfection. Eradication in affected countries therefore seems unrealistic (van den Berg *et al.,* 2000)

The distribution of very virulent strain of IBDV (vvIBDV) is now almost world-wide with the exception of North America, Australia and New Zealand (van den Berg *et al.*, 1991). In Nigeria, vvIBDV exists and can cause mortality of 50% - 100% in pullets, cockerels and local chicks (Okoye, 2005). The vvIBDV strain break the protection given by high levels of maternal antibodies (MDA) and also challenge the protection given by current intermediate vaccines (van den Berg *et al.*,2000; Okoye, 2005). The isolation, antigenic analysis and pathogenicity studies of viruses from field cases of IBD are needed continually so that changes in the wild virus population the kind of disease they cause and their pathogenicity can be detected(Lukert and Saif, 2003).

## 1.1 Statement of Research Problems

Very virulent strains of IBDV emerged in Europe in 1987 but the virus was already here (Nigeria); the first outbreak was recorded in 1975 (Okoye, 2005). Of the three pathotypes of serotype 1 IBDV that exist in the field, vvIBDV is capable of infecting chickens in the presence of maternally derived or higher level of vaccinal antibodies causing bursal damage and very high mortalities with severe economic losses (Musa *et al*., 2010; 2012). It has become practically impossible to control IBD in Nigeria due to poor biosecurity practices in most Nigerian households and poultry farms (Musa *et al*., 2010).

In Nigeria, recurrent outbreaks have shown consistently higher mortality rates which could be as a result of a vvIBVD that had become more pathogenic with successive passage in chickens during the natural course of the disease outbreak (Musa *et al*., 2012). All these necesitate continous field monitoring of IBDVs in Nigeria so that changes can be detected.

The lesions of IBD are well documented and very severe in Nigeria (Okoye and Uzoukwu, 1982b; Okoye, 1984c; Okoye and Uzoukwu, 1984; Okoye1985a). The most devastating lesions observed are congestion and paint-brush haemorrhage on the muscles of the breast, thigh and legs. The proventricular mucosa shows haemorrhage which classically appears in the form of a band or red zone at the proventricular- gizzard junction. There is haemorrhagic enteritis. The caecal tonsil is swollen, haemorrhagic and may contain cheesy materials. The bursa of Fabricius may show congested or haemorrhagic mucosa containing creamy fluid. The kidneys are swollen, congested, granular and often haemorrhagic.

So many researchers have tried to identify the actual cause of haemorrhage in IBD. Skeeles *et al.* (1979) associated complement to the haemorrhage and other lesions seen in IBD. Dos-Santos *et al*. (2004) suggested an immune mediated damage to the blood vessels which is

mainly due to the effect of macrophage derived tissue necrotic factor (TNF) and interleukins. Shibatani *et al*. (1997) suggested the possibility of disseminated intravascular coagulation (DIC) to be the cause of haemorrhage in IBD.

**1.2 Justification**

Infectious bursal disease is recognized as an important disease of young chickens worldwide (Oladele *et al.*, 2005) and has been a major threat to the poultry industry (Okoye, 2005). The first step in the control of a disease is the proper understanding of its pathogenesis. If the pathogenesis is well understood, critical control points for intervention will be easily developed. Haemorrhage appears to be one of the most important lesions associated with acute IBD in Nigeria. Blood clotting or haemostasis is a physiological process that stops bleeding. The haemostatic system includes blood platelets, endothelial cells and plasma coagulation factors. For a mechanically strong haemostatic blood clot to form, it is necessary to have both a sufficient number of functional platelets and a complete set of coagulation factors (Kerr, 2002). The pathogenesis of haemorrhage in IBD will therefore not be unconnected with a malfunctioning blood clotting mechanism.

Thrombocytopaenia is a condition in which there is an abnormally small number of platelet in circulating blood. Thrombocytopaenia has been described as the most common acquired haemostatic disorder in veterinary and human medicine (Kerr, 2002). Thrombocytopaenia has been reported in Gumboro disease (Zeryehun, 2012) and may be associated with the haemorrhage seen during the course of acute disease.

The possible involvement of clotting factors and thrombocytes in the haemorrhage observed in IBD caused by a Nigerian field isolate of IBDV therefore needs to be investigated.

## 1.3 Aim of the Study

To evaluate the role of coagulation factors on haemorrhage in cockerels experimentally infected with vvIBDV strain (IBDV37/Kaduna NG/ 2009)

## 1.4 Objectives of the Study

- To determine the incubation period and clinical signs in IBDV37/Kaduna NG/ 2009 infected chickens
- To determine the effect of infection with the virus on some haematological parameters in the chickens.
- To determine the thrombocyte count and activated partial thromboplastin time (coagulation time) in IBDV infected chickens
- To determine the gross and histopathological changes in the IBDV infected chickens.
- To correlate the clinical signs, gross and histopathological lesions observed during the course of the disease with the laboratory findings.

## 1.5 Research Questions

- Does infection with IBDV affect the haematology of chickens?
- Is the haemorrhage seen in IBD associated with abnormalities of thrombocytes and activated partial thromboplastin time?
- Is haemorrhage in IBD a major contributing factor to mortality?

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Infectious Bursal Disease

Infectious bursal disease (IBD) is one of the most important diseases of chickens because of the high mortality and immunosuppression associated with it (Okoye, 2005). It is an acute highly contagious viral infection of young chicken that has lymphoid tissues as its primary target (Lukert and Saif, 2003). Gumboro disease, avian nephrosis- nephritis, avian infectious bursitis are all synonymous to IBD (Nawathe and Lamorde, 1982). The disease has been recognized in Nigeria since 1969 (Ojo *et al.*, 1973) and further incidences were reported by Onunkwo (1975) and Nawathe *et al.* (1978) and many other authors (Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011).

## 2.2 Aetiology

Gumboro disease is caused by infectious bursal disease virus (IBDV) (Hitchner 1970; Saif. 1998; van den Berg 2000; Lukert and Saif, 2003; Okoye, 2005; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012). The virus is a member of the family *Birnaviridae* (Lukert and Saif, 2003, Mac Lachlan and Dubovi, 2011). The family was established to bring together viruses with two segments of double standard RNA (Murphy *et al.*, 1999)

The family *Birnaviridae* comprises three genera *Avibirnavirus*, *Aquabirvirus* and *Entomobirnavirus* (Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011). IBDV is the sole member of the genus *Avibirnavirus*. Members of the genus *Aquabirnavirus* include infectious pancreatic necrosis virus of salmonid fish and related viruses of mollusks and

crustaceans. Members of the genus *Entomobirnavirus* infect insects. The classification of the "*Picobirnaviruses*," that is viruses that resemble Birnaviruses but are smaller (30 - 40nm diameter compared with 60nm for the birnaviruses, and bi or trisegmented genomes) has not been resolved (Mac Lachlan and Dubovi, 2011).

### 2.2.1 Viral properties

The IBDV virus is non - enveloped, 55 – 60 nm in diameter (Isma'il and Saif, 1990; Mbuko *et al*., Mac Lachlan and Dubovi, 2011), hexagonal with a single shell having icosahedral symmetry. The genome is approximately 6 kilobase pairs (kbp) and consist of two molecules of linear double standard RNA, designated A and B. Segment A ranges from > 2.9 to < 3.4 kbp in size and contain two open reading frames (ORF), the largest of which encodes a polyprotein that is processed to form two structural proteins, VP2 and VP3 and a viral protease (designated as VP4) that autocatalytically cleaves the polyprotein (van den Berg, 2000; Mac Lachlan and Dubovi, 2011). VP2 is the major capsid protein (Mac Lachlan and Dubovi, 2011). It is considered to be the major host - protective antigen and contains the major antigenic site responsible for eliciting neutralising antibodies (van den Berg, 2000; Mac Lachlan and Dubovi 2011; Mahgoub, 2012). At least two neutralizing epitopes were located on this polypeptide. VP2 induces virus neutralising antibodies (Abs) that protect susceptible chickens from virulent IBDV. It is responsible for antigenic variation, tissue culture adaptation (Mahgoub, 2012), cellular tropism and binding (Mac Lachlan and Dubovi, 2011). The inner capsid protein, VP3 contains group - specific antigenic determinant and a minor neutralizing site (Mac Lachlan and Dubovi, 2011), some of which cross react in both serotype 1 and 2 (van den Berg, 2000). Segment A also encodes a 17 - KD non - structural protein, VP5, from the small ORF (Mundt *et al*, 1995,

Mahgoub, 2012). VP5 is a class 11 membrane protein with a cytoplasmic N - terminus and an extracellular C - terminal domain. It is highly basic, cysteine - rich and semi - conserved among all serotype 1 IBDV strains and it has been incriminated in the induced bursal pathology. Moreover, it has a role in virus dissemination from infected cells. VP5 accumulates within the cell membrane, resulting in its disruption and decreasing cellular viability. VP2 and VP5 have been shown to induce apoptosis in *in vitro* culture (Mahgoub, 2012). Segment B is 2.8 kb in length and encodes VP1, a 97 KD protein with polymerase activities (Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012). VP1 is the RNA dependent RNA polymerase of the virus (van den Berg, 2000). VP1 exist as a genome linked protein (VPg) that circularises segments A and B by tightly binding to their ends (Mac Lachlan and Dubovi, 2011). It plays a key role in the encapsidation of the viral particles (van den Berg, 2000). VP1 prevent IBDV replication in Vero cells (Mahgoub, 2012). Virions are relatively heat stable and their infectivity is resistant to exposure at pH 3 and to ether and chloroform.

### 2.2.2 Strain classification

The high mutation rate of the RNA polymerase of RNA viruses results in a genetic diversification that could lead to emergence in the field of viruses, with new properties allowing them to persist even in immune populations. In the case of IBDV, these mutations lead to antigenic variation and modification in virulence *in vivo* and attenuation *in vitro* (van den Berg, 2000).

In China, recent molecular indications for the emergence of variant vvIBDV have been reported (Cao *et al.*, 1998), but their biological and epidemiological relevance still needs to be established (van den Berg, 2000). Also in France, during their monitoring of the field,

Eterradossi *et al*.(1998) showed atypical antigenicity in some vvIBDVs due to critical amino acid changes in the second hydrophilic peak, but these strains were not shown to replace the more typical prevalent vvIBDVs (van den Berg, 2000).

A variety of phenotypic and molecular genetic procedures have been developed and used to classify isolates of IBDV. Serotyping of IBDV isolates using polyclonal antibodies in cross neutralization (VN) test has been very successful because it correlated very well with protection studies. The newer molecular genetic procedures have proved extremely useful for diagnosis and epidemiological studies but the use of these procedures for classification of isolates has caused some confusion mostly because of lack of documented criteria for interpretation of the results and the lack of correlation between serogrouping and molecular grouping (Lukert and Saif, 2003). The following are some of the procedures used to classify IBDV isolates.

*2.2.2a Antigenic types*

The first reported antigenic variation among IBDV isolates of European origin was by Mc Ferran in 1980 in Northern Ireland (Lukert and Saif, 2003). Two serotypes of IBDV were described and designated serotype 1 and 2 (Jackwood *et al.*, 1985; Saif 1998; van den Berg *et al.*, 2000; van den berg, 2000; Lukert and Saif, 2003 OIE, 2008; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012). Serotype 1 IBDV is pathogenic for poultry and serotype 2 is apathogenic and has been isolated from chickens and turkeys. The two serotypes are differentiated *in vitro* by the absence of cross - neutralisation and *in vivo* by the absence of cross - protection (van den Berg *et al.*, 2000; OIE, 2008). Thirty eight percent antigenic relatedness has been shown between several strains of serotype 1 and the designated

prototype of that serotype. Antigenic relatedness of 33% between 2 strains of serotype 2 was also reported indicating an antigenic diversity between both serotype 1 and 2 viruses (Lukert and Saif, 2003). Immunisation against serotype 2 viruses does not protect against serotype 1. The reverse situation cannot be tested because no virulent serotype 2 viruses are available for challenges (Jackwood *et al.*, 1985). Variant viruses of serotype 1 were described (Saif, 1984; Rosenberger and Cloud, 1986). Vaccine strains available at the time they were isolated did not elicit full protection against the variants, which are antigenically different from the standard serotype 1 isolates (Lukert and Saif, 2003).

In summary, there are currently 3 well documented antigenic types. These are classic (often called standard), variant serotype 1 and serotype 2 viruses. Subtypes of the three antigenic types have also been described (Lukert and Saif, 2003).

*2.2.2b Immunogenicity or protective types*

This classification is based on cross protection studies in live birds. The results obtained here are similar to those obtained by cross virus neuralisation (VN) studies used for antigenic classification (Isma'il and Saif 1991). There are two serotype 1 protective types, classic/standard and variant groups.

*2.2.2c Molecular genetic types*

These techniques have become popular because of their sensitivity, the time they save, the ability to use them on crude samples, or inactivated samples and they do not require replication of the virus. The most commonly used procedure is the reverse transcriptase/polymerase chain reaction restriction enzyme fragment length polymorphism RT/PCR-RFLP. Currently described molecular groups do not always correspond to

antigenic or protective groups and one has to be careful in interpreting the significance of this classification (Lukert and Saif 2003)

*2.2.2d Pathotypes*

Infectious bursal disease virus strains may be classified according to virulence (mortality and bursal lesion) (van den Berg *et al.*, 2000; van den Berg, 2000). Thus, strains of IBDV may be considered apathogenic (serotypes 2), mild, intermediate, intermediate plus, classical, variant and very or hypervirulent (vvIBDV) (van den Berg 2000). But there has been a great deal of confusion in these definitions. In particular, the term "very virulent" has been used to described both European hypervirulent strains and variant American strains that cause less than 5% mortality but are able to multiply to a higher degree in the bursal of Fabricius of vaccinated animals (van den Berg *et al.*, 2000; van den Berg, 2000). In the absence of the identification of specific virulence determinants, the only valuable criteria for the classification of IBDV strains as "pathotypes" should refer to their virulence (mortality or lesions) in 3 to 6-week-old SPF birds and not to any antigenic specificity (van den Berg, 2000).

## 2.3 EPIDEMILOGY

Infectious bursal disease is currently an worldwide problem (van den Berg 2000). Infection with serotype I IBDV is of worldwide distribution, occurring in all major poultry producing areas (Lukert and Saif, 2003). Chickens and turkeys are the natural hosts of IBDV (Lukert and Saif, 2003). Serologic evidence of natural infection with the virus has been reported in ducks, guinea fowl and ostrich but reports from both fields and experimental studies have shown conclusively that IBD is a disease of chicken only (Okoye, 2005) although

microscopic lesions were reported in association with infection in some of the other avian species (Saif, 1998). The disease in chickens is caused by serotypes 1 only, but they are susceptible to infection with serotype 2 viruses (Saif, 1998; OIE, 2008). Turkeys are also susceptible to both serotypes of the viruses but disease is not reported (Saif, 1998, van den Berg *et al.,* 2000). Clinical disease occurs mainly in young chicken (van den Berg *et al.,* 2000; Lukert and Saif, 2003) when the bursa is normally at its maximum development (Okoye and Uzoukwu, 1982 Okoye 1984 a van den Berg, 2000). Susceptible chickens younger than 3 weeks do exhibit subclinical disease with severe immunosuppression (van den Berg *et al.*, 2000; Lukert and Saif, 2003; Okoye, 2005) which may cause secondary problems due to the effect of the virus on the bursa of Fabricius (OIE, 2008). Few outbreaks have been reported in chickens of up to 20 weeks old (Okoye and Uzoukwu 1981; Durojaiye *et al.,* 1984). Pullets, cockerels and local chickens suffer higher mortality than broilers because light birds have higher bursal index than the heavy ones (van den Berg *et al.,* 2000; Okoye and Aba - Adulugba, 1998 and Okoye *et al.,* 1999).

Infectious bursal disease virus is highly contagious and its transmission occurs directly through contact, oral uptake and respiratory route. The virus is excreted in faeces of infected birds for 2 -14 days (Mac Lachlan and Dubovi, 2011) and indirectly through contact with contaminated feed and water (Okoye, 2005). There is no evidence of egg transmission of the virus (Saif 1998) and true carrier state does not exist in recovered birds (Lukert and Saif, 2003). Lesser mealworm (*Alphitobius diaperinus*) is recognized as a carrier of the virus (Saif, 1998; Lukert and Saif, 2003). The virus has been isolated from mosquitoes (Howie and Thorsen, 1981). Evidence of infection has been reported in wild rats (Okoye and Uche, 1985) and dogs (Pages - Mante *et al*., 2004; Zahoor *et al*., 2011),

cattle egrets, pigeons (Fagbohun *et al.,* 2000) and guinea fowls (Ambali *et al.,* 2004). There is no available evidence to further confirm that these species are vectors or reservoirs of the virus. Neutralising or precipitating antibodies have also been detected, *inter alia,* (*Among other things* -Latin) in various species of wild ducks, geese, tern, puffin, crow and penguin, which may mean that wild birds act as reservoirs or vectors (van den Berg, 2000).

## 2.4 PATHOGENESIS

Pathogenesis is the mechanism by which the virus to cause injury to the host with mortality, disease or immunosuppression as a consequence. After oral infection or inhalation, the virus replicates primarily in the lymphocytes and macrophages of the gut associated tissues (van den Berg, 2000) by 4 hours (Lukert and Saif, 2003) to 5 hours from where it enters the portal circulation leading to primary viraemia (Mac Lachlan and Dubovi, 2011). Within 11 hours pi, the virus is present in the lymphocytes of the cloacal bursal with production and release of large amount of virions resulting in a secondary viraemia and localization in other tissues including other lymphoid tissues (Mac Lachlan and Dubovi, 2011) leading to disease and death (Muller *et al.,* 1979). Similar kinetics is observed for vvIBDV but the replication at each step is amplified (van den Berg, 2000). The principal target organ of IBDV is the bursa of Fabricius (Saif 1998, van den Berg et *al.,* 2000). Maximum viral replication is supported by actively dividing, surface immunoglobulin M-bearing B lymphocytes or non-immunoglobulin bearing B lymphoblasts which are consequenty lysed by the infection. Stem cells however and peripheral B - cells do not support viral replication (van den Berg, 2000; Mac Lachlan and Dubovi, 2011). The stage of differentiation of B - lymphocytes in the bursa is therefore crucial in the pathogenesis of the disease. This also accounts for the paradoxical immune response to IBDV infection in which

immunosuppression co-exists with high anti-IBDV antibody titres. The mature and competent lymphocytes will expand as a result of stimulation by the virus whereas the immature lymphocytes will be destroyed (van den Berg *et al.,* 2000). Cells of the monocyte-macrophage lineage can also be infected in a persistent and productive manner, and play a crucial role in dissemination of the virus and in the onset of the disease (van den Berg, 2000). The specific viral tropism for lymphocytes at a certain stage of differentiation accounts for the age - dependent clinical disease in chickens (Mac Lachlan and Dubovi, 2011).

The exact cause of clinical disease and death is still unclear but does not seem to be related only to the severity of the lesion and the bursal damage (van den berg, 2000). Indeed after infection, some birds with few bursal lesions can be found dead, while others can survive despite extensive bursal damage. Prostration (with ruffled feathers, diarrhoea and inappetance) preceding death is very similar to what is observed in acute coccidiosis and is reminiscent of a septic shock syndrome. Macrophage could play a specific role in this pathology by an exacerbated release of cytokines such as tumor necrosis factor or interleukin 6 (Kim *et al.,* 1998; van den Berg, 2000). However, an intermediate role of T - helper cells in this pathophysiological mechanism should be investigated (van den Berg, 2000). Meanwhile several researches have demonstrated varying degrees of adverse effects of IBDV on cell mediated immunity (Panigrahy *et al.,* 1977; Sivanandan and Maheswaran, 1981; Craft *et al.,* 1990; Sharma *et al.,* 2001; Lukert and Saif, 2003). Another component of the immune system is the harderian glands, which is associated with local immune system of the respiratory tract. Research has shown that IBDV infection of chicken produces a drastic reduction in plasma cell content of the harderian gland (Dohm *et al.,* 1981) and

reduced antibody titre to *Brucella abortus* and sheep red blood cells (Dohms and Jaeger, 1988).

The depletion of lymphoid cells due to necrosis and apoptosis in bursa of Fabricius following IBDV infection creates an immunosuppression that is severe in younger birds (van den Berg *et al*., 2000) (van den Berg, 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012). Studies have confirmed that the immunosuppression induced by IBDV is caused, at least in part, by apoptosis (Vasconcelos and Lam, 1994; Ojeda *et al.,* 1997; Tanimura and Sharma, 1998; Nieper *et al*., 1999). A direct effect of viral proteins like VP2 and VP5 has been implicated in the induction of the mechanism (Fernandez - Arians *et al*., 1997; Yao *et al.*, 1998; Rodriguez - Le Compte *et al*., 2005; Mahgoub, 2012) but further investigations are needed to establish their exact role in the pathogenesis and immunosuppression noted by comparing them in strains with different virulence (van den Berg, 2000). A high level of apoptosis is evident in chicken peripheral blood lymphocytes infected with serotype 1 IBDV strain L (Vasconcelos and Lam, 1994). On the other hand, apoptotic cells have also been observed in virus negative bursal cells (Taminura and Sharma, 1998; Nieper *et al*., 1999) reinforcing the possible role of immunogical mediators in the process (van den Berg, 2000). Recovery from disease or subclinical infection will be followed by immunosuppression with more serious consequences if infection occurs early in life. What has been called "viral bursectomy" results in diminished antibody response and increase susceptibility to a wide range of opportunistic infectious agents, including *salmonella spp* and *E. coli.* In addition, the immunosuppression leads to diminished antibody production after vaccination, so that

outbreaks of diseases may occur even after vaccination. Very virulent virus strains also deplete cells in the thymus, spleen, and bone marrow (Mac Lachlan and Dubovi, 2011).

Haemorrhages in the thigh and breast muscles and the mucosa of proventriculus, are characteristic lesions seen in Gumboro disease; the pathogenesis of this bleeding has not been clearly elucidated. Skeeles *et al*. (1979) attempted to demonstrate that the haemorrhagic lesions were as a result of formation of immune complexes. They observed that histologic lesions in the cloacal bursa resemble an Arthus reaction (necrosis, haemorrhage and large numbers of polymorphonuclear leukocytes). This reaction is a type of localised immunologic injury caused by antigen-antibody- complement complexes that induce chemotactic factors, which cause haemorrhage and leukocyte infiltration. They found that 2-week-old and 8-week-old chicks produced rapid and high levels of antibody by 72 hours pi, but that 2-week-old chickens had very little complement compared with 8-week-old chickens. They postulated that the reason 2-week-old chicken did not develop Arthus type lesion was lack of sufficient complement. They also showed that complement was depleted in 8-week-old chicks at 3.5 and 7 days pi. compared with uninfected controls. A later study by Skeeles *et al*. (1980) with another IBDV isolate failed to substantiate the depletion of complement at 3 days pi. (Lukert and Saif, 2003). Previous researches (Kosters *et al.* 1972; Skeeles *et al.* 1979; 1980; Zeryehun *et al*., 2012) found increased clotting times in IBDV infected chickens and suggested that such coagulopathies would contribute to the haemorrhagic lesions observed with this diseases. Skeeles *et al*. (1980) found that 17-days-old chickens did not exhibit clothing defect but at 42 days, they had greatly increased clothing times and become clinically ill (Lukert and Saif, 2003). Panigrahy *et al.* (1986) and Zeryehun *et al.* (2012) observed thrombocytopaenia in SPF chicken with vvIBDV but

Lima *et al.* (2005) could not establish thrombocytopaenia. The pathogenesis of haemorrhage in chickens infected with IBDV is almost certainly viral strain and age dependent but is obviously not clearly understood.

## 2.5 CLINICAL SIGNS

The incubation period is short; normally two to three days (Saif 1998; van den Berg, 2000; van den Berg *et al*., 2000; Lukert and Saif 2003; Mac Lachlan and Dubovi, 2011 and Mahgoub, 2012). In fully susceptible flocks, the disease appears suddenly, with a high morbidity rate, usually approaching 100% (Lukert and Saif, 2003), whilst mortality is variable (van den Berg *et al.,* 2000). Mortality may be nil but can be as high as 20 to 30 %. In Nigeria and other countries where vvIBDV strain exists, mortalities could approach 50 – 100 % in pullets, cockerels and local chickens (Okoye, 2005). Outbreaks caused by the classical pathogenic strains of the virus usually show much lower mortalities. One of the earliest signs of infection in a flock is the tendency for some birds to pick at their own vents (Lukert and Saif, 2003). Other signs include distress, depression, ruffled feathers, anorexia, diarrhea, trembling and dehydration (Mac Lachlan and Dubovi, 2011; Mahgoub, 2012). Picking of vent and trembling which was earlier described by some workers have not been reported in Nigeria (Okoye and Uzoukwu, 1982; 1983). Acute IBD has a spiking mortality curve. The disease starts with high mortality of up to 70 % which peak by the fifth day and drops by the seventh day (Okoye, 2005). Immunosuppression may persist, increasing susceptibility to other viral agents or bacteria (Mac Lachlan and Dubovi, 2011). Initial outbreaks on farms are usually the most acute. Recurrent outbreaks in succeeding broods are less severe and frequently go undetected. Many infections are silent, owing to age of

birds, infection with avirulent field strains, or infection in the presence of MDA (Lukert and Saif, 2003).

The clinical signs of IBD vary considerably from one farm, region, country or even continent to another (van den Berg *et al*., 2000). Schematically, the global situation can be divided into three principal clinical forms, as follows:

a) The classical form, as described from the early 1960s, is caused by the classical virulent strains of IBDV. Specific mortality is relatively low, and the disease is often subclinical, occurring after a decline in the level of passive antibodies (Faragher, 1972);

b) The immunosuppressive form, principally described in the USA, is caused by low-pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variant E or GLS strains, which partially resist neutralisation by antibodies against the so-called 'classical' viruses (Jackwood and Saif, 1987; Snyder, 1990);

c) The acute form, first described in Europe, and then in Asia, is caused by 'hypervirulent' strains of IBDV, and is characterised by an acute progressive clinical disease, leading to high mortality rates on affected farms ( Chettle *et al.,* 1989; Stuart, 1989; van den Berg *et al*., 1991 ).

## 2.6 PATHOLOGY

### 2.6.1 Gross lesion

Birds that succumb to the infection are dehydrated with darkened discolouration of pectoral muscles. Renal changes resulting from severe dehydration may be prominent in birds that die or are in advanced stages of the disease (Lukert and Saif 2003). The kidneys are swollen (van den Berg, 2000), whitish containing deposits of urate crystals and cell debris

(van den Berg *et al*., 2000).The bursa of Fabricius is the principal diagnostic organ (Cheville 1967; Okoye and Uzoukwu 1982b; 1984; Saif, 1998; van den Berg *et al.*, 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi 2011; Mahgoub, 2012).

Necropsy performed on birds that died during the acute phase revealed hypertrophic, hyperaemic and oedematous bursae. All these result in the enlargement of the bursa (Mac Lachlan and Dubovi, 2011). By day 4, the bursa is usually about twice its normal size and weight and then begins to recede. By day 5, the bursa returns to its normal weight, but continues to atrophy, and from day 8 forward, it is approximately one - third its original weight (Cheville, 1967; Lukert and Saif, 2003). A yellowish gelatinous transudate covers the serosal surface of the bursa of Fabricius and there is prominent appearance of longitudinal striations representing the bursal folds. The normal white colour turns to cream colour. The infected bursa often shows necrotic foci with petecchial and ecchymotic haemorrhages on the mucosal surface. Occasionally, extensive haemorrhages throughout the entire bursa have been observed and in these cases birds may void blood in their droppings (Lukert and Saif, 2003). Certain variants from the USA are reported to cause rapid atrophy of the bursa without a previous inflammatory phase (van den Berg *et al.*, 2000). In cases where infection is caused by vvIBDV, gross lesions are also observed in other lymphoid organs (thymus, spleen, caecal tonsil, Harderian glands, Peyer's patches and bone marrow). In an experiment in which SPF chickens were infected with vvIBDV, Inoue *et al.* (1994) observed slight haemorrhage and severe atrophy in the thymus. Other researchers have documented similar thymic lesions (Lukert and Saif, 2003; Okoye 2005).

The spleen may be slightly enlarged and very often has small grey foci uniformly dispersed on the surface (Lukert and Saif, 2003) but later atrophic (Okoye, 2005). Generally infection

with vvIBDV cause more severe pathology and affect wide spread lymphoid organs when compared with the other strains.

Frequently hemorrhage is present in the thigh and pectoral muscles and at the junction between proventriculus and gizzard.

### 2.6.2 Microscopic lesions

Histopathologic changes are more severe in the cloacal bursa (Lukert and Saif, 2003). As early as 1 day pi, there was degeneration and necrosis of lymphocytes in the medullary area of bursal follicles. Lymphocytes were soon replaced by heterophils, necrotic debris and hyperplastic reticuloendothelial cells. Haemorrhages often appear but are not a consistent lesion. All lymphoid follicles were affected by 3 or 4 days post-infection and this result in depletion of lymphoid cells. Depletion of lymphoid cells is due to necrosis and apoptosis (van den Berg 2000; Mac Lachlan and Dubovi, 2011). Severe oedema, hyperaemia and marked accumulation of heterophils has been observed and this result in increase in bursal weight at the early part of the infection (Lukert and Saif, 2003; Mahgoub, 2012). As the disease evolves, the surface epithelium disappears and cystic cavities develop in the medullary areas of follicles (van den Berg *et al.,* 2000). During this stage of bursal atrophy, necrosis and phagocytosis of plasma cells occurred and there is fibroplasia in interfollicular connective tissue (Lukert and Saif, 2003). The permanent loss of lymphocytes in the bursa leads to atrophy and premature involution of the organ. This is the genesis of the immunosuppression which may be transient or permanent in birds that recovered from IBD (Okoye, 1983; Okoye and Shoyinka, 1983; Okoye, 2005). Regeneration of lymphocytes may occur later (Saif, 1998) in mildly pathogenic IBDV.

The thymus exhibited some cellular reaction. Inoue *et al.* (1994) found that evidence of lymphocyte necrosis in the inner cortex appear at 2 days pi in 4-day-old and 2-week-old chickens infected with IBDV. At 3 and 4 days pi, many altered lymphocytes were detected throughout the entire cortex and lymphocyte depletion was prominent in areas of the cortex. Lymphocyte depletion had markedly progressed at 5 days pi in the whole cortex. At 7 days pi, cortical atrophy was greatest but visible lymphocytes remained focally and diffusely in the outer cortex. At 14 days pi, the cortex showed apparent repopulation and recovery. In the medulla, macrophage and plasma cells infiltration and microscopic haemorrhage were prominent from 5 to 7 days pi.

In the spleen, hyperplasia of the reticulo- endothelial cells around the adenoid sheath arteries has been observed in the early stages of infection (Lukert and Saif 2003). At day 3 pi, focal areas of necrosis of lymphoid nodules and periarteriolar lymphoid sheath were observed (Ley *et al*., 1983). Okoye (1984) reported more severe tissue destruction in areas near the splenic capsules. Complete lymphocytes regeneration was observed in spleen and thymus unlike in the cloacal bursal (Ley *et al.*, 1983; Okoye, 1984; Lukert and Saif, 2003).

The Harderian gland has been found to be affected severely following IBDV infection. Many necrotic foci with pyknotic nuclei and leukocyte infiltration in the lymphoid areas have been observed (Ley *et al.*, 1983). Reduction in plasma cell infiltration has also been observed (Lukert and Saif, 2003).

In the caecal tonsils at day 3 pi, birds infected with IBDV have exhibited lymphoid cell loss from the lymphoid nodules with macrophage infiltration and an occasional crypt containing

necrotic materials and heterophils (Ley *et al.,* 1983) but the organ was normal by day 7 pi (Okoye, 1984).

Histologic lesions of the kidney are non - specific and probably occur because of severe dehydration of affected chickens (Lukert and Saif, 2003). Helmboldt and Garner (1964) observed large cast of homogeneous materials infiltrated with heterophils. Ley *et al.* (1983) presumed this homogeneous materialkyv to be urate.

Ley *et al.* (1983) observed multifocal periportal accumulation of lymphoid cells, mostly perivascular in the liver 3 day pi. Sinusoids appeared slightly hyperaemic with apparently increased numbers of macrophages and heterophils. There was evidence of mild fatty change of hepatocytes. They also observed multifocal hepatic necrosis and ectopic lymphoid foci. By day 14 pi, no lesion was observed in the liver of IBDV infected chickens.

### 2.6.3 Ultrastructural lesions

In an experiment on SPF chicken conducted by Luengo *et al*. (2001), the first ultra-structural lesion observed was at 3 days pi. Degeneration and necrosis of lymphocytes in the follicles of the medullary area of the bursa of Fabricius together with condensation and margination of nuclear chromatin was observed. Bursal cells were widely separated due to accumulation of intercellular oedema fluid and infiltration of macrophages and heterophils. Viral particles were observed in the cytoplasm of lymphocytes and macrophages. Lipid droplets in the cytoplasm and phagolysosome of macrophages, lymphoid cells and reticular cells were frequently observed in addition to the lesions described above at 7 days pi. Virions of 50 to 55 nm in diameter with an electron - dense core where seen in the

cytoplasm of lymphocytes and macrophages. At 24 days after challenges, they observed neither evidences of viral particles nor lymphocytes regeneration by transmission electron microscope.

Naqi and Millar (1979) followed the sequential changes in the surface epithelium of the cloacal bursal of IBDV infected chicks by scanning election microscopy. They observed a reduction in number and size of microvilli on epithelial cells at 48 hours pi. There was gradual loss of the button follicles normally seen at the surface and by 72 hours most had involuted. By 96 hours, there were numerous erosions of the epithelial surface. The surface was intact by day 9 pi, but follicles were involuted, leaving deep pits (Lukert and Saif, 2003).

Remarkable alterations were also detected in the thymus of chickens inoculated with vvIBDV (inoue *et al.,* 1994). Two and three days after inoculation with this virus, some lymphocytes were observed to be scattered throughout the cortex and had crescent-shaped aggregates abutting the nuclear membrane. Morphologically, alterations of these cortical lymphocytes corresponded with the characteristics of apoptosis. At 4 and 5 days pi, these alterations were observed more frequently and apoptotic bodies were also observed in the medulla. Clusters of virus particles showing crystalline arrangement were detected in altered cells which were characteristics of infiltrated B-lymphocytes showing development of rough surfaced endoplasmic reticulum. Also, single virus particles were found in the cytoplasmic vacuoles of epithelial reticular cells (inoue *et al.,* 1994).

Ley *et al.* (1983) followed the changes in the kidney of SPF infected with IBDV using transmission electron microscope and observed that at 7 days pi, thickened endothelial cell

layers and thickened glomerular basement membrane (GBM) were present. Podocyte foot processes in these area contained several membrane - bound structures. Within these structures were multiple vesicles with bilaminar limiting membranes. In a sample taken at day 3 pi, there were an electron dense accumulation (subendothelial deposit) between the thickened endothelial layer and the GBM. A similar dense deposit was seen in a sample from 7 pi. Adjacent to the deposit was a cell highly suggestive of heterophils. The endothelial cell layer was not intact in the area between the heterophils and the GBM.

### 2.7 Diagnosis

Acute clinical outbreaks of IBD in fully susceptible flocks are easily recognized and presumptive diagnosis can be readily made (Lukert and Saif, 2003). Flock history, clinical signs and lesions (Butcher and Miles, 2012) are useful in arriving at preliminary diagnosis (Saif 1998).

#### 2.7.1 Clinical diagnosis

Clinical diagnosis of the acute form of IBD is based on disease evolution (rapid onset, high morbidity, spiking mortality curve and rapid recovery within 5-7 days from clinical signs (Lukert and Saif, 2003). it relies on the observation of clinical signs and post mortem lesions; in particular the bursa of Fabricius (van den Berg *et al.,* 2000). Infection of very young chicks or chicks with maternal antibody, are usually subclinical and are diagnosed retrospectively at necropsy with observation of macroscopic and histologic bursal atrophy. Infection of chickens of any age with variant strains of IBDV will be detected only by histopathology of the cloacal bursa or by virus isolation (Lukert and Saif, 2003).

### 2.7.2 Histological diagnosis

Histological diagnosis is based on the detection of changes occurring in the bursa of Fabricius as earlier described. The ability to cause histological lesions in non - bursal lymphoid organs such as the thymus, the spleen or bone marrow, has been reported as a characteristic of hypervirulent IBDV strains. The histological approach has the advantage of allowing for diagnosis of both acute and chronic or subclinical forms of the disease (van den Berg *et al.,* 2000).

### 2.7.3 Serological diagnosis

Immunological methods are the best in the confirmation of clinical and subclinical disease because of the difficulty in isolating the virus (OIE, 2008). Current serological test cannot distinguish between the antibodies induced by pathogenic IBDV and those induced by attenuated vaccine viruses (van den Berg *et al.,* 2008). Also with the exception of the virus neutralization test (VN), all the commonly used test procedures for viral antigens and antibodies detect only group specific antigens without differentiating serotypes (Saif, 1998; Lukert and Saif, 2003). Despite these limitations of serology in the diagnosis of IBD, confirmation of clinical disease or detection of subclinical disease is best done by these methods as IBDV is difficult to isolate (OIE, 2008) .The quantification of IBDV - induced antibodies is important in order to determine the appropriate date for vaccination or in laying hens to verify the success of vaccination. Serology is likewise essential to confirm the disease free status of SPF flocks (van den Berg *et al.*, 2000). Ager gel immunodiffusion test (AGID), Enzyme linked immunosorbent assay (ELISA) and VN test are the commonly used serodiagnostic techniques for the detection of either viral antigens or antibodies.

The AGID test is the most useful of the serological test for the detection of specific antibodies in serum, or for detecting viral antigen or antibodies in bursal tissues (OIE, 2008). It is the simplest, but least sensitive techniques (van den Berg *et al.,* 2000). Results are obtained after an incubation period of 48 hours. Variability in results may be due to the investigator, as well as the nature of the viral strain used as an antigen (van den Berg *et al.*, 2000). AGID can be used to detect antigen in the bursa of Fabricius. This can be very useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely replaced by the ELISA (OIE, 2008).

The ELISA procedure is presently the most commonly used serological test for the evaluation of IBDV antibodies in poultry flocks (Lukert and Saif, 2003). The ELISA procedure has the advantage of being a rapid test with the results easily entered into computer software programs. With these programs, one can establish an antibody profile on breeder flocks that will indicate the flocks' immunity level and provide information for developing proper immunisation programs for both breeder flocks and their progeny. Different protocols have been described for the detection of serotypes of IBDV using an antigen-capture enzyme linked immunosorbent assay (AC-ELISA) (OIE, 2008).

Prior to the use of the ELISA, the most common procedure for antibody detection was the constant virus-diluting serum virus neutralisation test (VN) performed in a microtiter system (Skeeles *et al.,* 1979). The VN test is the only serological test that will detect the different serotypes of IBDV and it is still the method of choice to discern antigenic variations between isolates of this virus (Lukert and Saif, 2003). The test is more laborious

and expensive than the AGID (OIE, 2008) requiring specialized equipments and five days incubation (van den Berg *et al.*, 2000).

The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. The VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA and AGID is more variable depending on the source of the ELISA reagents. When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. A formula has been devised that allows ELISA titres to be used to calculate the optimal age for vaccination, which will vary depending on the vaccine used. Nonspecific positive reactions may occur with most ELISA because they are usually designed for monitoring vaccine responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks, the possibility that a serotype 1 ELISA antigen also detects antibodies induced by a natural infection with serotype 2 IBDV cannot be ruled out, however this possible cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA (OIE, 2008).

### 2.7.4 Virological diagnosis

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus is difficult to isolate (Lukert and Saif, 1997) The cloacal bursa and spleen are the tissues of choice for the isolation of IBDV but the bursa is the most commonly used. Others contain the virus at a lower concentrations and probably only because of the viraemia (Lukert and Saif, 2003). Isolation and identification of IBDV involve the aseptic removal of bursae of Fabricius, maceration in antibiotic treated broth and centrifugation to remove the large particles. The supernatant fluid can then be used for detection of viral antigen (van den Berg *et al.,* 2000; Lukert and Saif, 2003; OIE, 2008). This suspension can be used to carry out the AGID, a technique which is based on comparison between the suspension to be tested and a specific antiserum or a monoclonal antibody (van den Berg *et al.,* 2000). The appearance of precipitation lines signifies the presence of viral antigen (Hirai *et al.*, 1971). Agglutination tests using latex beads coated with an anti IBDV monoclonal antibody or sheep red blood cells coupled with anti - IBDV immunoglobulin are also possible (van den Berg *et al.,* 2000).

Antigen capture as revealed by ELISA (AC - ELISA) consists of capturing the viral antigens present in the suspensions using anti-IBDV antibodies and a polystyrene support (van den Berg *et al.,* 2000). Different protocols have been described for the detection of serotype 1 IBDV using this technique (Kwang *et al.,* 1987). It includes detecting viral antigens through a sandwich ELISA and an anti-IBDV antibody conjugate with peroxidase or with an anti IBDV antibody followed by an adapted anti-species conjugate. It has been found that antigen- capture with polyclonal antibody was more sensitive than with

monoclonal antibody (Lukert and Saif, 2003; OIE, 2008). The use of monoclonal antibodies in the detection stages allows for more precise antigenic characterization of the captured viruses, (van den Berg, 2000)

The viral antigens may also be detected by direct and indirect immunoflourescence (van den Berg, *et al.*, 2000; Lukert and Saif, 2003; OIE, 2008) or by immunoperoxidase staining of bursal follicles (Cho *et al.*, 1987; Okoye, 2005). No viral antigen is detectable from the tenth day (van den Berg *et al.,* 2000). The use of monoclonal antibodies for detection of the virus enhances the specificity of the test (Cho *et al.*, 1987).

The filtrated homogenate of bursa of Fabricius described is used to inoculate embryonating eggs or to prepare cell cultures. For viral isolation in embryonating eggs, eggs originating from hens, free of anti-IBDV antibodies are required. Chorioallantoic membrane (CAM) of an 11-day old embryo was found to be most sensitive route for isolation of the virus. The yolk sack route gave an intermediate result and the intra-allantoic route is the least sensitive (Hitchner, 1970; Lukert and Saif, 2003; van den Berg *et al*., 2000; OIE, 2008). The specificity of the lesion observed must be demonstrated using mono-specific anti-IBDV serum in an embryo revealed virus neutralisation assay. The embryonating egg may be the most sensitive substrate for the isolation of IBDV (Lukert and Saif, 2003). Mc Ferran *et al.*, (1980) reported that 3 of 7 chicken isolates of IBDV fail to grow in chicken embryos fibroblast (CEF) cells; however, they could be propagated in embryonating eggs. Lesions observed with serotype 1 IBDV include dwarfing of embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhage. The liver is usually mottled and swollen. The spleen is enlarged and the kidneys are mottled and swollen. The heart has a pale "parboiled" appearance (OIE, 2008; Lukert and Saif, 2003). Isolation of IBDV in

chicken has been done in the past but is no longer recommended due to animal welfare concerns (OIE, 2008).

The IBDV has been shown to replicate in B lymphocytes. Either primary cells derived from the cloacal bursa or continuous cell line of B-cell origin would therefore be the cells of choice for the isolation of the virus (Lukert and Saif, 2003). Other cell culture of chicken embryo origin have also been shown to support the propagation of IBDV. They include chicken embryo kidney cells (Lukert and Davis, 1974). In addition to cells of chicken origin the virus has been grown in turkey and duck embryo cells (Mc Nulty *et al.*, 1979), mammalian cell lines derived from rabbit kidney (RK 13) (Rinaldi *et al.*, 1972), monkey kidney (vero) (Hirai and Shimakura, 1974) and Baby grivet monkey kidney cells (BGM-70) (Jackwood *et al.*, 1987). Of the mammalian cell line compared for their ability to support several strains of IBDV serotype 1 and 2 by Jackwood *et al.*, (1987), cytopathic effects were most pronounced in the BGM-70 cultures. Some strains of the virus are very fastidious, cell cultures containing 50% bursal lymphocytes and 50% CEF have been used to successfully isolated and serotype IBD viruses (Lukert, 1986). The fibroblast serve as a matrix for the lymphocytes and the infected lymphocytes are detected by immunoflourescence. If the infection of cell cultures occurs, and CPE observed, the virus should be tested against IBDV antiserum in a tissue culture virus neutralization test. One study (Hassan *et al.*, 1996) compared antigen capture enzyme immunoassay with cell cultures and determined that cell culture was more sensitive than AC-ELISA.

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (Jackwood, 1990; van den Berg, 2000; Lukert and Saif, 2003; OIE 2008; Mac Lachlan and Dubovi, 2011). The most frequently used

molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reactions (RT-PCR) (Lukert and Saif, 2003; OIE 2008; Mac Lachlan and Dubovi, 2011; Mahgoub, 2012) followed by restriction enzyme digestion or restriction fragment length polymorphism (RFLP) of the amplified fragment (van den Berg, 2000). DNA probes labeled with $^{32}P$, biotin or digoxigenin have been used on prints of infected tissues to detect multiple virus strains of serotype 1 and 2 (van den Berg *et al.*, 2000). Molecular techniques can detect the genome of IBDV which is unable to grow in cell culture because it is not necessary to grow the virus before amplification. In a study using several procedures for detection of the virus in bursa of experimentally infected chickens, the RT-PCR was the most sensitive (Abdel- Alim and Saif, 2001; Lukert and Saif, 2003).

**2.8 Differential Diagnosis**

Gumboro disease has to be differentiated from other diseases that share some features with it. Diarrhoea is one of the clinical signs of IBD. Diseases like velogenic visceral Newcastle disease, salmonellosis, colibacillosis, fowl cholera, spirochaetosis and coccidiosis (Okoye, 2005) also produce diarrhoea clinically and are differentiated from IBD at necropsy by the presence of bursal lesions.

The sudden onset, morbidity, ruffled feathers and droopy appearance of the birds in initial disease outbreaks are suggestive of acute outbreaks of coccidiosis. In some cases there is blood in the dropping that would lead one to suspect coccidiosis. The muscular haemorrhages and enlarged oedematous or haemorrhagic cloacal bursae would however, suggest IBD (Lukert and Saif, 2003).

Certain nephrotoxic strains of infectious bronchitis virus, cause nephrosis (Winter- field and Hitchner, 1962; van den Berg *et al.*, 2000; Lukert and Saif, 2003). These cases can be differentiated from infections bursal disease by the fact that there are no changes in the cloacal bursa, and deaths usually are preceded by respiratory signs (Lukert and Saif, 2003)

The muscular haemorrhages and mucosal haemorrhages seen at the junction of proventriculus and gizzard are similar to those reported for haemorrhagic syndrome and could be differentiated on the basis of bursal changes that accompany IBDV infections, (Lukert and Saif, 2003).

Marek's disease has been found to be associated with bursal atrophy (Jakowski *et al.*, 1969). The histologic lesions are distinct from what is found in IBD. The visceral form of Newcastle disease has been observed to cause marked degeneration of lymphocytes in the medullary region of the cloacal bursa (Lukert and Saif, 2003; Okoye, 2000) but necrosis and later replacement of lymphocytes by heterophils are additional features of IBD.

Grimes and King (1977) reported that experimental infection of 1-day-old, SPF chickens with a type 8 avian adenovirus produce small bursae and atrophy of bursal follicles at 2 weeks PI. Several other organs such as the liver, spleen, pancreas and kidney were grossly affected and intranuclear inclusion bodies were observed in the liver and pancreas (Lukert and Saif, 2003). Bursal atrophy is a lesion seen occasionally in chicken infections anaemia but histopathology of this disease distinguishes it from IBD.

### 2.9 Treatment

No therapeutic or supportive treatment has been found to change the course of IBDV infection (Cosgrove, 1962; Parkhurt, 1964). Because of the rapid recovery of the affected

flocks, treatment might appear highly effective if non-treated controls were not maintained for comparison. There are no reports in the literature concerning the use of some of the newer antiviral compounds and interferon inducers for the treatment of IBD (Lukert and Saif, 2003). Meanwhile ascorbic acid dietary supplementation has been shown to significantly reduce IBD mortality from 87% to 20% in USA (Pardue, 1987), similar research has demonstrated no effect under Nigerian tropical climate (Okoye *et al.*, 2001).

**2.10 Prevention and control**

The epidemiology of this infection has not been studied extensively, but it is known that contact with infected fomites readily cause spread of the infection (Lukert and Saif, 2003). The very high resistance of IBDV as has been described by Benton *et al*., (1967) account for persistence of the virus in the environment, particularly on contaminated farms, despite disinfection and will increase the likelihood that it will be carried over from one flock to a succeeding flock. Eradication in affected countries therefore seems unrealistic (van den Berg *et al.,* 2000). Prevention of IBD requires rigorous hygienic measures and medical prophylaxis. Sanitary precautions should include all-in/all-out farming method, cleaning and disinfection of premises and observance of a 'down time' (a period of rest between depopulation and restocking) (van den Berg *et al.,* 2000). Effective insect and pest control should be instituted.

Different consanguineous lines of poultry show highly variable susceptibility to experimental infection with the same strain of IBDV. The results of crosses between resistant and susceptible lines show that resistance is a dominant heredity characteristic. However, the genes responsible for resistance have not been identified, and genetic

selection for resistance has not yet been practice (Bumstead *et al.,* 1993; Bumstead, 1998; van den Berg *et al.,* 2000; Mac Lachlan and Dubovi, 2011).

At one time, before the development of attenuated vaccine strains, intentional exposure of chicks to infection at an early age was used for controlling IBD. This could be advised on farms that had a history of the disease, and the chicks normally would have maternal antibodies for protection. Also, young chicks less than 2 weeks of age did not normally exhibit clinical signs of IBD. When the severe immunosuppressive effect of early IBD infections was discovered, the practice of controlled exposure with virulent strains became less appealing. On many farms, the cleanup between broods is not thorough, and due to the stable nature of the virus, it easily persists and provides an early exposure by natural means (Lukert and Saif, 2003).

Immunization of chickens is the principle method used for the control of IBD in chickens (Saif, 1998; van den Berg, 2000; Lukert and Saif, 2003; Okoye, 2005; Mac Lachlan and Dubovi, 2011; Butcher and Miles, 2012). No Vaccine can however solve the problem if sanitary precautions are not taken (van den Berg, 2000). An ideal vaccine must offer the complete balance between efficiency and innocuity; the vaccine must not cause disease or bursal lesion, must not be immunosuppressant or excreted and must confer long -lasting immunity even in birds with a high level of maternal immunity (Thornton, 1977). Unfortunately such a vaccine does not exist (Mc Ferran, 1993; van den Berg, 2000).

Two types of vaccines are mostly available for the control of IBD. These are live attenuated vaccines and inactivated oil emulsion adjuvanted vaccine (Thorton and Pattison, 1975; OIE, 2008). A live recombinant vaccine expressing IBDV antigens has also been licensed (Darteil *et al*., 1995).

To date, IBD vaccines have been made with serotype1 IBDV only although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 viruses (Resonberger and cloud, 1986). Cross protection studies have shown that inactivated vaccines prepared from 'classical' serotype 1 virus require a high antigenic content to provide good protection against some of its variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses are now licensed (OIE, 2008).

Live IBD vaccines are very widely used. They are produced from fully or partially attenuated strains of virus through serial passages in embryonated eggs. Based on their degree of attenuation, they are classified as mild, intermediate or intermediate plus (hot) (van den Berg, 2000; OIE, 2008).

Depending on the degree of attenuation, the vaccine strains cause histological lesions of varying severity to the bursae of SPF chickens. The hot strains induce histological lesions which are comparable to those caused by pathogenic strains, the only difference being that the hot strains do not cause mortality (van den Berg, 2000).

Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only

minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first (van den Berg *et al.*, 2000; OIE, 2008).

The mild strains are used chiefly for the vaccination of breeder flocks. These are very sensitive to interference by homologous maternal antibodies, and are administered when these antibodies have disappeared, i.e. between the fourth and eighth week of age, depending on whether the grandparent flocks have or have not been vaccinated with an oil-emulsion inactivated vaccine before lay (van den Berg *et al.*, 2000).

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period (Haddad *et al*., 1997; van den Berg, 2000; Lukert and Saif, 2003; OIE, 2008). This novel technology utilizes specific hyperimmune neutralising antiserum (or "

virus neutralizing factor" (VNF)) with a vaccine virus under conditions that are not sufficient to neutralise the vaccine virus but which are sufficient for delaying the pathologic effects of the vaccine alone. This allows young chicks to be vaccinated more effectively in the presence of passive immunity even with a strain that would be too virulent for use *in ovo* or at hatching. Although some questions still remain concerning the batch variation and risks of contamination of VNF, the residual pathogenicity of the vaccine strain and mechanism involved in the delay of the immune response (Jeurissen *et al.,* 1998), this technology is very promising for the future control of IBDV (van den Berg, 2000).

A vaccine using recombinant viruses expressing the Vp2 protein of IBDV such as fool pox virus (Bayliss *et al*., 1991; Heine and Boyle, 1993), herpes virus of turkey (HVT) (Darteil *et al*., 1995; Tsukamoto *et al.,* 1999) or fowl adenovirus (Sheppard *et al*., 1998) have been described and have proven efficiency in laboratory tests. The advantages of these vaccines are the absence of residual pathogenicity, sensitivity to MDA and risk of selection of mutants as well as the possibility of use *in ovo* and differentiation between infected and vaccinated animals (van den Berg *et al.,* 2000). The use of HVT as a viral vector recombinant vaccine has been licensed recently in Europe. There is however limited information available on the use of this vaccine (OIE, 2008).

Live IBDV vaccines are compatible with other avian vaccines. However, the strains that cause serious lesions to the bursa of Fabricius may also provoke immunosuppression, exacerbate the pathogenicity of other immunosuppressive viruses (Marek's disease virus [MDV] and chicken anaemia virus [CAV]) and jeopardise the immunisation of poultry against other diseases. Registration procedures for these vaccines must include tests to verify the absence of interference with other vaccinations as well as the absence of

reversion to virulence in the course of serial passages in three- to six-week-old SPF chickens (van den Berg *et al*., 2000).

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (Cullen and Wyeth, 1976). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous routes. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 4°C and 8°C. It should not be frozen or exposed to bright light or high temperature. Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way, the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth and Cullen, 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. However, the chicks are not protected from other highly pathogenic strains that may inflict high mortality rates at later stages (Wyeth and Cullen, 1979; van den Berg and Meulemans, 1991). It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent

cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine (van den Berg *et al*., 2000; OIE, 2008)

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study Area

This study was conducted in the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, and in the Department of Haematology and Blood Transfusion, Ahmadu Bello University Teaching Hospital, Zaria.

## 3.2 Experimental Chickens

One hundred Near Black cockerels were obtained from Farm Support Hatcheries®, Ibadan, Nigeria, at day old. They were housed on a deep litter in the Poultry research facility of the department of veterinary pathology and fed with chick mash (Vital Feeds®), and water was provided *ad libitum.* They were acclimatised for 28 days before they were allocated at random into two groups of 50 birds each; the IBDV infected and uninfected (control), and kept separately.

## 3.3 Preparation of Viral Inoculum

Infectious bursal disease virus strain IBDV37/Kaduna NG/2009 (GenBank accession number: JX424062) in form of 50% (w/v) bursal homogenate was used for the study. A fresh clarified 50% (w/v) bursal homogenate was obtained following inoculation of four-week-old cockerels that were devoid of maternal antibodies against IBDV with the experimental challenge IBDV. The birds were euthanised three days post-inoculation (pi) and their bursae were harvested aseptically. The bursae were pooled, weighed with the addition of equivalent volume of phosphate buffered saline, pH 7.4 and homogenized with

sterile mortar and pestle. The homogenate was centrifuged at 1250 g for 30 min and the supernatant was harvested, dispensed into aliquots and stored at -25 $^{0}$C. The bursal supernatant obtained was tested for the presence of IBDV antigen by agar gel immunodiffusion test according to the method of Willey *et al*. (2011)

### 3.4 Experimental Design

The cockerels in the infected group were each inoculated intraocularly with a drop (approximately 0.02 ml) of bursal tissue supernatant containing the IBDV at 35 days of age in each opened eye. Those in the control group were not inoculated. Following inoculation, the chicks were observed daily for any clinical signs and records were taken. Four birds from the inoculated group and two from the control group were sampled randomly at the following post inoculation (pi) periods: 6, 12, 24, 36, 48, 60, 72, 84, 96, 120, 168 hours pi. Four and half millilitre of blood was collected from the heart of each bird using a sterile 23 gauge needle and 5 ml hypodermic syringe. During each time, the birds were sacrificed by injecting air embolus into the heart and then necropsied.

### 3.5 Haematology

Two millilitres of the blood collected was transferred into a 4 ml tube containing 1.5 mg potassium ethylene diamine tetra acetic acid ($K_2$ EDTA). The blood was used to determine the packed cell volume and haemoglobin concentration by the method of Samour (2006); the haemoglobin was estimated as oxyhaemoglobin using an automated haemoglobinometer (XF-1C Hemoglobin meter®, China); total protein was determined by the use of refractometer. Thrombocyte count was determined, while performing the differential white blood cell count using the following formular (Samour, 2006):

$$\left(\frac{No.of\ Thrombocytes}{100}\right) \times \text{WBC} = \text{Thrombocyte} \times 10^9/\text{L}$$

The total leukocyte and differential white blood cell count were determined using the method described by Sinkalu *et al.* (2010). Briefly, the blood films were made according to the standard two-slide wedge technique used in mammalian haematology. Leishman's staining technique was done as described by Cheesbrough (2000). The slide was covered with the undiluted stain, and then diluted twice the volume with water and allowed to stain for 10 minutes. The slides were then washed off the stain with tap water, air-dried and observed microscopically under oil immersion. One hundred leukocytes, including heterophils (H), lymphocytes (L), monocytes, (M), basophils (B) and eosinophils (E) were counted on each slide. The differential white blood cell count is expressed as a percentage of the individual cell group. Total leucocytes counts were calculated using the formular (Campbell, 1995):

$$\textit{Total white blood cell count (WBC)/mm}^3 = \frac{\boldsymbol{number\ of\ cells\ counted \times 10 \times 32 \times 100}}{(\%\ \boldsymbol{H} + \boldsymbol{E}) \times \boldsymbol{18}}$$

The percentage of each cell group is then converted into absolute numbers by reference to the total WBC using the following formular (Samour, 2006):

$$\frac{(\boldsymbol{percentage\ of\ white\ blood\ cell\ counted \times total\ WBC})}{\boldsymbol{100}} = \boldsymbol{Absolute\ No. \times 10^9/L}$$

### 3.5 Evaluation of Coagulation Factors

Another 2.5 ml of blood was emptied into a tube containing 1 part of 3.8 % sodium citrate and 9 part of blood. The plasma was harvested by centrifuging the blood at 2240 × g for 15

minutes. The harvested plasma was transferred into labeled tubes using a plastic pipette and used for the determination of Activated partial thromboplastin time using a semi-automated analyzer (semi-automated blood coagulameter, start 4, Stago®, France) with standard reagents as earlier described (Rizzo *et al.*, 2008). Briefly, 100 µl of prewarmed sample was dispensed into a cuvette. Another 100 µl of APTT reagent I containing coagulation activator was added together with a magnetic bead. The cuvette was incubated in the incubation column of the coagulometer. After an alarm, the cuvette was transferred into the test column. After another alarm, 100 µl of APTT reagent II containing CaCl was added and observed for clot formation.

### 3.6 Necropsy and Histopathology

At necropsy, the bursa of Fabricius, spleen, thymus liver, kidney, proventriculus, gizzard and skeletal muscles were examined grossly for any lesion and findings were recorded. Sections of these organs were taken and fixed in 10% buffered neutral Formalin, processed by paraffin embedding, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). The sections were examined with a light microscope.

### 3.7 Statistical analysis

Mean values of packed cell volume, haemoglobin concentration, total protein, thrombocytes count, total leukocytes, differential leukocyte count and APTT were calculated and expressed as mean ± standards error of mean (SEM). The values were analyzed using Graph pad prism version 6.0. Student's t-test was used to compare the mean values of the IBDV inoculated groups and the uninoculated control. Values of $p < 0.05$ were considered significant.

# CHAPTER FOUR

# RESULTS

## 4.1: Clinical Signs and Mortality

At 48 hours post-inoculation (hpi), clinical signs of IBD such as depression and ruffled feathers (Plate I) appeared in 2.7 % of the chickens in the infected group. At 36 hpi, the proportion increased to 10 %. Severe depression, ruffled feathers anorexia (69 %) and diarrhea (54 %), characterized by greenish yellow watery faeces, were observed in chickens in the infected group 72 hpi. At 96 hpi, all the chickens (100 %) in the IBDV infected group showed severe depression, ruffled feathers and diarrhoea. This proportion declined gradually and by 168 hpi, all survivors appeared apparently healthy (Figure 1).

Mortality began at 84 hpi with 4.5 % of chickens in the IBDV infected group. It peaked at 96 hpi with 23.5 % mortality and declined to 11 % at 120 hpi. As early as 168 hpi, survivors appeared apparently normal (Figure 2). No clinical signs or mortality were observed in the non-infected control group throughout the period of the experiment.

Plate I. Chicken infected with infectious bursal disease virus showing depression and ruffled feathers.

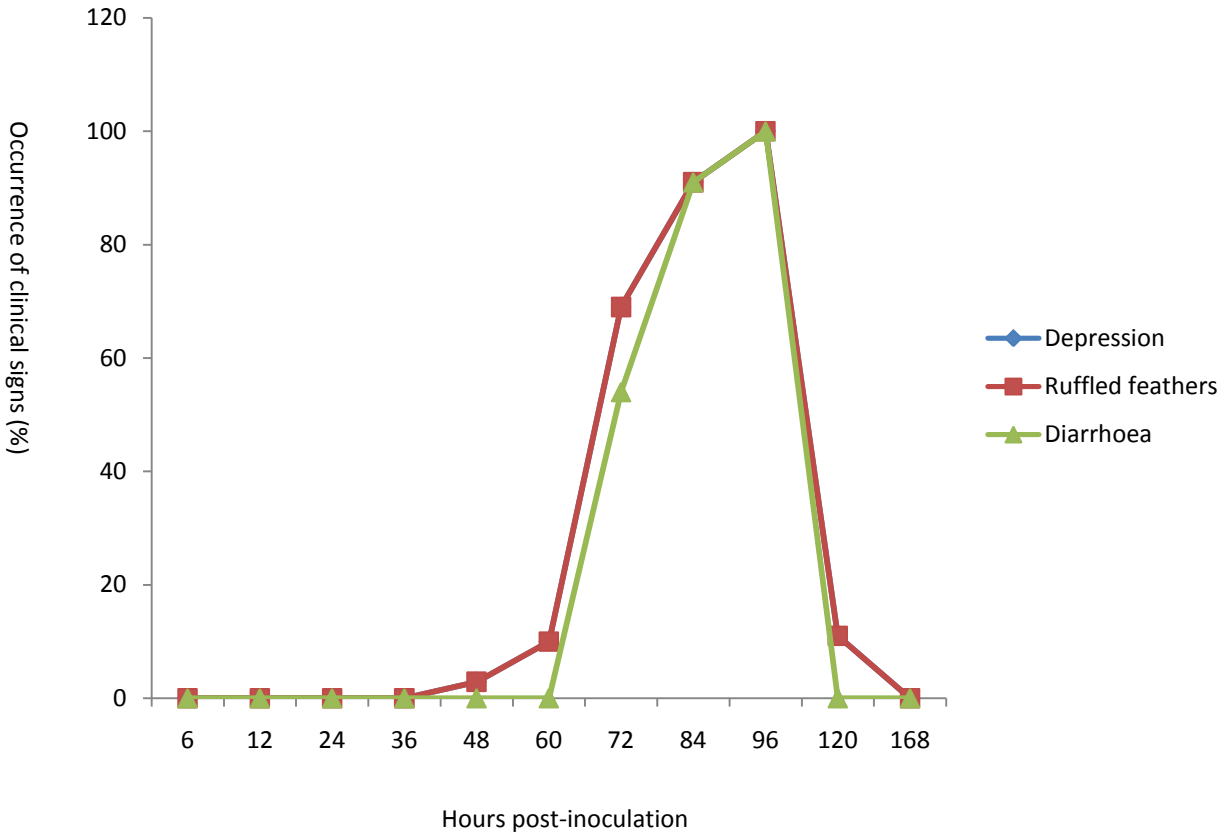


Figure 1: Percentage occurrence of clinical sign in IBDV infected Chickens

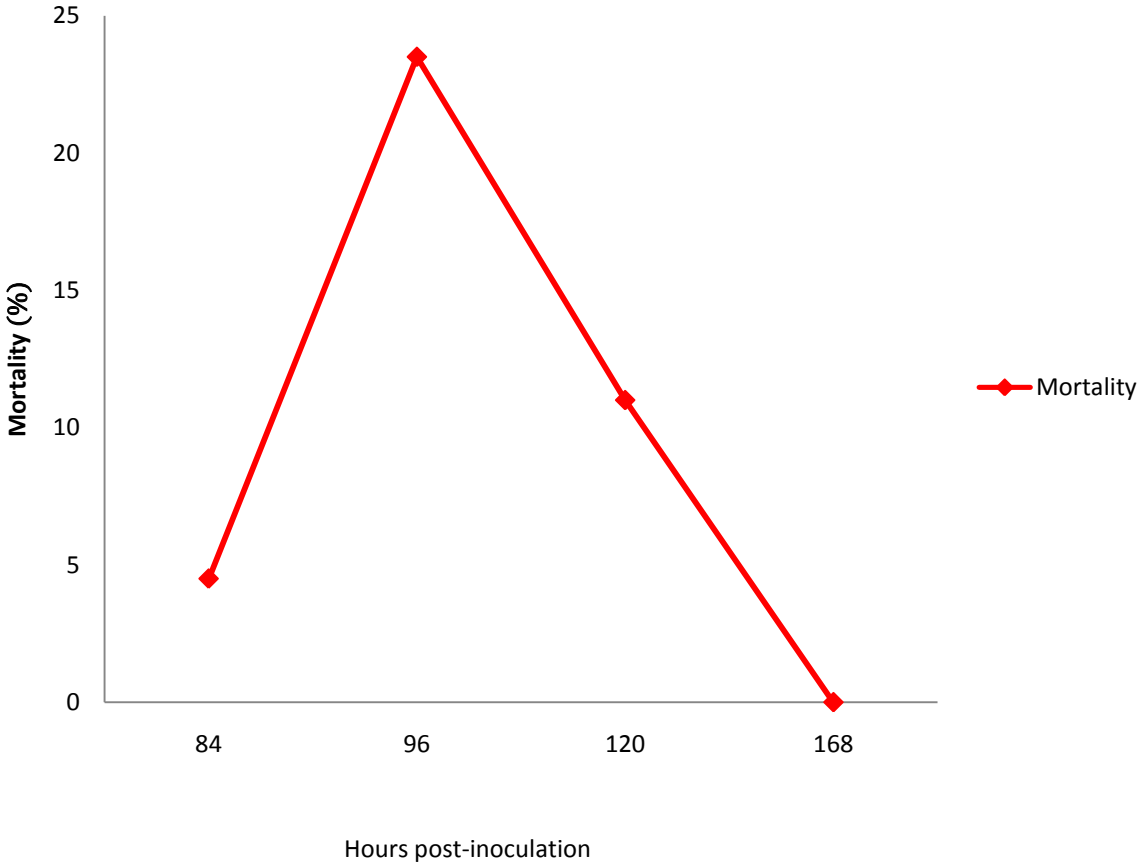


Figure 2: Mortality in chickens infected with infectious bursal disease virus

## 4.2: Haematology

### 4.2.1: Packed cell volume

The PCV in the IBDV infected chickens decreased from the initial mean of 31.50 ± 0.50 at 6 hpi to 25.25 ± 0.48 at 36 hpi. It then increased and fluctuated between 28% and 26% up to 120 hpi after which it declined to 24.67 ± 1.33 at 168 hpi. There was no significant difference ($p > 0.05$) between the IBDV infected and uninfected control groups.

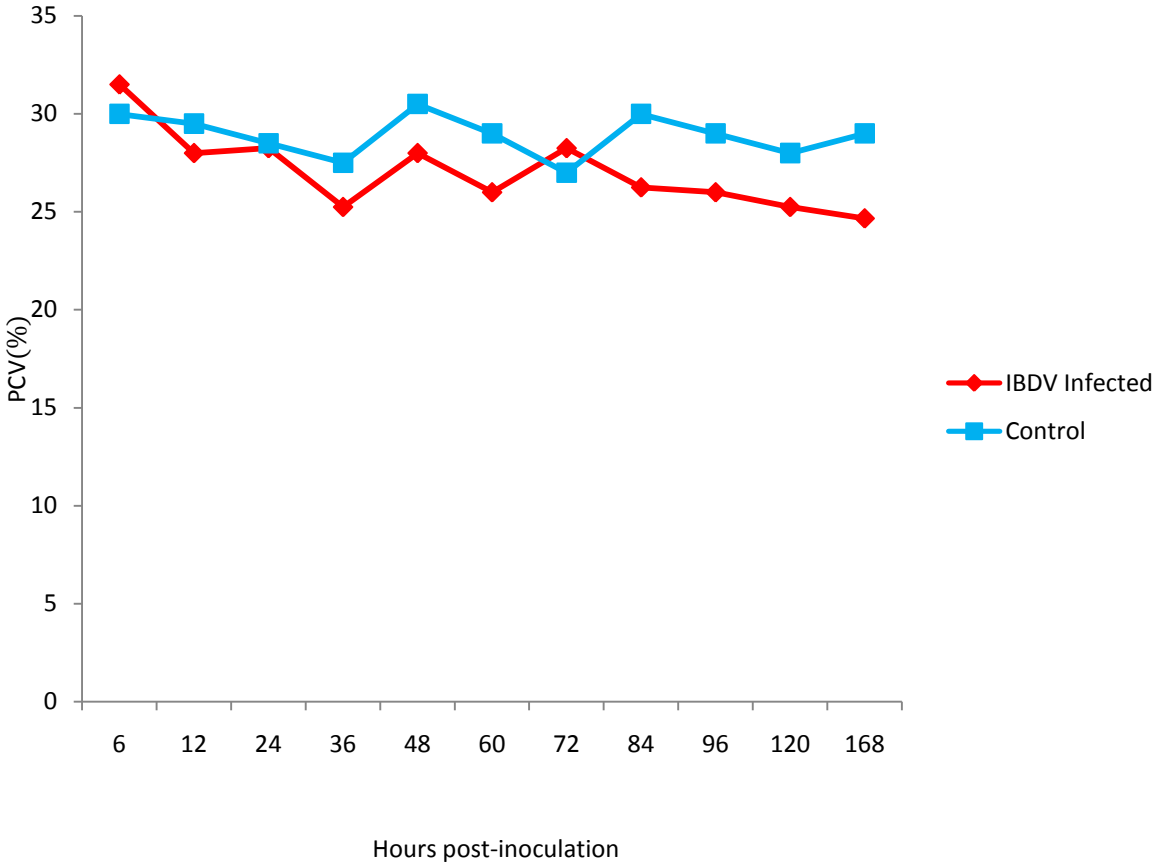


Figure.3 Packed Cell Volume of chickens infected with infectious bursal disease virus and un-infected chickens (Control)

### 4.2.2: Haemoglobin concentration

The mean haemoglobin concentration (g/dL) did not show any difference with the uninfected control values until at 36 hpi when a non-significant ($p > 0.05$) decrease ($15.8 \pm 5.70$) was observed in the IBDV infected chickens. It then rise again reaching the peak of $16.8 \pm 0.75$ at 72 hpi and eventually declined to the value of $15.3 \pm 1.50$ at 168 hpi. There was no statistical significance in the mean values between the two groups.

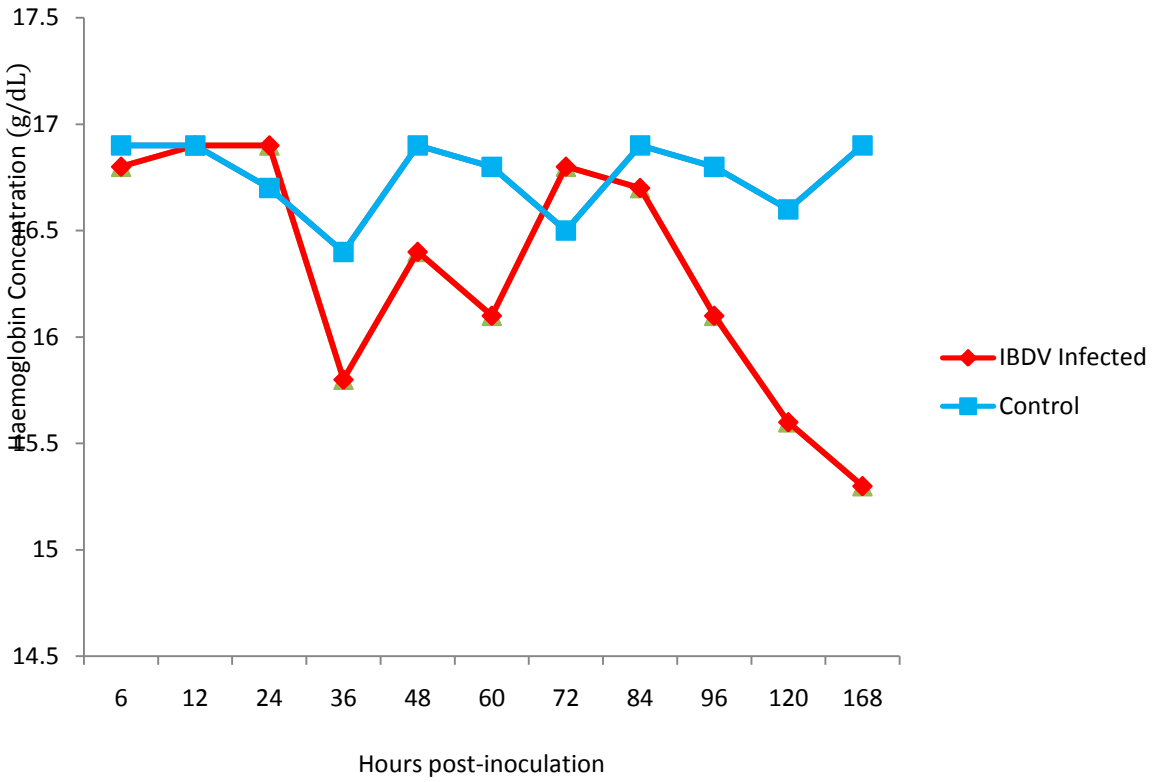


Figure 4: Haemoglobin Concentration in chickens infected with infectious bursal disease virus and un-infected (control) chickens

### 4.2.3: Total protein

There was a decrease in the mean total protein value from 2.95 g/dL at 6 hpi to 2.50 g/dL at 36 hpi. The values then maintain a raise until the end of the experiment. Although the mean values obtained for the IBDV infected group were higher than the values obtained for the uninfected control group, there were no significant differences ($p > 0.05$) between the values.

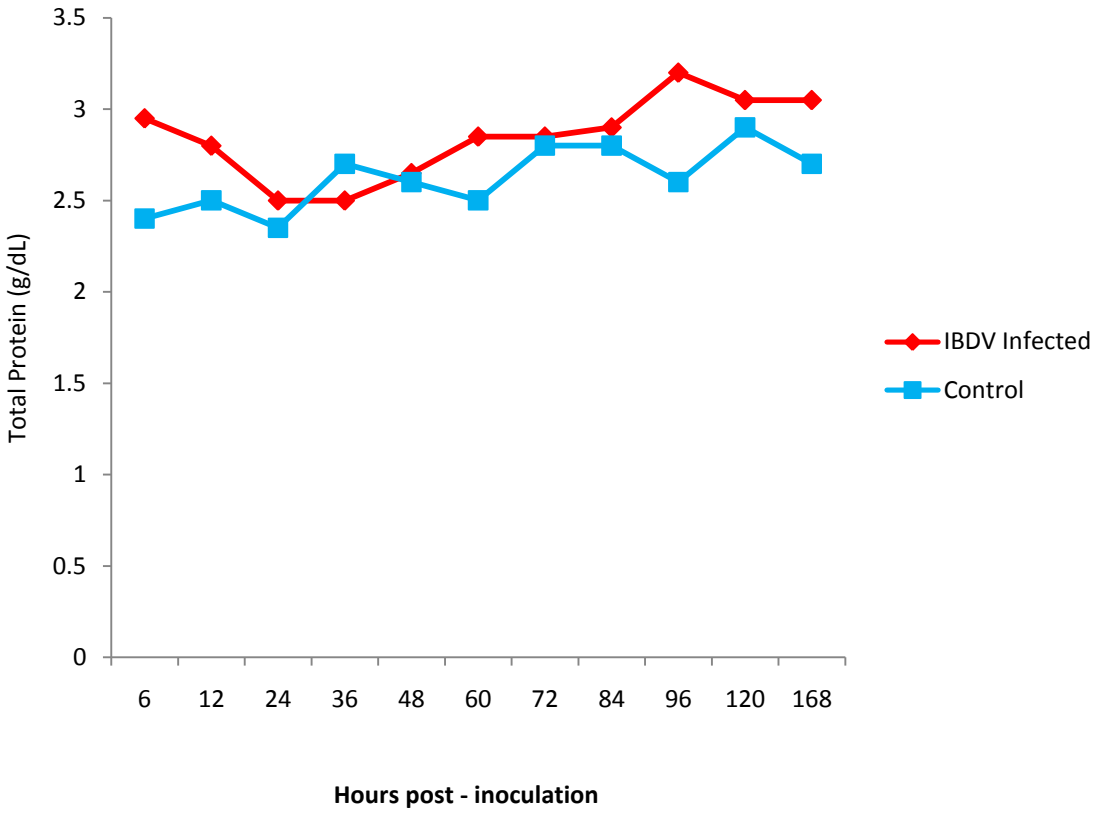


Figure 5: Total Protein level in chickens infected with infectious bursal disease virus and un-infected (control) chickens

### 4.2.4: Thrombocyte count

The mean thrombocyte count of the IBDV infected group was comparable with that of the uninfected control until at 36 hpi when sharp increase was observed in the infected group. This increase in thrombocyte count was followed by a significant fall beginning from 48 hpi to reach a minimum level ($2.55 \pm 0.14$) ($p < 0.05$) at 84 hpi. This low thrombocyte profile continued until at 168 hpi when the values sharply rose to $12.27 \times 10^{3}\mu l^{-1}$ ($p>0.05$).

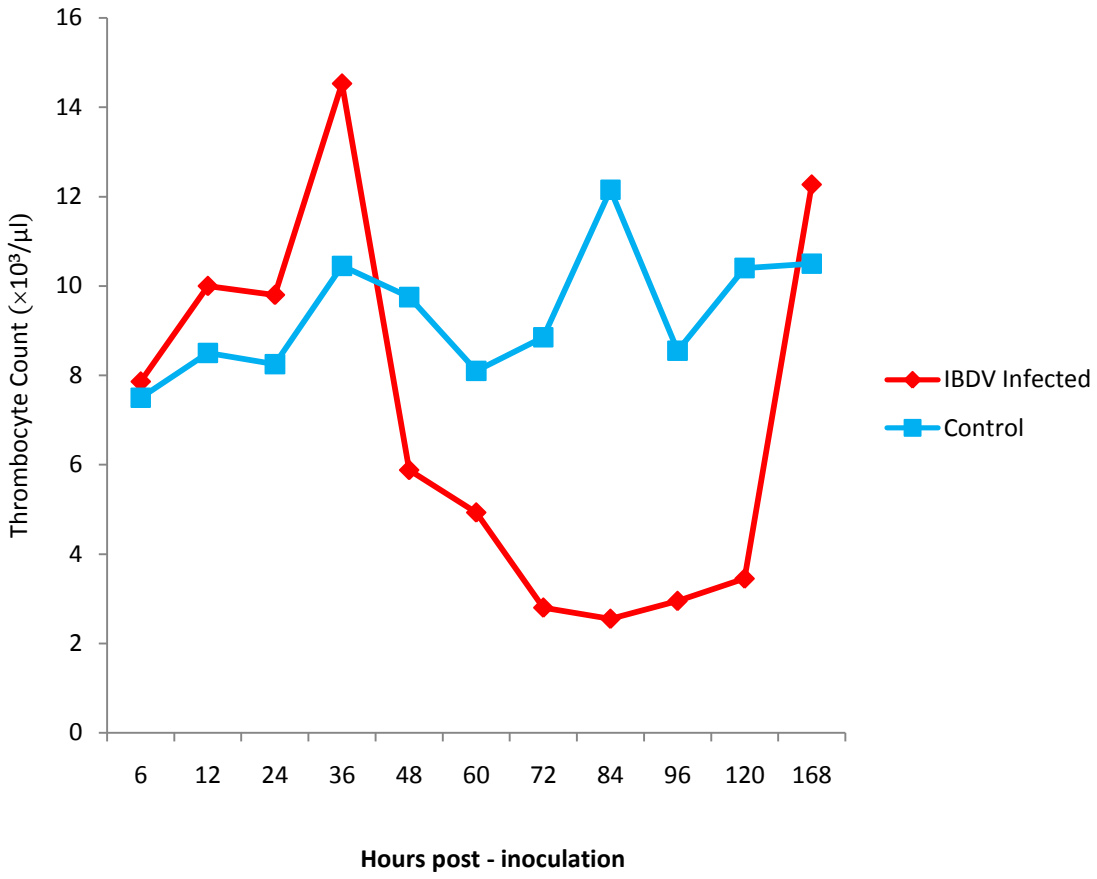


Figure 6: Thrombocyte Count in chickens infected with infectious bursal disease virus and un-infected (control) chickens

### 4.2.5: Leukocye count

The total leukocyte count of the IBDV infected group increased from17.8$\times 10^9$/L at 6 hpi to 22.2 $\times 10^9$/L at 12hpi ($p>0.05$). It then begins to decline until at 48 hpi ($p > 0.05$) when it increased again steadily until the end of the experiment. Like the total leukocyte count, the lymphocyte count increased slightly ($p > 0.05$) at 12 hpi and then begins to decline reaching its lowest level at 60 hpi ($p < 0.05$). It thereafter increased steadily peaking at 168 hpi. There was no much difference in the heterophils count between the values in the uninoculated control and the IBDV infected group except at 60 hpi ($p > 0.05$). The value then rose spontaneously and remained fairly constant between 72 hpi and 84hpi ($p > 0.05$) and then declined to fluctuate between 1.9 $\times 10^9$/L and 1.8 $\times 10^9$/L.

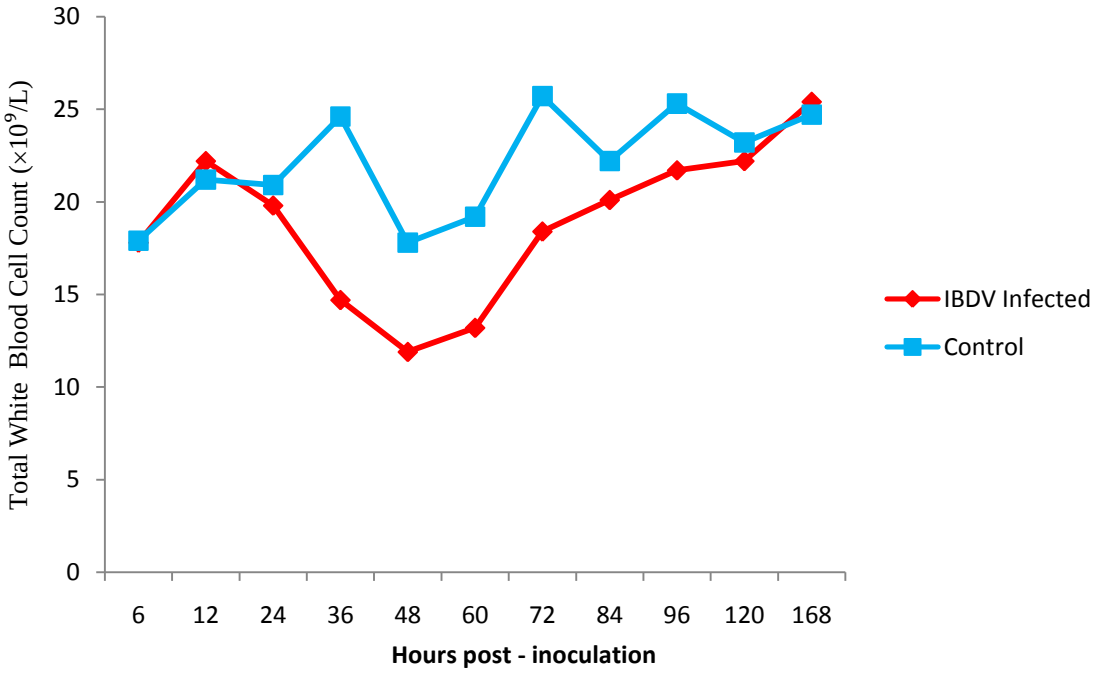


Figure 7: Total White Blood Cell Count in chickens infected with infectious bursal disease virus and un-infected (control) chickens

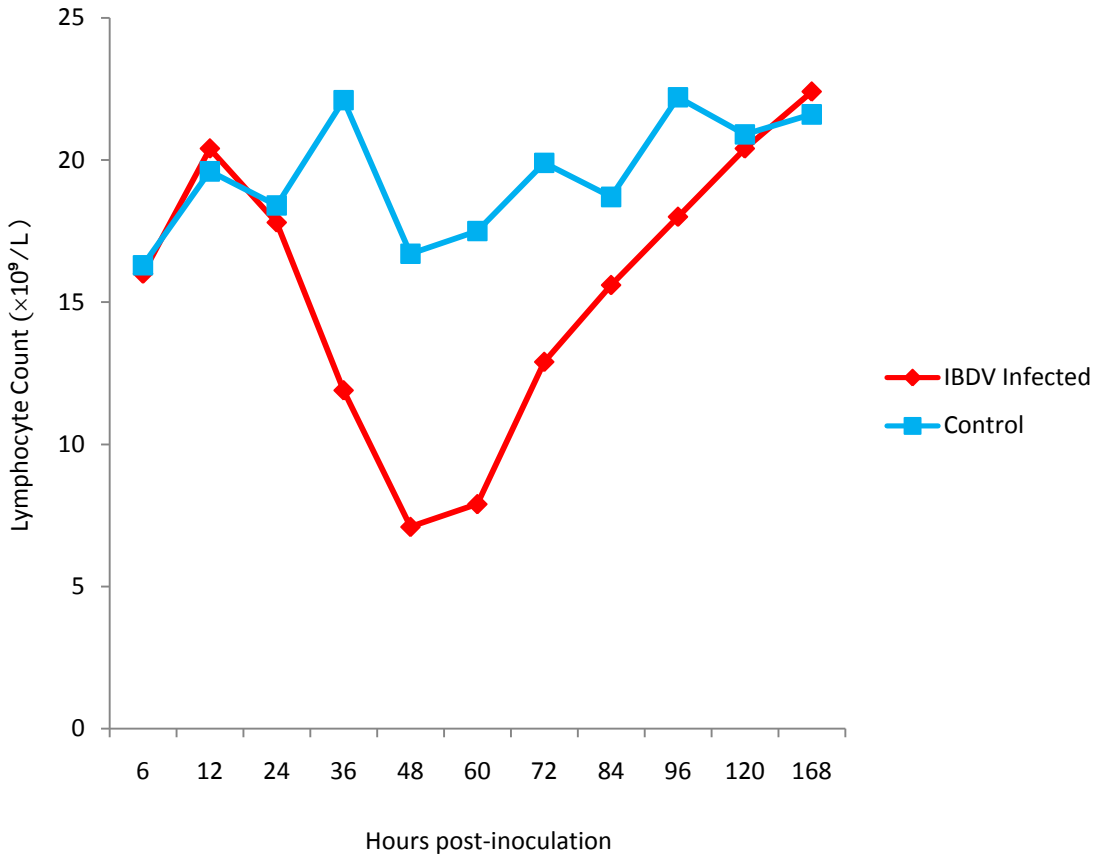


Figure 8: Lymphocyte Count in chickens infected with infectious bursal disease virus and un-infected (control) chickens

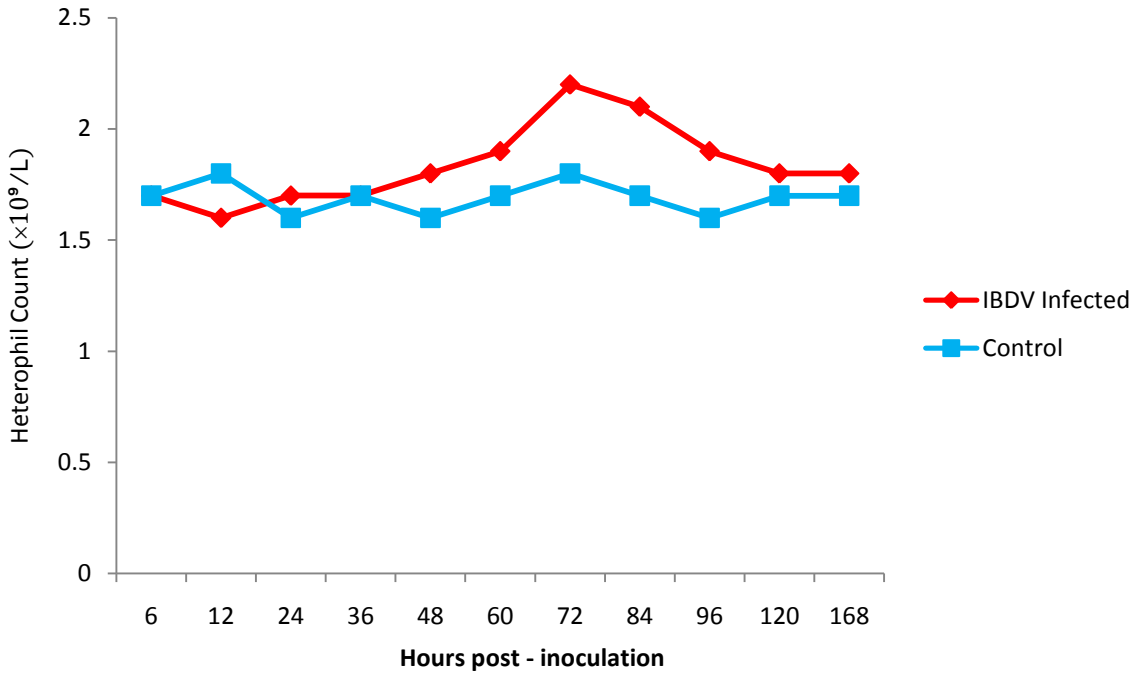


Figure 9: Heterophil Count in chickens infected with infectious bursal disease virus and un-infected (control) chickens

### 4.3: Coagulation Factors

#### 4.3.1: Activated partial thromboplastin time (APTT)

The mean APTT in the IBDV infected chickens remained comparable with the corresponding uninnoculated control group up to 60 hpi ($126.5 \pm 2.2$) when it becomes significantly prolonged ($p < 0.05$). The APTT value remained prolonged up to 168 hpi when it decreased to $112.5 \pm 6$ ($p > 0.05$).

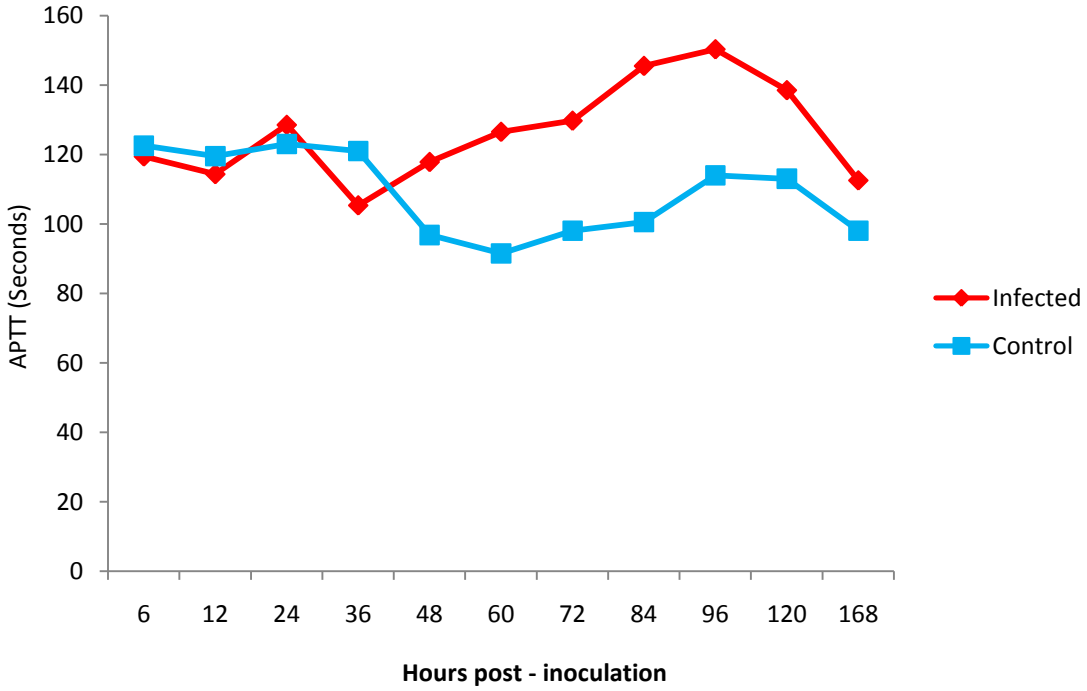


Figure 10: Activated partial thromboplastin time of the chickens infected with infectious bursal disease virus and un-infected (control) chickens

### 4.2. Necropsy

There were no apparent gross lesions in the IBDV infected group until 72 hpi, when the bursae become slightly oedematous, hyperaemic (plate II), with a gelatinous yellowish exudate covering the serosa surface. On the mucosal surfaces, the bursae showed mild petecchial haemorrhages. At 84 hpi, bursal haemorrhage became extensive in the dead and 2 of the 4 sacrificed birds (Plate III). In these birds, both the serosal surfaces and mucosa plicae (Plate IV) of the bursa showed significant congestion and hemorrhages. There were petecchial and ecchymotic haemorrhages in the thigh (Plate V), and keel muscles (Plate VI) of the sacrificed (3 of 4) birds as well as the dead ones. There was haemorrhage at the proventricular-gizzard junction (Plate VII) of the sacrificed (3 of 4) and all the dead birds. In both dead and sacrificed birds, the kidneys appeared congested and the spleens were enlarged.

At 96 hpi, while the bursae were still enlarged, hemorrhage was more severe in both sacrificed and dead birds. Variable degrees of hemorrhages were present in the bursae, keel and thigh muscles (skeletal muscles), and proventricular-gizzard junction (Figure 11) with the highest proportion being in dead birds when compared with sacrificed birds (figure 12). There were cheesy materials in the bursae of all the chickens examined. The kidneys and spleen were congested. There was no gross lesion in the thymus.

At 120 hpi, mild hemorrhages were observed in the bursae and thigh of both sacrificed and dead birds. The liver was congested and the spleen enlarged. The bursae atrophied to about $^{1}/3$ of its size when compared with the control (Plate VIII). Atrophy of the bursae was the

only significant lesion observed at 168 hpi. No observable gross pathological lesion was noticed in birds in the uninfected control group.

Plate II. Hyperaemic, oedematous bursa from IBDV infected chicken (72 hpi)

Plate III. Severe haemorrhage and oedema in a bursa from IBDV infected chicken (84 hpi)

Plate IV. Haemorrhagic bursal plicae of IBDV infected chicken at 84 hpi

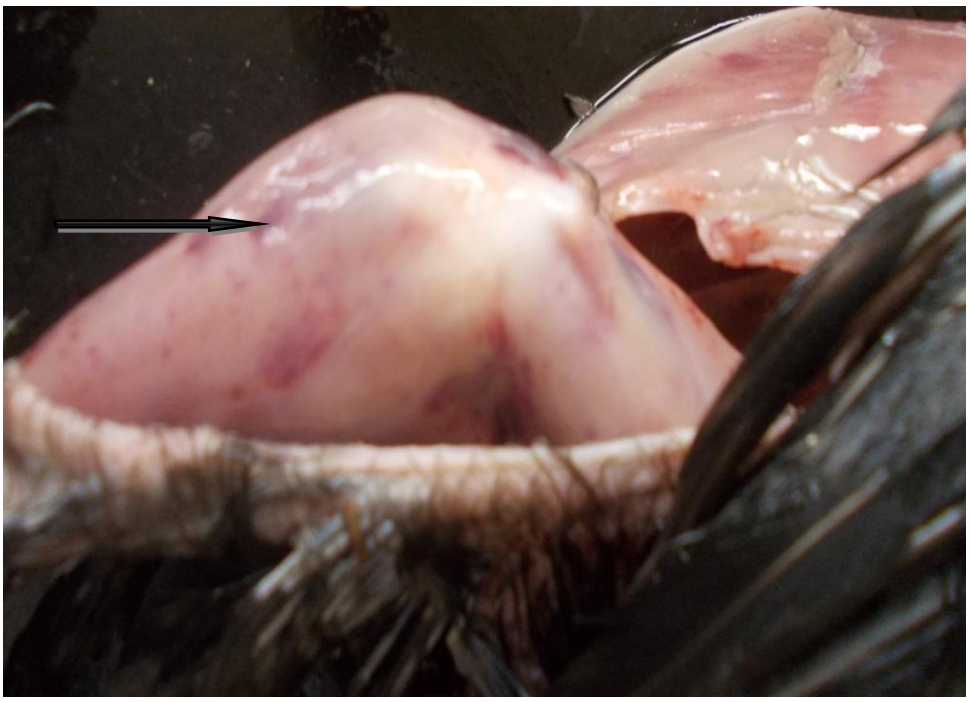

Plate V. Petechial and ecchymotic haemorrhages in thigh and leg muscles (arrow) of chicken in the IBDV infected group

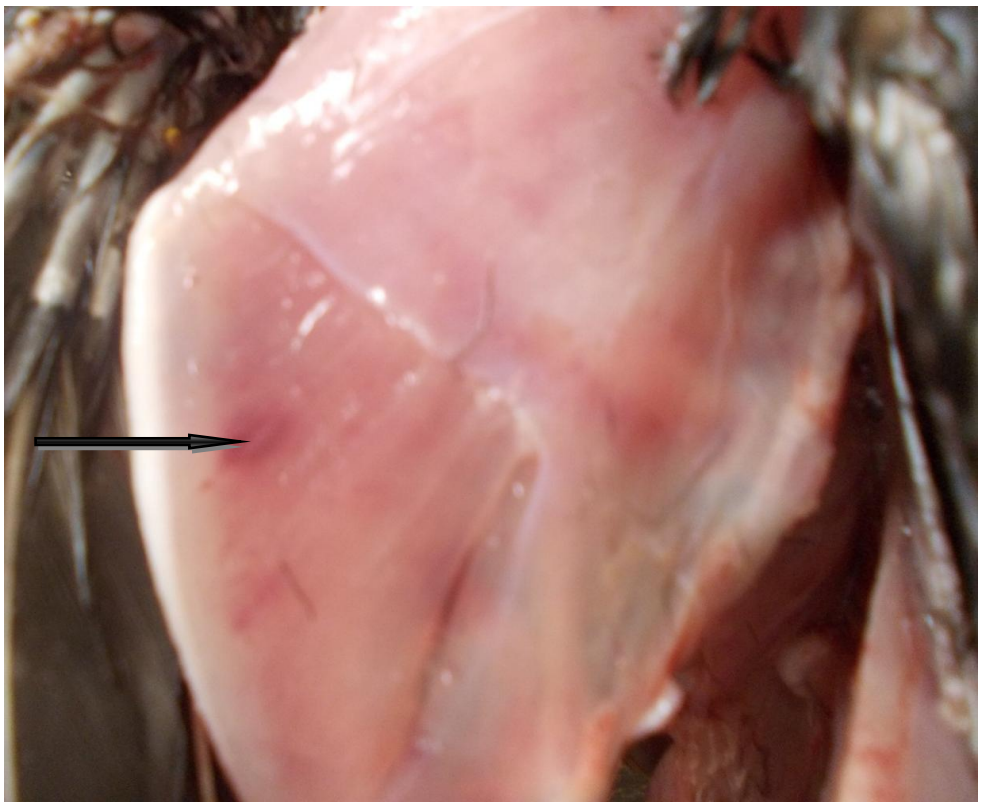

Plate VI. Ecchymotic haemorrhages in pectoral muscles (arrow) of a bird in the IBDV infected group

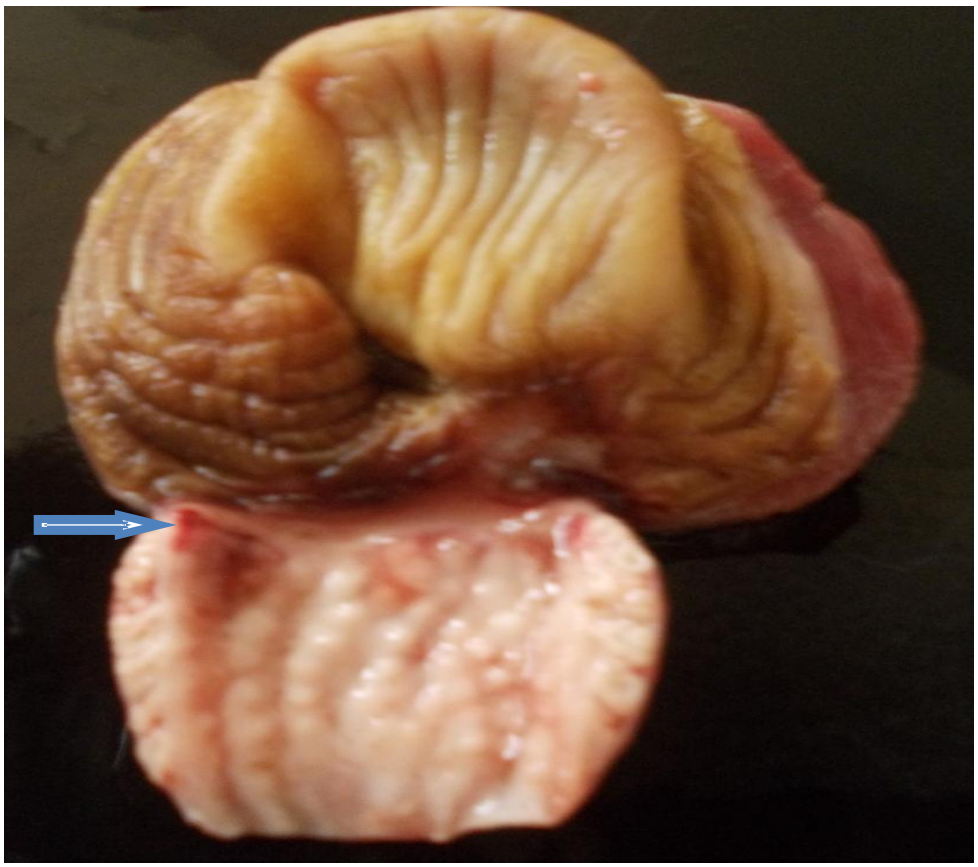

Plate VII. Haemorrhagic proventricular-ventricular junction (arrow) in IBDV infected chicken


Plate VIII. Bursal atrophy (A) in IBDV infected chickens compared to the controls

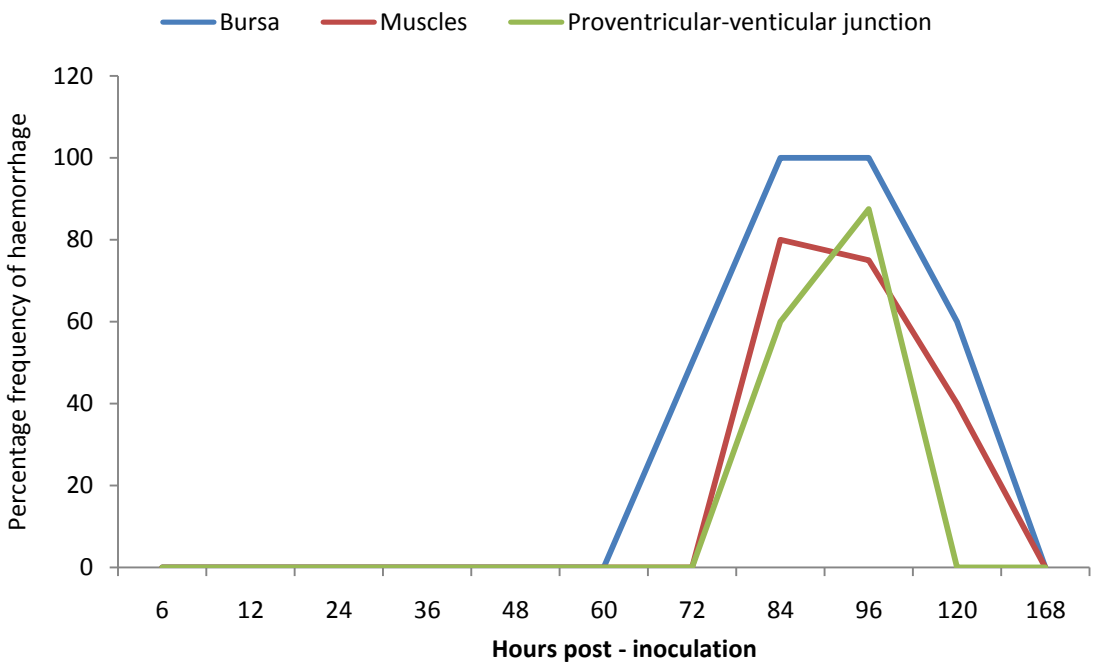


Figure 11: Frequency of haemorrhage in Bursa of Fabricius, Muscles and Proventricular-ventricular Junction in the IBDV inoculated chickens

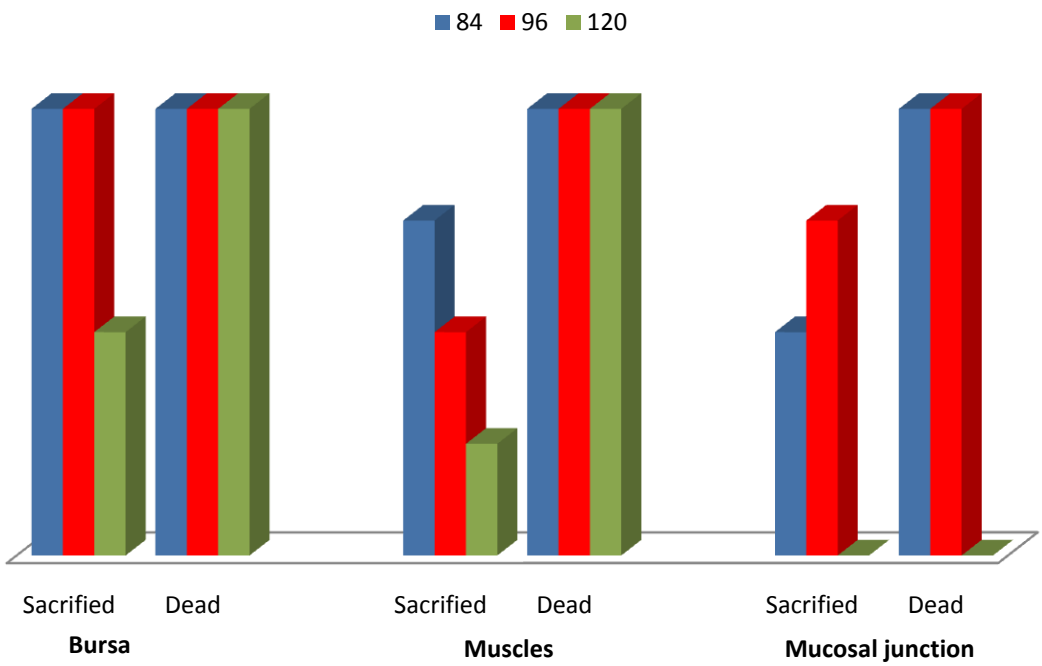


Figure 12: Percentage Frequency of Haemorrhage in Organs of Sacrificed Chickens Compared to Dead Ones at 84, 96 and 120 hpi

## 4.4 Histopathology

Bursa of Fabricius

The first observable microscopic lesion in the bursa of Fabricius of the infected chickens was a slight depletion of lymphocytes in the medullary areas of the bursal follicles 60 hpi (Plate X). At 72 hpi, necrosis and depletion of lymphocytes has become more pronounced in the follicular medulla and foci of lymphocyte necrosis have spread to the cortical areas. The interfollicular spaces also become widened. By 96 hpi, the lymphoid follicles were only sparsely populated with lymphoid cells (Plate XI). The undifferentiated epithelial layer between the cortex and medulla was clearly visible. The bursal epithelial layer has become thickened and folded. At 120 hpi, lymphoid necrosis progressed and areas of coagulative necrosis were observed within the follicles forming cysts filled with lightly eosinophilic debris. At 168 hpi, necrosis and depletion of lymphoid cells were observed not only in the lymphoid follicles but also in the interfollicular space. The depletion of lymphoid cells created vacuoles which were seen within the follicles and in the interfollicular connective tissues (Plate XII).

Spleen

Lymphoid cell necrosis and depletion was observed in both the germinal follicles and the periarteriolar lymphoid sheaths (PALS) in the IBDV infected group from 60hpi to 96hpi (Plates XIII and XIV). Beyond this period, the spleen appeared to have regenerated as no significant changes were observed.

Liver

There was a slight congestion within the sinusoids almost throughout the experiment (Plate XV) except at 168hpi when it appeared normal.

Kidney

At 84 and 96 hpi, the kidneys showed congested intertubular spaces and the presence of intratubular eosinophilic materials was noticed (Plate XVI and XVII).

Muscles

Haemorrhage was observed in the keel and thigh muscles of the IBDV infected birds between 72 hpi and 96 hpi (Plate XVIII)

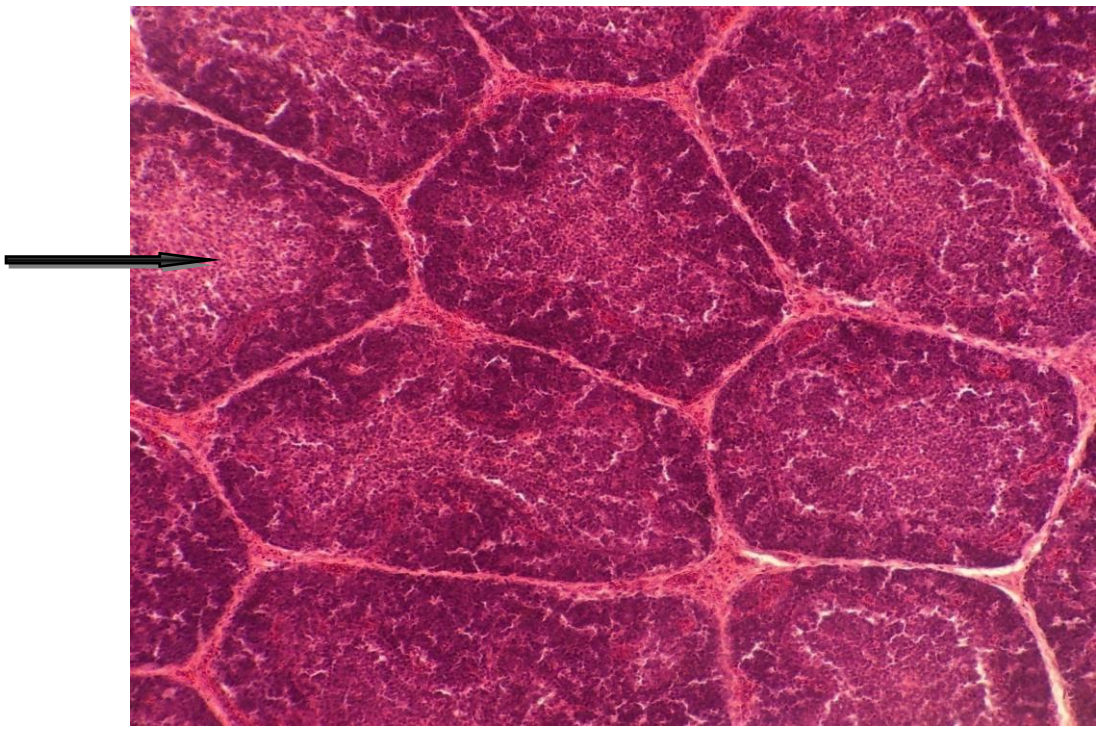

Plate IX. Bursal follicles from IBDV infected chicken, 60hpi showing mild lymphoid cell necrosis in the medullary regions (arrow).(H and E ×200)

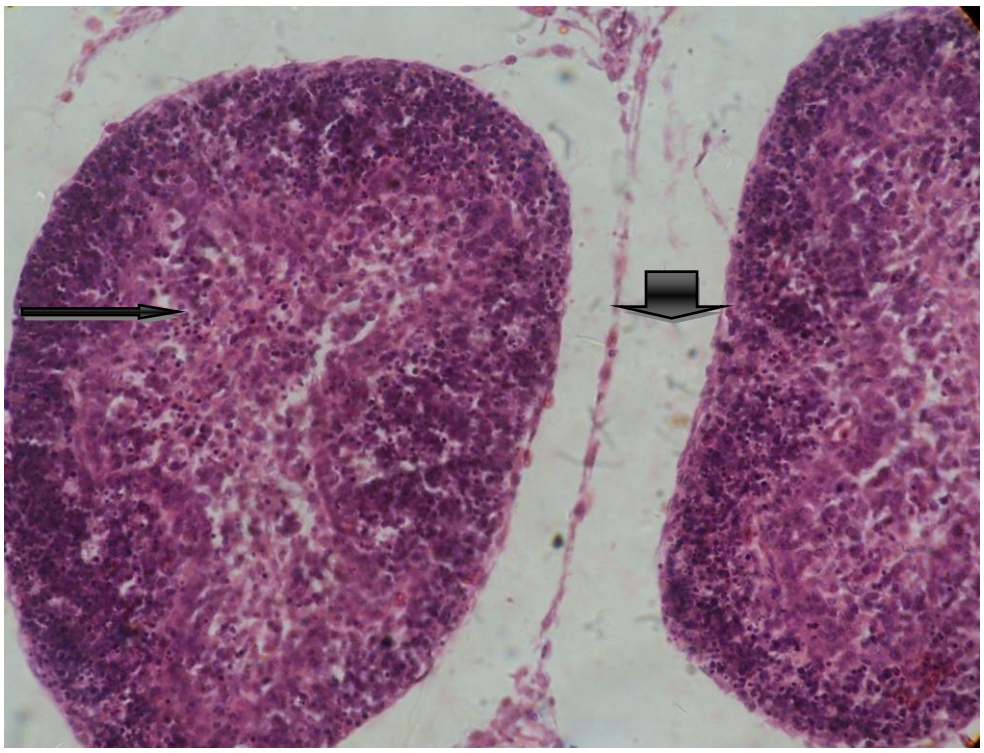

Plate X. Bursal follicles from IBDV infected chicken, 96hpi showing generalised lymphoid cell necrosis (arrow) and interfollicular oedema (arrow head). (H and E ×400).

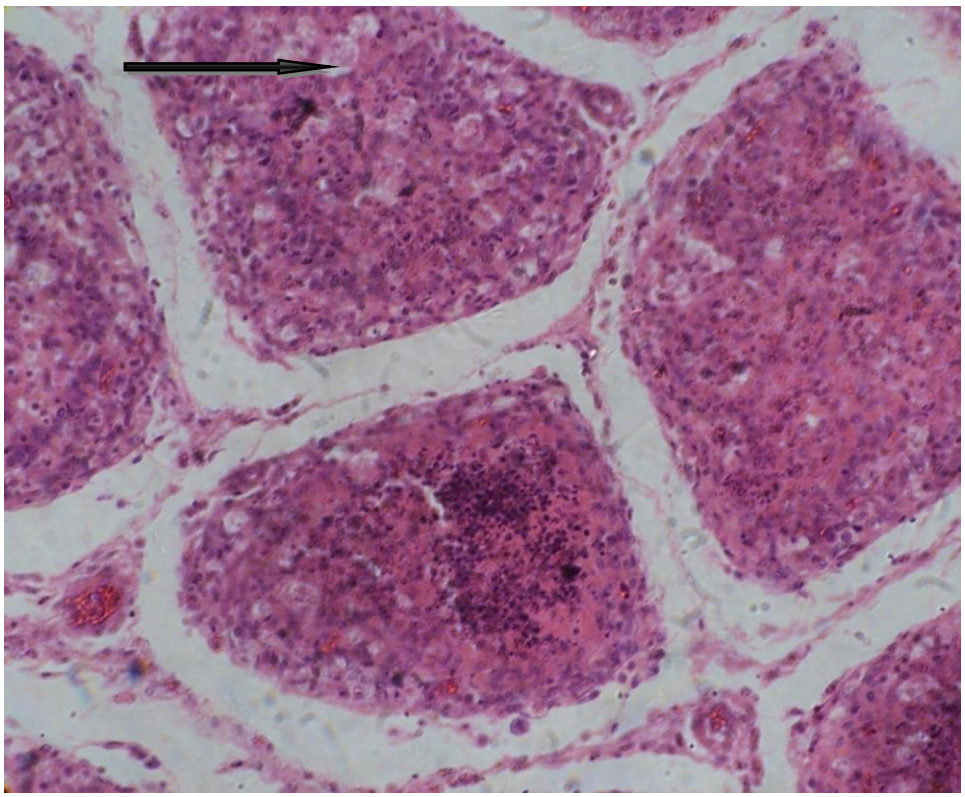

Plate XI. Bursal follicles from IBDV infected chicken, 168hpi showing severe lymphoid necrosis and formation of cysts (arrow). (H and E ×200).

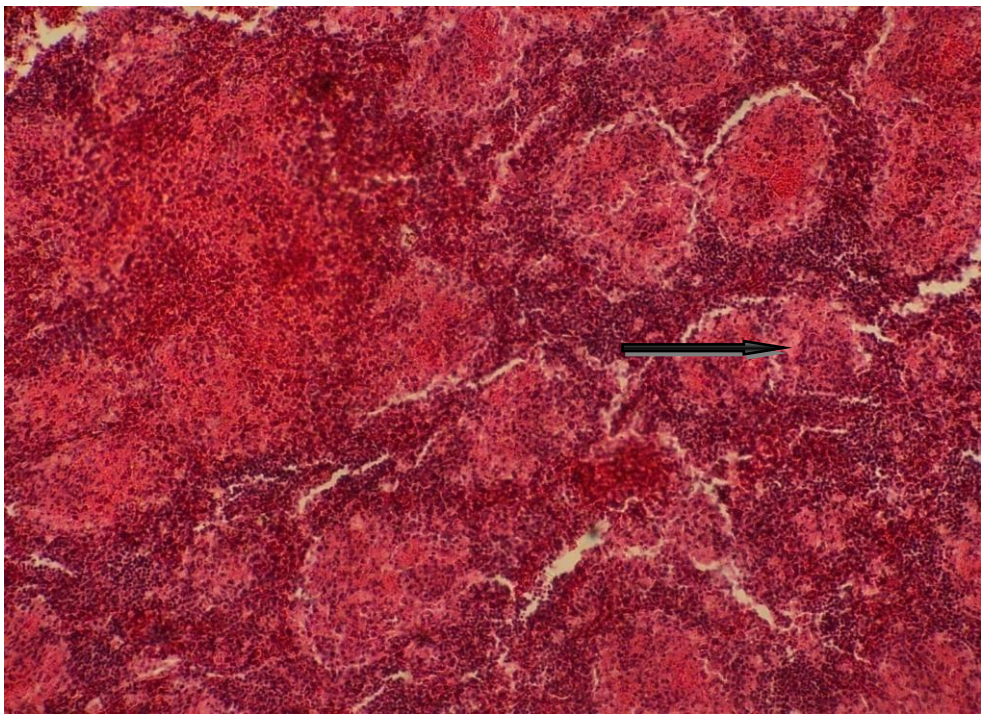

Plate XII. Mild lymphoid necrosis in the spleen (arrow) of an IBDV infected chicken 60hpi. (H and E ×200).

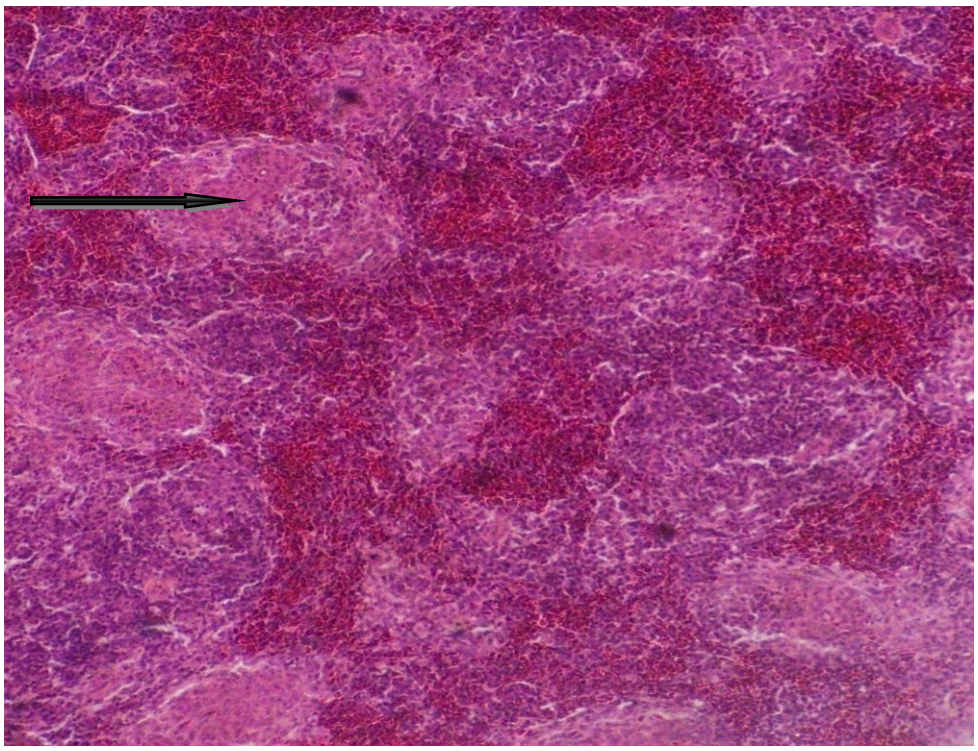

Plate XIII. Severe lymphoid necrosis in the spleen (arrow) of an IBDV infected chicken 72hpi. (H and E ×200).

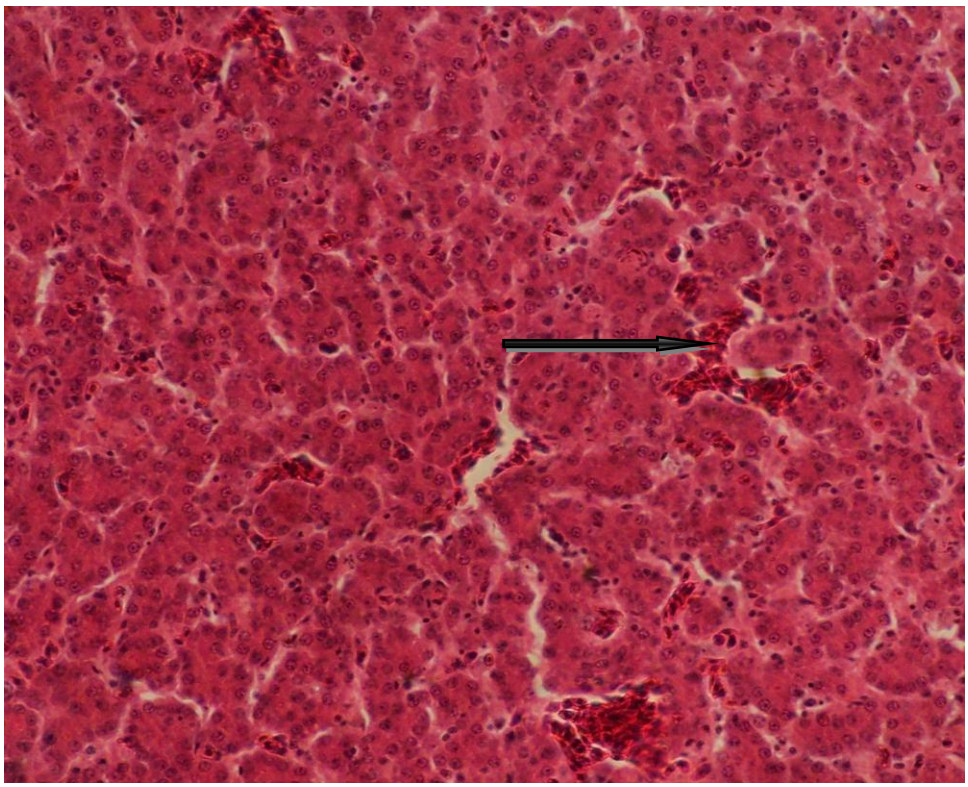

Plate XIV. Congestion in the liver (arrow) of an IBDV infected chicken. (H and E ×200).

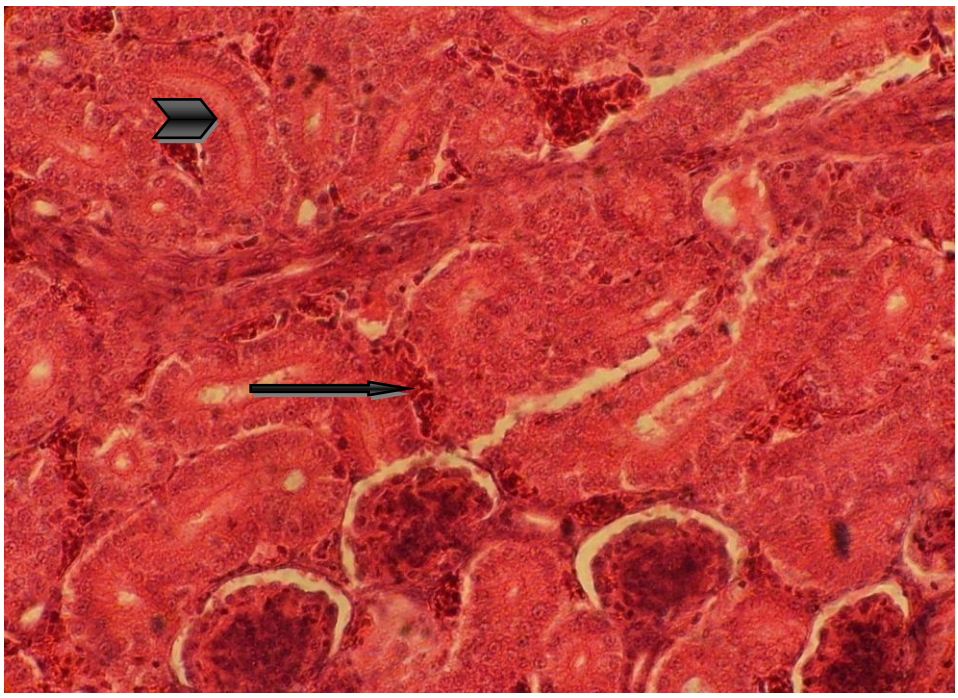

Plate XV. Congested intertubular spaces (arrow) and eosinophilic materials (arrow head) in the lumen of tubules in the kidney of an IBDV infected chicken. (H and E ×400).

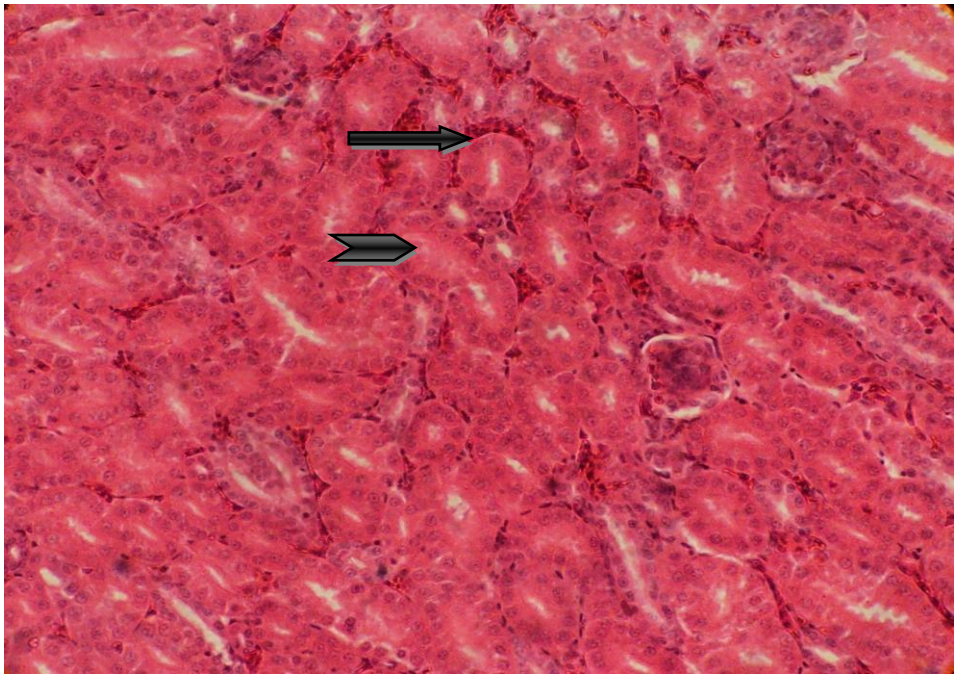

Plate XVI. Congested intertubular spaces (arrow) and eosinophilic materials (arrow head) in the lumen of tubules in the kidney of an IBDV infected chicken. (H and E ×200).

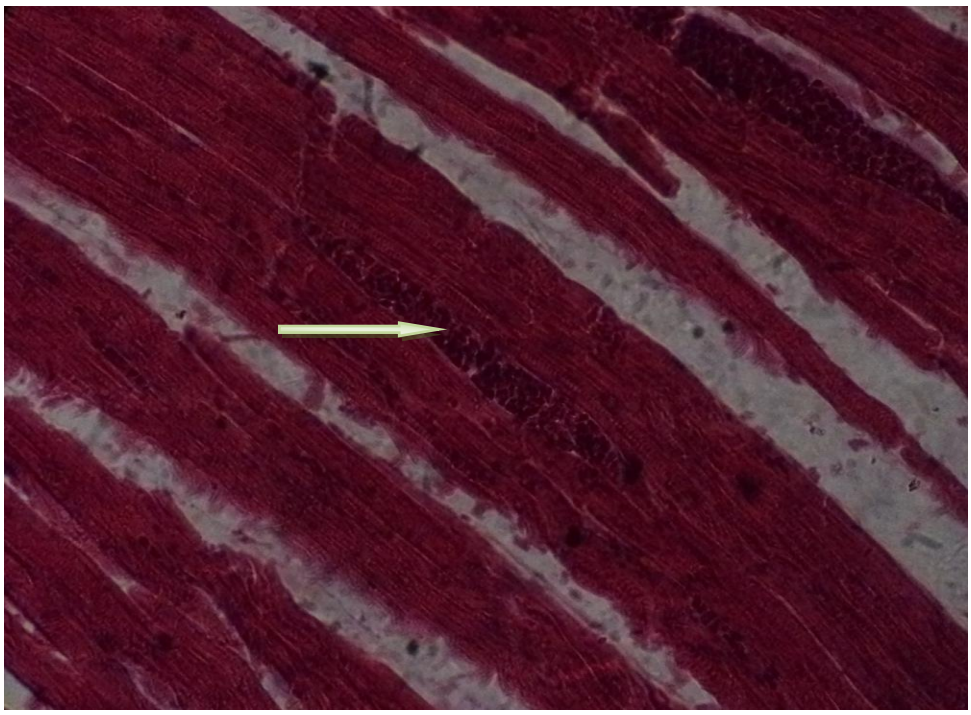

Plate XVII. Haemorrhage in thigh muscle (arrow) of an IBDV infected chicken. (H and E ×200).

# CHAPTER FIVE

# DISCUSSION

The clinical signs, gross and histopathological changes observed in the chickens infected with IBDV in this study are consistent with previous reports (Okoye and Uzoukwu,1981; Zeryehun *et al*., 2012). These findings re-affirm that IBDV strain IBDV 37/Kaduna NG/2009 successfully infected and caused IBD in the chickens. The clinical disease observed in the infected chickens in this study was however milder than the field case from which the virus was isolated. This is in line with earlier reports by Helmbolt and Garner (1964), Chineme and Cho (1984) as well as Oladele *et al*. (2010) that the clinical and pathological changes in experimental IBDV infection of chickens were with rare exceptions milder than in spontaneous disease.

This study demonstrated a significant reduction in thrombocyte concentration starting 72 hpi which is responsible, at least in part, for the haemorrhage observed in the IBDV infected group of chickens noting that thrombocytopaenia at this point coincided with the onset of haemorrhage and prolonged APTT. This could be as a result of consumption coagulopathy steming from localised damage to blood vessels (Dos Santos *et al*., 2004). Zeryehun *et al*. (2012) made a similar finding but Lima *et al*. (2005) obtained a contrasting result in which thrombocytopaenia was absent. The discordant findings could be as a result of differences in the severity of damage to endothelial lining of blood vessels and thus the degree of haemorrhage. Another possibility is that of the direct effect of IBDV on thrombocyte resulting in its depletion. This is based on findings that thrombocyte represent a target for IBDV (Skelees *et al*., 1980; van den Berg, 2000).

Packed cell volume (PCV) is an important haematologic parameter because it provides an easy and objective way of estimating the erythrocyte mass in a sample. The finding in this study that the mean PCV in the infected chickens remained slightly below the control values is consistent with the findings of Chineme and Cho (1984) and Lima *et al*. (2005). Dehydration resulting from diarrhoea and anoraexia, which characterised the disease, is believed to cause haemoconcentration and subsequent masking of the degree of fall in PCV. It is noteworthy that the significant haemorrhages observed in the bursa, keel, and thigh muscles and at the proventricular-gizzard junction might have caused decrease in the PCV which in the face of dehydration was masked. The fact that there was no significant difference in the value of haemoglobin throughout the period of observation between the IBDV infected and control groups also ruled out the possibility of anaemia in the IBDV infected chickens in the face of dehydration. This finding is not unique to this study as previous researches had reported similar observations (Chineme and Cho, 1984; Oladele *et al*., 2005 and Lima *et al*., 2005).

Leukopaenia observed beginning at 36 hpi is the consequence of corresponding lymphopaenia. This result is in agreement with the findings of Cheville (1967) who reported severe panleukopaenia during the severe inflammatory stage of IBD and also showed that blood smears after this period are less severely affected and by day 9 after inoculation, the white blood cells type appeared to be normal.

The lymphopaenia observed from 36 hpi to 72 hpi is probably due to the multiplication of IBDV and subsequent necrosis of bursal lymphoid cells as suggested by Ley *et al*. (1983). It has been shown that the target cell for IBDV is the non-immunoglobulin bearing B-lymphoblast or IgM bearing B-lymphocyte (Mac Lachlan and Dubovi, 2011). These cells

are destroyed before they migrate into systemic circulation. The steady increase in heterophil count beginning at 48 hpi may be as a result of the ongoing inflammatory response to the necrotizing effects of the virus on the lymphocytes and other tissues in the body as seen grossly and histologically. Empirical evidences have shown that, once inflammation is established, an orchestra of chemical mediators modulates many events. Cytokines released from local mononuclear cells make their way to the bone marrow, where they increase the rate of release of maturing neutrophils and the rate of production by increasing stem-cell entry, proliferative events, and maturation events. The net result is that the marrow response dramatically increases the delivery rate of neutrophils to blood. In summary, a complete cycle of consumption, production and release is activated, with the goal of delivering a supply of neutrophils to the inflammatory lesion until it resolves. Similar mechanisms probably exist in birds but have not received detailed study (Latimer and Bienzle, 2010).

The values obtained in the uninnoculated control chickens for total leukocyte count were within the normal reference interval (NRI) reported by Wakenell (2010), i.e. 12-30$\times 10^9$/L but the lymphocyte and heterophils were slightly above and below this NRI, respectively. This difference may be as a result of difference in breed, age, diet, climate or environment of chickens. The lymphocyte-heterophil ratio however demonstrated the already established findings that lymphocyte are the dominant leukocytes in the blood of chickens (Latimer and Bienzle, 2010).

The observed delayed coagulation time or a prolonged activated partial thromboplastin time are further evidences of consumption coagulopathy that had occurred. This finding is consistent with that of Chineme and Cho (1984) who also reported delayed coagulation

time coinciding with hypocalcaemia in IBDV infected chickens which led to their conclusion that the deficiency of calcium, an important factor in blood clotting was responsible. Zeryehun *et al*. (2012) observed delayed whole blood recalcification time, prothrombine time and activated partial thromboplastin time at day 3 and 4 after infection with IBDV and attributed it to a coagulopathy affecting the surface induced (intrinsic), tissue factor (extrinsic) and common clotting pathway. Kosters *et al.* (1972) and Skeeles *et al.* (1979; 1980) in separate researches found increased clotting time in IBDV infected chickens and suggested that such coagulopathies would contribute to haemorrhagic lesions observed in this disease.

The actual cause of haemorrhage in the bursa and other non-lymphiod organs in this study has not been determined. Skeeles *et al*. (1979) postulated that haemorrhagic lesions were as a result of formation of immune complexes. They proposed that histologic lesions in the cloacal bursa are a type of localised immunologic injury caused by antigen-antibody-complement complexes that induced chemotactic factors which caused haemorrhage and leukocyte infiltration. This explanation remained doubtful as infection with another IBDV isolate in a different experiment failed to substantiate the involvement of complement (Skeeles *et al*., 1980). In our study, histologic evidences of damage to blood vessels were observed as erythrocyte extravasation was noticed in the skeletal muscles. This phenomenon could be explained by the findings of Dos-Santos *et al*. (2004) which suggested that the supposed immune mediated damage to the blood vessels in IBDV infection was mainly due to the effect of macrophage derived tumour necrosis factor (TNF) and interleukins.

Haemorrhage and congestion are major gross lesions observed during necropsy in birds infected with IBDV. Though the frequency of haemorrhage has been found to be higher in the bursa of Fabricius, variable degrees of congestion and haemorrhage were observed in the keel and thigh muscles and at the proventricular-gizzard mucosal junction 84 hpi. Petechial and ecchymotic haemorrhage (Lukert and Saif, 2003; Zeryehun *et al.*, 2012) in the bursa and mucosa of proventricular-gizzard junction and even paintbrush haemorrhage (Okoye, 2005) in the muscles of the breast, thigh and legs has been reported. The frequency of haemorrhage in the bursa, skeletal muscles and the proventricular-gizzard mucosa was found to be higher in the dead birds than the sacrificed ones. This finding highly suggests that haemorrhage is a contributing factor for mortality in IBD.

The enlargement of the bursa observed early in the infected chickens is believed to be due to inflammatory response to tissue damage by IBDV in the bursa of Fabricius of such birds. The oedema and hyperaemia observed grossly and the widening of the interfollicular space resulting from oedema and massive infiltration by heterophils were supportive of this argument. These findings are consistent with earlier reports on the pathogenesis of IBD elsewhere (Cheville, 1967; Ley *et al*., 1983; Chineme and Cho, 1984 and Oladele *et al*., 2010).

Histopathologic examination of lymphoid and non-lymphoid tissues from the chickens infected with IBDV showed that the predominant lesions were in the bursa of Fabricius. This suggests that the target cells for IBDV are concentrated in the bursa of Fabricius and seem to affirm earlier speculations by Ley *et al*. (1983) that the unique sensitivity of bursal lymphoid cells to IBDV suggests the possibility of specific viral receptors on a B-cell subpopulation that is most abundant in the bursa but may also occur in other lymphoid

organs. Recent findings (Mac Lachlan and Dubovi, 2011) had shown that only non-immunoglobulin-bearing B lymphoblast or IgM-bearing B lymphocyte support viral replication whereas stem cells and peripheral B cells do not. Mac Laclan and Dubovi (2011) had suggested that the cloacal bursa plays a key role in the pathogenesis of IBD. Earlier researches had shown that bursectomised birds survived an otherwise lethal infection without developing clinical signs or disease (Mahgoub, 2012). Cloacal bursa may also be responsible for the age-related susceptibility of chicken to the virus as clinical disease is common in birds at an age when it is most viable (Muller *et al*., 2003). The selective viral tropism for only B - lymphocytes at certain stage of differentiation may also account for the age-dependent clinical disease in chickens.

Non-bursal lymphoid organs examined in this study were the spleen and thymus. The spleen showed a mild degree of lymphoid cell necrosis and depletion, which could be attributable to IBDV's low affinity for these organs. This is because preliminary cell-culture studies (Becht, 1980) with pure populations of bursal lymphoid cells yielded IBDV titres as high as $10^8$ PFU/ml whereas spleen, thymus or circulating lymphoid cell culture yielded virus titre just above background levels. However, the absence of significant lymphoid necrosis in thymus in this study did not necessarily preclude viral presence in the organ; this may be due to a probable reduction in the virulence of the virus. To further buttress the findings in this study, IBDV was earlier demonstrated in the thymus using fluorescent antibody technique even though there was no evidence that the virus actually replicates in the thymic cells (Ley *et al*. 1983; Sharma *et al*., 2000).

The observed lymphocyte depletion could be the result of phagocytosis of the necrotic lymphocytes by macrophages in the follicles. Also the formation of cysts in the bursa by

120 hpi following the necrosis and clearance by phagocytic cells and their eventual contraction could have contributed to the bursal atrophy. Another plausible explanation for the bursal atrophy at the later part of the disease was fibroplasia that occurred in the interfollicular connective tissue which possibly caused the collapse of the cystic cavities particularly in the medullary areas. Bursal atrophy has been reported in most clinical cases of IBD beginning at about 5 days after infection (Cheville, 1967, Okoye and Uzoukwu, 1984; Mahgoub, 2012).

The clinical implication of the depletion of bursal B cells and the subsequent bursal atrophy at the terminal stage of IBD as observed in this study is that the affected chickens would have a diminished antibody response and increased susceptibility to secondary infection in recovered cases. The resulting immunosuppression leads to diminished antibody production following vaccination against other viral infections with subsequent outbreaks of diseases. Paradoxically, birds that recovered from the disease had been shown to have high anti-IBDV antibody titres (Mahgoub, 2012). This is because, fully differentiated antigen specific B-lymphocytes are unaffected during the course of the disease and are stimulated to release anti-IBDV antibodies.

The development of hyperplastic thickened and folded bursal epithelia is of interest and is typical of previous reports (Cheville, 1967; Chineme and Cho, 1984) but cannot be explained immediately. Cheville (1967) suggested that the bursal epithelial layers, which differentiate to give origin to bursal lymphoid tissue during embryonic development (Ackerman and Knoff, 1964) is so altered in the course of viral infection that it reverted to the type of endodermic epithelium from which it originated. Later, Chineme and Cho, (1984) described the epithelial hyperplasia as a mucin-secreting glandular structure.

Oluwayelu *et al*. (2002) in their study on the pathology of infectious bursal disease in 11-week-old crossbreeds of Harco cocks and indigenous Nigerian hens reported degeneration in the epithelial cells of the renal tubules and ducts, but no evidence of such changes were observed in the present study. However, congested intertubular spaces were observed. The discrepancies in these findings may be attributed to difference in the breed and age of the birds and strain variation in viral innoculum which have bearing on the pathogenesis of the disease. Findings similar to ours were previously reported by Helmboldt and Garner (1964) and Ley *et al*. (1983). The intratubular oesinophilic material observed in this study was believed to be urate cast just as it was earlier suggested (Ley *et al*., 1983). The presence of urate in the tubular lumen of nephron in IBDV infected chicken is indeed a consistent finding in most reports. The intertubular congestion observed in this study could be the result of the urate stasis, which distended the wall of the tubule. The accumulation of this material could be a result of decrease water intake, diarrhoea and resulting dehydration with consequent decrease in renal blood perfusion. The slight congestion observed around sinusoidal space in the liver of infected chickens was a non-specific change that could have resulted from a variety of insults. Similar finding had been reported by Peters (1967) and Ley *et al*. (1983).

# CONCLUSION AND RECOMMENDATIONS

## Conclusion

Thrombocyte count reduced significantly $7.86 \pm 0.79 \times 10^3/\mu l$ at 6 hpi to $2.55 \pm 0.14 \times 10^3/\mu l$ at 84 hpi ($p < 0.05$)

The activated partial thromboplastin time was significantly prolonged from 60 hpi, reaching its plateau at 96 hpi before it dropped at 168 hpi.

The prolonged activated partial thromboplastin time coupled with thrombocytopaenia is important factors associated with haemorrhage in IBD.

There is positive correlation between the degree of haemorrhage and mortality. This makes haemorrhage a factor associated with mortality in IBD.

This study also showed that vvIBDV Nigerian field isolates caused severe depletion of bursal lymphoid cells and later bursal atrophy.

## Recommendations

1. Further work should be done to examine the involvement of tissue factor pathway in the clotting defect observed in IBD caused by Nigerian field isolates.
2. Further studies should be done to determine the actual cause of thrombocytopaenia in IBD.
3. The effects of haematinics should be tried in birds with Gumboro disease.
4. Further work should be done to evaluate the effects of IBDV on other haematological paerameters not studied in this research

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## APPENDIX I

Table 1: Shows the packed cell volume (%) values of the IBDV infected chickens and the pre-innoculation values throughout the experiment (Mean ± SEM)

| Sampling time (hours) | Control | IBDV Infected |
|---|---|---|
| 6 | 30.00 ± 1.00 | 31.50 ± 0.50 |
| 12 | 29.50 ± 0.50 | 28.00 ± 1.08 |
| 24 | 28.50 ± 0.50 | 28.25 ± 1.49 |
| 36 | 27.50 ± 1.50 | 25.25 ± 0.48 |
| 48 | 30.50 ± 2.50 | 28.00 ± 0.41 |
| 60 | 29.00 ± 0.00 | 26.00 ± 1.29 |
| 72 | 27.00 ± 3.00 | 28.25 ± 1.25 |
| 84 | 30.00 ± 0.00 | 26.25 ± 2.39 |
| 96 | 29.00 ± 0.00 | 26.00 ± 1.00 |
| 120 | 28.00 ± 1.00 | 25.25 ± 1.65 |
| 168 | 29.00 ± 0.00 | 24.67 ± 1.33 |

## APPENDIX II

Table 2: Haemoglobin concentration (g/dL) values of the IDBV infected chickens and uninfected control group (Mean ± SEM).

| Sampling time (hours) | Control | IBDV Infected |
|---|---|---|
| 6 | 16.9 ± 1.00 | 16.8 ± 1.75 |
| 12 | 16.9 ± 0.05 | 16.9 ± 0.48 |
| 24 | 16.7 ± 1.50 | 16.9 ± 0.47 |
| 36 | 16.4 ± 1.00 | 15.8 ± 5.70 |
| 48 | 16.9 ± 1.50 | 16.4 ± 6.34 |
| 60 | 16.8 ± 0.50 | 16.1 ± 1.00 |
| 72 | 16.5 ± 1.00 | 16.8 ± 0.75 |
| 84 | 16.9 ± 0.00 | 16.7 ± 0.56 |
| 96 | 16.8 ± 0.00 | 16.1 ± 0.33 |
| 120 | 16.6 ± 0.50 | 15.6 ± 2.70 |
| 168 | 16.9 ± 1.00 | 15.3 ± 1.50 |

## APPENDIX III

Table 3: Total protein (g/dL) of the IBDV infected group and control (Mean ± SEM)

| Sampling time (hours) | IBDV infected | Control |
|---|---|---|
| 6 | 2.40 ± 0.20 | 2.95 ± 0.21 |
| 12 | 2.50 ± 0.30 | 2.80 ± 0.14 |
| 24 | 2.35 ± 0.25 | 2.50 ± 0.24 |
| 36 | 2.70 ± 0.10 | 2.50 ± 0.53 |
| 48 | 2.60 ± 0.04 | 2.65 ± 0.21 |
| 60 | 2.50 ± 0.30 | 2.65 ± 0.23 |
| 72 | 2.80 ± 0.20 | 2.85 ± 0.15 |
| 84 | 2.80 ± 0.20 | 2.90 ± 0.06 |
| 96 | 2.60 ± 0.60 | 3.20 ± 0.30 |
| 120 | 2.90 ± 0.10 | 3.05 ± 0.36 |
| 168 | 2.70 ± 0.20 | 3.05 ± 0.14 |

## APPENDIX IV

Table4: Mean thrombocyte count ($\times 10^3 \mu l^{-1}$) of the IBDV infected group and pre-innoculation (Mean ± SEM)

| Sampling time (hours) | Control | IBD infected |
|---|---|---|
| 6 | 7.50 ± 1.50 | 7.86 ± 0.79 |
| 12 | 8.50 ± 0.00 | 10.00 ± 0.69 |
| 24 | 8.25 ± 0.85 | 9.80 ± 2.25 |
| 36 | 10.45 ± 0.65 | 14.53 ± 3.83 |
| 48 | 9.75 ± 1.65 | 5.88 ± 0.70 |
| 60 | 8.10 ± 2.00 | 4.93 ± 1.00 |
| 72 | 8.85 ± 2.45 | 2.80 ± 0.30 [a] |
| 84 | 12.15 ± 3.05 | 2.55 ± 0.14 [a] |
| 96 | 8.55 ± 0.45 | 2.95 ± 0.49 [a] |
| 120 | 10.40 ± 2.30 | 3.45 ± 0.20 [a] |
| 168 | 10.50 ± 0.00 | 12.27 ± 5.37 |

[a] = statistically significant compared with the control group($p < 0.05$ )

## APPENDIX V

Table 5: Total white blood cell count ($\times 10^9$ /L) of the IBD and un-inoculated control group (Mean ± SEM)

| Sampling time (hours) | IBDV Infected | Control |
|---|---|---|
| 6 | 17.8 ± 1.8 | 17.9 ± 1.6 |
| 12 | 22.2 ± 1.1 | 21.2 ± 2.4 |
| 24 | 19.8 ± 2.3 | 20.9 ± 2.1 |
| 36 | 14.7 ± 2.1 | 24.6 ± 2.4 |
| 48 | 11.9 ± 3.1 | 17.8 ± 3.1 |
| 60 | 13.2 ± 2.9 | 19.2 ± 2.5 |
| 72 | 18.4 ± 2.7 | 25.7 ± 2.3 |
| 84 | 20.1 ± 1.4 | 22.2 ± 1.8 |
| 96 | 21.7 ± 2.1 | 25.3 ± 1.3 |
| 120 | 22.2 ± 1.4 | 23.2 ± 2.1 |
| 168 | 25.4 ± 1.2 | 24.7 ± 1.8 |

## APPENDIX VI

Table 6: Lymphocyte count ($\times 10^9$/L) of the IBDV infected and un-inoculated control groups (Mean ± SEM)

| Sampling time (hours) | IBDV Infected | Control |
|---|---|---|
| 6 | 16.0 ± 2.1 | 16.3 ± 3.2 |
| 12 | 20.4 ± 1.8 | 19.6 ± 1.1 |
| 24 | 17.8 ± 0.1 | 18.4 ± 2.3 |
| 36 | 11.9 ± 0.8 [a] | 22.1 ± 0.9 |
| 48 | 7.1 ± 1.2[a] | 16.7 ± 1.2 |
| 60 | 7.9 ± 1.4[a] | 17.5 ± 1.4 |
| 72 | 12.9 ± 0.7 [a] | 19.9 ± 1.0 |
| 84 | 15.6 ± 1.9 | 18.7 ± 1.8 |
| 96 | 18.0 ± 0.2 | 22.2 ± 1.6 |
| 120 | 20.4 ± 2.1 | 20.9 ± 1.5 |
| 168 | 22.4 ± 1.8 | 21.6 ± 1.6 |

[a =] statistically significant compared with the control group($p < 0.05$ )

## APPENDIX VII

Table 7: Heterophil count ($\times 10^9$/L) of the IBDV infected and un-inoculated control group (Mean ± SEM)

| Sampling time (hours) | IBDV Infected | Control |
|---|---|---|
| 6 | 1.7 ± 0.0 | 1.7 ± 0.1 |
| 12 | 1.6 ± 0.1 | 1.8 ± 0.1 |
| 24 | 1.7 ± 0.0 | 1.6 ± 0.2 |
| 36 | 1.7 ± 0.5 | 1.7 ± 0.0 |
| 48 | 1.8 ± 0.2 | 1.6 ± 0.2 |
| 60 | 1.9 ± 0.0 | 1.7 ± 0.2 |
| 72 | 2.2 ± 0.6 | 1.8 ± 0.1 |
| 84 | 2.1 ± 0.1 | 1.7 ± 0.0 |
| 96 | 1.9 ± 0.1 | 1.6 ± 0.0 |
| 120 | 1.8 ± 0.6 | 1.7 ± 0.3 |
| 168 | 1.8 ± 0.2 | 1.7 ± 0.1 |

## APPENDIX VIII

Table 8: Activated partial thromboplastin time (APTT) (second) of the IBDV infected chicken and control groups (Mean ± SEM)

| Sampling time (hours) | IBDV infected | Control |
|---|---|---|
| 6 | 119.4 ± 1.5 | 122.5 ± 0.5 |
| 12 | 114.3 ± 5.7 | 119.5 ± 1.5 |
| 24 | 128.5 ± 4.0 | 123.0 ± 1.0 |
| 36 | 105.3 ± 16.2 | 121.0 ± 3.0 |
| 48 | 117.8 ± 5.3 | 96.8 ± 1.6 |
| 60 | 126.5 ± 2.2 [a] | 91.5 ± 0.5 |
| 72 | 129.7 ± 3.8 [a] | 98.0 ± 1.0 |
| 84 | 145.5 ± 5.6 [a] | 100.5 ± 1.5 |
| 96 | 150.3 ± 2.2 [a] | 114.0 ± 1.3 |
| 120 | 138.5 ± 3.1 [a] | 113.0 ± 1.0 |
| 168 | 112.5 ± 6.7 | 98.0 ± 1.0 |

[a=] statistically significant compared with the control group($p < 0.05$ )

## APPENDIX IX

Table 9: Percentage frequency of haemorrhage in the bursa of Fabricius, skeletal muscle and proventricular- gizzard mucosal junction in the IBDV inoculated chickens.

| Sampling time (hours) | Bursa (%) (a/b) | Muscles (%) (a/b) | proventricular-gizzard junction (%) (a/b) |
|---|---|---|---|
| 6 | 0(0/4 ) | 0(0/4) | 0(0/4) |
| 12 | 0(0/4) | 0(0/4) | 0(0/4) |
| 24 | 0(0/4) | 0(0/4) | 0(0/4) |
| 36 | 0(0/4) | 0(0/4) | 0(0/4) |
| 48 | 0(0/4) | 0(0/4) | 0(0/4) |
| 60 | 0(0/4) | 0(0/4) | 0(0/4) |
| 72 | 50(2/4) | 0(0/4) | 0(0/4) |
| 84 | 100(5/5) | 80(4/5) | 60(3/5) |
| 96 | 100(8/8) | 75(6/8) | 87.5(7/8) |
| 120 | 60(3/5) | 40(2/5) | 0(0/5) |
| 168 | 0(0/4) | 0(0/4) | 0(0/4) |

a = Number of chickens with lesion

b = Number of chicken examined

## APPENDIX X

Table 10: Percentage frequency of hemorrhage in organs of sacrificed chickens compared to dead ones.

| Sampling time (hours) | Chickens | Bursae (%) (a/b) | Muscles (%) (a/b) | Mucosal Junction(%) (a/b) |
|---|---|---|---|---|
| 84 | Sacrificed | 100($4^a$/$4^b$) | 75(3/4) | 50(2/4) |
| | Dead | 100(1/1) | 10(1/1) | 100(1/1) |
| 96 | Sacrificed | 100(4/4) | 50(2/4) | 75(3/4) |
| | Dead | 100(4/4) | 100(4/4) | 100(4/4) |
| 120 | Sacrificed | 50(2/4) | 25(1/4) | 0(0/4) |
| | Dead | 100(1/1) | 100(1/1) | 0(0/1) |

a = Number of birds examined

b= Number of birds with lesion

## APPENDIX XI

Table 11: Percentage morbidity and mortality observed in the IBDV infected Chickens post inoculation

| Sampling time (hours) | Depression (%) (a/b) | Ruffled feather (%) (a/b) | Diarrhoea (%) (a/b) | Mortality (%) (a/b) |
|---|---|---|---|---|
| 6 | 0(0 /50) | 0(0/50) | 0(0/50) | 0(0/50) |
| 12 | 0(0/46) | 0(0/46) | 0(0/46) | 0(0/46) |
| 24 | 0(0/42) | 0(0/42) | 0(0/42) | 0(0/42) |
| 36 | 0(0/38) | 0(0/38) | 0(0/38) | 0(0/38) |
| 48 | 2.9(1/34) | 2.9(1/34) | 0(0/34) | 0(0/34) |
| 60 | 10(3/30) | 10(3/30) | 0(0/30) | 0(0/30) |
| 72 | 69(18/26) | 69(18/26) | 54(14/26) | 0(0/26) |
| 84 | 91(20/22) | 91(20/22) | 91(20/22) | 4.5(1/22) |
| 96 | 100(17/17) | 100(17/17) | 100(17/17) | 23.5(4/17) |
| 120 | 11(1/9) | 11(1/9) | 0(0/9) | 11(1/9) |
| 168 | 0(0/4) | 0(0/4) | 0(0/4) | 0(0/4) |

a= no of chickens that showed signs

b= total no. of chicken present