PATHOLOGICAL AND SEROLOGICAL EVALUATION OF SOME INFECTIOUS BURSAL DISEASE VACCINES AND NIGERIAN FIELD VIRUS STRAIN ON SOME IMMUNE ORGANS OF COCKERELS

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MAY, 2015

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DVM (UDUS), 2007
M. Sc./VET.MED./27111/2012-2013

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN VETERINARY PATHOLOGY

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MAY, 2015

DECLARATION

I declare that the work in this thesis entitled **''Pathological and serological evaluation of some infectious bursal disease vaccines and Nigerian field virus strain on some immune organs of cockerels''** has been carried out by me in the Department of Veterinary Pathology. 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.

Abdullahi Abdullahi RAJI

_____ _____ _____

Signature Date

CERTIFICATION

This Thesis entitled **''PATHOLOGICAL AND SEROLOGICAL EVALUATION OF SOME INFECTIOUS BURSAL DISEASE VACCINES AND NIGERIANFIELD VIRUS STRAIN ON SOME IMMUNE ORGANS OF COCKERELS''**by Abdullahi Abdullahi RAJI 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 scientific knowledge and literary presentation.

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ACKNOWLEDGMENTS

All praise and glory belongs to Allah alone. The creator of the universe, the preserver and maintainer of all its affairs, the administrator of all its workings, the will behind its phenomena and disposer of all activities to their successful completion.

To my great wonderful parents, may Allah reward them abundantly for their prayers, love, parental care, support and moral upbringing throughout my life.

I am deeply thankful to my supervisors, Dr. Bisallah Mohammed, Professor S. B. Oladele and Professor L. Saidu.I would like to find a better way to express my gratitude but, I could not find it, may the Lord Almighty reward you.

I sincerely and deeply appreciate Dr. Jibril Adamu for his assistance, guide, constructive criticism and provision of the stock of vaccine virus candidate used in this research.

I wish to thank Prof. C. N. O. Njoku for his mentoring and encouragement for the pursuance of Veterinary Pathology.

The efforts of Drs. A.M. Talba, H.A. Bako, Babashani Muhammad, Lateefah Jumoke Alabi, M.S. Yahya, A. Aliyu, M. B. Bello and Mikail Audu, are highly acknowledged. Moreover, I do appreciate the efforts of members of staff of the Department of Veterinary Pathology, Veterinary Histopathology Laboratory and Veterinary Public Health and Preventive Medicine Laboratoryfor their tireless support and cooperation in the entire period of the laboratory aspect of this work.

I also acknowledge the patience of my beloved wife (Zainab) and children (Haleemah, AbdulHaleem and Maimunah) especially during my long stays during the period of this study.

I sincerely appreciate the assistance of Hybrid feed Ltd., for the supply of chick feed used in this research.

ABSTRACT

Infectious bursal disease (IBD) is an economically important disease to the poultry industry, with adverse effects on the immune system of the birds. The efficacy of most of the vaccines used in the control of IBD and pathology associated with the use of these vaccines in Nigeria is not clearly understood. Thisstudy was designed to compare pathological and serological changes in cockerels vaccinated with different IBD vaccines and those infected with the field infectious bursal disease virus (IBDV) strain. Serology, gross pathology and histopathologic changes caused by 3 intermediate vaccines MB®, Bursine 2®,Infectious Bursal Disease Vaccine® and Cevac® IBD L (Intermediate Plus strain), were evaluated and compared with those of field IBDV strain and control. Serum antibody titres, gross and histopathologic changes were monitored at 1, 2, 3, 4 and 5Days postvaccination/challenge (Dpv/c) and on the 7^{th}, 10^{th}, 14^{th} and 21^{st} days post vaccination/challenge (dpv/c). The titres of maternally derived antibody (MDA) are protective against IBD for a period ranging from 27 to 40 days post hatch was determined based on calculation of optimal vaccination time (OVT). ELISA test follow-up post vaccination/challenge (pv/c), gave a detectable antibody titre (3.492±0.72 to 4.480±0.03) indicating protection or MDA against IBDV in the vaccinated and control groups respectively, and infection with IBDV in the challenge group.At 3 Dpv/c, petechial and ecchymotic haemorrhages in thigh, keel muscles, and spleen were observed in all the groups with degree of variation except the control. Cevac® IBD L and field virus had a significant difference ($p<0.05$) in spleen relative weight (SRW) and spleen body weight ratio (S:BW). Bursa body index (BB Index) presented 0.3-0.7 index (relative transient atrophy) and a strong atrophy (< 0.3) with the field IBDV strain on 7Dpv/c to

14Dpv/c. Histopathologic lesion scores recorded from bursa, spleen and thymus, showed that birds vaccinated or challenge withMB®, Cevac® IBDL and challenge had similar degree of injury, whereas Bursine 2® and Infectious Bursal Disease Vaccine®were slightly different, but not statistically significant ($p>0.05$). In general, bursa, spleen and thymus lesions scores were statistically significant ($P<0.05$). These findings suggest that intermediate vaccines are capable of causing bursal injuries. Bursallesions and rise in antibody titre against IBDV after vaccination/challenge were similar, and may be confused with field virus-induced lesions.The intermediate plus strain or an intermediate with moderate to high residual pathogenicity should be used in vaccinating birds against IBD in highly IBD endemic areas.

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ABBREVIATIONS, DEFINITIONS, GLOSSARY AND SYMBOLS

AGP	Agar Gel Precipitation
B:BW ratio	Bursa Body Weight Ratio = bursa weight (mg) / body weight (g)
BB index	BB Index = B:BW ratio of the tested chicken / mean B:BW ratio of the controls.
BF	Bursa of Fabricius
CAM	Chorio-Allantoic Membrane
CE	Chicken Embryo
CEB	Chicken Embryo Bursa
CEF	Chicken Embryo Fibroblast
CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo Kidney
CIBDV	Classical Infectious Bursal Disease Virus
DPC or dpc	Days Post Challenge
Dpv/c	Days post vaccination or challenge
Deventer Formula	Vaccination age = {(log2 IBDV antibody ELISA titre of the bird (%)-log2 breakthrough titre of the vaccine) x t ½} + age at sampling + correcting value 0-4 In which

	Bird (%): titre of the bird (at sampling) that represents a certain percentage of the flock (in this study: 75 %) that is desired to be susceptible to the vaccine at the time of the application
	Breakthrough: breakthrough (ELISA) titre of the vaccine to be used (for this vaccine in the ELISA system used, a titre of 125)
	$t_{1/2}$: half life time of the antibodies (ELISA titre) in the type of chickens that were sampled (Layers: 5.5days)
	Age at sampling: age of the birds at sampling
	Correcting value 0-4: extra days when the sampling was done at 0-4 days post hatch.
ELISA	Enzyme Linked Immunosorbent Assay
FRET	Flourescence Resonance Energy Transfer
Hpv/c	Hours post vaccination or challenge
IBD	Infectious Bursal Disease (also named Gumboro Disease)
IBDV	Infectious Bursal Disease Virus
MDA	Maternally Derived Antibodies
OIE	Office des Internationale Epizootics (World Organisation for Animal Health)
ORF	Open Reading Fragment
RFLP	Restriction Fragment Length Polymorphimism
rHVT	recombinant vaccine based on the Herpes Virus of Turkey.
rHVT-F	rHVT vector vaccine containing an inserted gene encoding for the “F” (Fusion) protein of NDV (Newcastle Disease Virus),

	designed to induce protection against the Newcastle Disease (ND).
rHVT-VP2	rHVT vector vaccine containing an inserted gene encoding for the “VP2” (Viral Protein 2) of IBDV, designed to induce protection against Gumboro Disease.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
SSIU	Scientific Support and Investigation Unit
vIBDV	virulent Infectious Bursal Disease Virus
VN	Virus Neutralisation
VP2	Viral Protein 2
vvIBDV	very virulent Infectious Bursal Disease Virus

CHAPTER ONE

INTRODUCTION

1.1Infectious Bursal Disease

Infectious bursal disease (IBD) is a highly contagious, immunosuppressive infection of immature chickens (Faragher, 1972; Abdu *et al.*, 1986; McFerran, 1993) caused by a birnavirus (Lukert and Saif, 1991; Lukert and Saif, 2003). The syndrome, which emerged in 1957 (Cover, 1960) was formally documented by Cosgrove (1962) in broiler flocks located near the town of Gumboro, Delaware USA, hence the common eponym 'Gumboro disease'. Originally the condition was referred to as 'nephritis-nephrosis syndrome of chickens' because of prominent kidney lesions, but lesions in the bursa of Fabricius were also recognized by pathologists (Lasher and Shane, 1994). Rapid spread of the infection occurred, and most broiler growing areas in the United States were affected by 1960. Infectious bursal disease, as it became known, was diagnosed in the UK in 1962 (Edwards, 1981). By the mid 1970s IBD was present in most countries with established poultry industries including Australia (Firth, 1974). New Zealand was free of the disease during the mid 1980s (Jones, 1986), but IBD was diagnosed in late 1993 and became endemic within months of the initial outbreak in Waikato (Lasher and Shane, 1994).

Despite confusion regarding the identity of the causal agent, which was originally isolated by Winterfield *et al.* (1962), Koch's postulates were satisfied by Snedeker *et al.* (1967), who isolated a virus from the bursa of Fabricius of affected birds and subsequently produced the disease. This agent was distinct from the nephropathogenic coronavirus (Gray strain of infectious bronchitis virus) isolated from Delmarva broilers in 1960 (Winterfield and Hitchner, 1962) responsible for the nephritis-

nephrosis syndrome. After initial classification as a picornavirus (Cho and Edgar, 1969) and then a reovirus (Lukert and Davis, 1974), the IBD agent was designated a birnavirus (Dobos, 1979) based on the presence of double stranded RNA (Muller *et al.*, 1979) and unique biophysical characteristics (Dobos *et al*., 1979).

Two serotypes of infectious bursal disease virus (IBDV) have been identified: serotype 1 is pathogenic to chickens and varies markedly in virulence, whereas serotype 2, which has been isolated from both ducks and turkeys, is non-pathogenic (Rosenberger *et al.,* 1998). Pathotype serotype 1 IBDV strains can be grouped into classical virulent, antigenic variant and very virulent (vv) strain which is associated with high mortality ranging from 60-100% (Ture *et al.,* 1998; van den Berg, 2000).

The clinical features of the syndrome include whitish or watery diarrhoea, followed by anorexia, depression, trembling, severe prostration, and death. At necropsy, the birds exhibit dehydration, haemorrhages in the leg and thigh muscles, urate deposits in kidneys and enlargement of the bursa of Fabricius (Cosgrove, 1962). The bursa of Fabricius may appear oedematous, turgid and sometimes haemorrhagic and turns atrophic within 7 to 10 days post infection (dpi).

The lesser mealworm, *Alphitobius diaperinus* may be reservoir hosts of infectious bursal disease virus (Mc Allister *et al.,* 1995). Infectious bursal disease virus has also been isolated from *Aedes vexans* mosquitoes; however, the virus was not pathogenic to susceptible Leghorn pullets (Howie and Thorsen, 1981). The virus was also isolated from dog faeces and it had pathogenic effect on five specific pathogen free chicks (Pages-Mante, 2004). Okoye and Uche (1986) also detected IBDV by agar gel precipitation from different tissues of rats captured on poultry farms.

From 1987, acute IBDV cases with up to 30% and 60% mortality in broilers and pullets flocks, respectively became commonly reported in Europe and the first reports were made by Chettle *et al*. (1989) and van den Berg *et al*. (1991). Surprisingly, some of these acute outbreaks occurred in broilers where all the hygienic and prophylactic measures had been taken. These findings indicated a dramatic change in the field situation. These vv strains have now spread all over the world (Eterradossi *et al.,* 1999). In West Africa, severe acute IBD is hardly new. For ten years prior to the emergence of European vvIBDV strains, several reports had mentioned unusually high IBD-induced mortalities in local or imported chickens (Gyening and Corkish, 1977; Okoye, 1987). However, the unusual severity of these African cases was attributed as much to concurrent aggravating factors, such as climatic stress or co-infection with parasites, bacteria and other viruses (Eterradossi *et al.,* 1999).

Transmission of infectious bursal disease may be by direct contact between infected and susceptible flocks through the oro faecal route. Infected chickens shed IBDV one day after infection and can transmit the disease for at least 14 days (Vindevogel *et al.,* 1976). There are neither experimental data nor field observations to suggest that IBD can be transmitted vertically (Lukert and Saif, 1997). Indirect transmission of virus most probably occurs through fomites (feed, clothing and litter) or through airborne dissemination of virus-laden feathers and poultry house dust (Benton *et al.,* 1967).

Pathogenicity of IBDV has been associated with virus distribution bursal and non-bursal lymphopoietic and haematopoietic organs such as spleen, thymus, bone marrow etc. In particular, atrophy of the thymus has been associated with the acute phase of the disease (Sharma *et al.,* 1993).

Infectious bursal disease virus has been suggested to be part of an aetiologic complex causing proventriculitis in broilers (Huff *et al.* 2001). The vvIBDV strains are able to cause greater decrease in thymic weight index and more severe lesion in ceacal tonsils, thymus, spleen, and bone marrow, but the bursal lesions are similar (Lukert and Saif, 1997).

1.2STATEMENT OF RESEARCH PROBLEMS

Worldwide, the economic impact of infectious bursal disease is well known with losses counted as mortality, decreased production efficiency, andsecondary bacterial infections decrease response to vaccination as well as expenses of disease control (Musa*et al.,* 2012). Infectious bursal disease has been recognised in Nigeria since 1969 (Ojo *et al.,* 1973) and further incidence described by Onunkwo (1975) and Nawathe *et al.* (1978). Even today, the disease persists in virulent form, usually causing high mortality and could approach 50-100% in pullets, cockerels and local chicks (Okoye, 2005). Although the disease was first reported from the Western State of Nigeria, it has become nation-wide (Nawathe and Lamorde, 1982). Once Gumboro disease occurs, the farms remain permanently 'Gumboro farms' (Nawathe and Lamorde, 1982). An estimated economic loss of over three billion naira was reported during a three year study (2009-2011) by Musa *et al.*(2012).

Vaccination, the method of choice in protection has been failing using the local IBDV vaccine. Therefore, several IBDV vaccine types are now commercially available (Winterfield *et al.,* 1978). In terms of virulence these vaccines range from "mild" to intermediate and intermediate- plus (hot). Since the response to intermediate vaccines is less affected by maternal antibodies they are superior to the "mild" vaccines in

providing immunity to commercial chickens with maternal antibodies (Guittet *et al.*, 1992).While vaccination is the most practical procedure for the 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 even in vaccinated flocks (Abdu 1986;1987; Onunkwo and Okoye, 1991; Hassan *et al.,* 2002; Musa *et al.,* 2012). Hence, it was speculated that the vaccine types used were inappropriate given that an important cause of vaccination failure is the vaccine type used (Otsyina *et al.,* 2009). The efficacy and safety of a vaccine depends on its ability to elicit adequate antibody titre which should be of sufficient duration and not cause disease or other adverse reactions (Mc Mullin, 1985). Unfortunately, the efficacy and safety of most of the vaccines used in the control of IBD in Nigeria is poorly understood. The pathology associated with the use of these imported IBD vaccines in Nigeria is also not clearly understood.

1.3JUSTIFICATION OF THE RESEARCH

During the 63rd General Session of the OfficeInternational des Epizooties (OIE, 1995), it was estimated that IBD has considerable socio-economic importance at the international level, as the disease is present in more than 95% of the Member Countries (Eterradossi, 1995).The poultry industry in Nigeria has expanded over the years and indices have always indicated affordability in animal protein supply, food security and poverty alleviation in both rural and urban populace (Musa *et al.,* 2012). Infectious bursal disease has negatively affected this industry (Okoye, 1984; Abdu, 1986; Islam *et al.,* 2005; Musa *et al.,* 2010). Unfortunately, the control of infectious bursal disease in Nigeria presents a complex problem because of its highly endemic and ubiquitous (Nawathe and Lamorde, 1982).

Vaccine types and vaccination procedures, maternal derived antibody (MDA) in the chicks, pathogenicity of IBD field virus strains are factors that determine the efficacy of IBD vaccine/ vaccination (Sainsbury, 2000; Hair-Bejo *et al.,* 2004). Vaccination is effective in controlling infectious bursal disease when appropriate vaccination practices are followed (Islam *et al*., 2005).

Some vaccines are capable of inducing similar or more bursal lesions than those caused by field virus strains (Moraes *et al.,* 2004). The degree of histological damage of the bursa of Fabricius is directly proportional to immunosuppression and also to the age at which it was induced (Ivan *et al.,* 2001).

In Nigeria, different companies are marking a large number of imported IBD vaccines without knowing their efficacy, ability to overcome the maternal antibodies, and their congruency to local strains.

There have been reports of lesions caused by IBD vaccines in the bursa of Fabricius and its ability to recover (Okoye, 1987; Abdu *et al.,* 1988). This study will reveal the pathology of the bursa of Fabricius and other immune organs caused by some IBD vaccines and field virus; in order to provide information that will contribute to the selection of the best vaccine to be used in the control of IBD in Nigeria.

1.4AIM OF THE STUDY

To compare pathological and serological changes in cockerels administered different IBD vaccines with those infected with field virus strain of IBD.

1.5OBJECTIVES OF THE STUDY

The objectives were:

1. To compare the pathologic lesions of bursa of Fabricius, thymus and spleen of cockerels administered different IBD vaccines with those infected with afield strain of IBD.
2. To evaluate immunological response of cockerels administered different infectious bursal disease vaccines with those infected with field virus strain of IBD.

1.6 RESEARCH QUESTIONS

1. What are the similarities between IBDV vaccine and the IBD field virus strain onthe immune systems?
2. Are there similarities in immune response to different IBD vaccines and infection with field strain of IBD virus?

CHAPTER TWO

LITERATURE REVIEW

2.1Infectious Bursal Disease

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD) that affects young chickens about 3-6 weeks of age (Lukert and Saif, 2003). It is a highly contagious and acute viral disease that is characterized by the destruction of lymphoid cells in the bursa of Fabricius (BF) of fowls and rarely of turkeys (Cheville, 1967; Faragher, 1972; Hopkins *et al.,* 1979; Chettle *et al.,* 1985; Abdu *et al.,* 1986; Lukert and Saif 2003). The disease has the following synonyms: avian-nephrosis; Gumboro disease; infectious bursitis and avian infectious bursitis (Cosgrove, 1962; Hitchner, 1970; Faragher, 1972; Ibragimov, 1976 and Rodon, 1982).

Ever since the disease was recognized over 50 years ago, it continues to pose a threat to the commercial poultry industry (Okoye, 2005). The economic impacts of the disease are manifold including losses due to morbidity and mortality, immunosuppression in the surviving chickens since the infection exacerbates infections with other disease agents, reduction in the chicken's ability to respond to vaccination and risk of introduction to exotic places from importing infected poultry products (Eterradossi, 1995; van den Berg, 2000 and Shamaila, 2005). The economic impact of the disease is influenced by pathogenicity of the strain of the virus, susceptibility and breed of flock, other prevalent pathogens, environmental and management practices (van den Berg, 2000).

The causative agent is a bisegmented, double stranded RNA virus that belongs to the family Birnavirideae (Dobos *et al.*, 1979; Muller *et al.*, 1979; Lukert and Saif, 2003, Mac Lachlan and Dubovi, 2011). Two distinct serotypes have been recognized. Pathogenic strains are grouped into; serotype 1 viruses, while serotype 2 strains are non-pathogenic (Rosenberger *et al.*, 1998; Ture *et al.*, 1998 and van den Berg, 2000).

Initially,recognized strains were named Classical strains. Mutations in the RNA genome of the virus have resulted in the appearance of variant strains in the US and very virulent strains all over the world.

Comparing the disease outcome of one of the classical virulent IBDV (vIBDV) strains (F52/ 70) to the variant/very virulent IBDV (vvIBDV) strains, there was an increase in the mortality rate, from 50 % with vIBDV to 90 % with vvIBDV, which was accompanied by more-severe immunosuppression in the lymphoid organs (Mahgoub, 2012).

Infectious bursal disease virus (IBDV) infection also lowers the egg production, leads to deterioration of egg shell and internal egg quality. Infectious bursal disease virus (IBDV) infection results in immunosuppression which is marked by higher viral respiratory infections and increased mortality due to airsacculitis and colisepticaemia at the end of the growing period in broilers (Muller *et al.*, 2003).

Significant progress has been made in recent years that led to better understanding of morphology and molecular biology of this virus. Specific and sensitive diagnostic tools are now available and effective vaccines have been prepared for combating the disease (Shamaila, 2005).

2.2Aetiologic Agent of Infectious Bursal Disease

2.2.1Description of the virus

The virus responsible for IBD is a member of the family *Birnaviridae*, within the *Avibirnavirus* genus (Muller *et al*., 1979; Murphy *et al.*, 1999). It is nonenveloped with a single-shelled icosahedral capsid and has a diameter between 58nm - 60nm (Hirai and Shimakura, 1974: Kibenge *et al.*, 1988 and van den Berg, 2000). The genus name *Birnavirus* was proposed to describe viruses with 2 segments of double stranded RNA (Murphy *et al*., 1999). Other viruses included in this group are infectious pancreatic necrotic virus (IPNV) of fish, Tellina virus, oyster virus, blotched snakehead virus (BSVN) (Dobos *et al.*, 1979) and crab virus of bivalve mollusks belonging to Aquabirnavirus, while Drosophila X virus *(Drosophila melanogaster)* belongs to genus Entomobirnavirus. All of these contain two segments of double stranded RNA surrounded by a single protein capsid of icosahedral symmetry (Kibenge *et al.*, 1988).

2.2.2Types and subtypes of infectious bursal disease virus

Two serotypes of IBDV are recognized based on serum neutralization. Following isolation of variants, McFerran *et al*. (1980) described types 1 and 2 which could be differentiated by serological criteria. Parallel studies in the US also demonstrated two major serotypes, designated 1 and 2, isolated from turkeys (Jackwood *et al.*, 1982). Comperative studies have confirmed that the US types 1 and 2 are equivalent to the European types 1 and 2, respectively (McNulty and Saif, 1988). Although antibodies to both types 1 and 2 occur in both domestic fowl and turkeys, only infection with type 1 in chickens results in clinical signs. The major difference between types 1 and

2 involves the higher molecular weight of VP2 in type 2 viruses (Kibenge *et al.*, 1988).

The two serotypes are differentiated in *vitro* by the absence of cross-neutralisation, and in vivo, by the absence of cross-protection (McFerran *et al*., 1980; Jackwood *et al.*, 1982; Jackwood *et al.*, 1984; Becht *et al*., 1988; Ismail *et al.*, 1988).

Serotype 1 viruses can be further categorized into 4 groups on the basis of their pathogenicity: classical strains, variants, attenuated strains and very virulent strains (Lasher and Davis, 1997). Different pathotypes of the virus and the cell culture adapted strains differ markedly in virulence. The three criteria currently being used for the characterization of IBDV strains include antigenicity, genetic relatedness and pathogenicity.

Classical IBDV has traditionally affected poultry worldwide since the first reported incident from Gumboro. Classical strains cause bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20 –30% in specific pathogen free (SPF) chicken (Lim *et al.*, 1999).

Variant strains appeared in the US in 1983. These strains were antigenically different from classical strains and caused a rapid and severe bursal atrophy (Vakhari, 1994) and in contrast to classical strains produced no clinical signs of illness. Antigenic variants have been recognized by their ability to escape cross-neutralization by antiserum against the classical strains (Lim *et al*., 1999).

Attenuated strains have been generated by adapting the classical and variants strains to chicken embryo fibroblasts (CEF) or other cell lines (Lim *et al*., 1999). Since they are not pathogenic they have been used as live vaccines (Sharma, 2005).

The emergence of the very virulent strains during the 1980's in Europe, Japan and China resulted in dramatic losses to the poultry industry(van den Berg, 2000). Very virulent strains have been characterized by severe clinical signs and high mortality ranging from 60-100%. Very virulent strains can break through the immunity provided by the maternal antibodies(van den Berg, 2000). The vvIBDVs produce similar signs as the classical strains and the same incubation period of 4 days but the acute phase is more severe and more generalized in the affected flocks (van den Berg, 2000).

2.2.3Structure of the infectious bursal disease virus

Infectious bursal disease virus particles have a non-enveloped, icosahedral capsid with a diameter of about 58nm - 60nm (Kibenge *et al.,* 1988; van den Berg, 2000). The structure of the virus is based on a $T = 13$ lattice and the capsid subunits are predominantly trimer clustered (Böttcher *et al*., 1997). Characterization of the viral genome as a bi-segmented dsRNA (Müller *et al*., 1979) allowed placing IBDV into a new family of viruses, the *Birnaviridae* (Dobos *et al*., 1979); where it represents the prototype member of the genus *Avibirnavirus* (Leong *et al*., 2000). Meanwhile, the complete nucleotide (nt) sequences of the two genome segments A and B have been established (Mundt and Müller, 1995), and nt sequence data are now available for many IBDV strains. A large open reading frame (ORF) in segment A (Hudson *et al*., 1986; Bayliss *et al*., 1990) encodes a polyprotein that is co-translationally processed into the major structural proteins VP2 and VP3 forming the viral capsid, and into VP4 (Müller and Becht, 1982). TheVP2 and VP3 form the outer and inner capsid of the virus, respectively (Böttcher *et al*., 1997; Caston *et al*., 2001). The domains for

homotypic interactions of the structural proteins have been mapped (Tacken *et al*., 2003). The VP4 is a virus-encoded protease that shares a number of features with bacterial Lon proteases that utilizes the serine-lysine (Ser-652 and Lys-692) catalytic dyad (Birghan *et al*., 2000; Lejal *et al*., 2000). In VP2, a precursor–product relationship exists as only the larger protein (pVP2) can be demonstrated in infected cells and further proteolytic cleavage transforms the precursor into mature VP2, present in the complete virus particle (Müller and Becht, 1982). The cleavage sites between pVP2–VP4 (511LAA513) and VP4–VP3 (754MAA756) have been established (Sanchez and Rodriguez, 1999). It looks as if the final processing of pVP2 to VP2 is controlled by the correct scaffolding of VP3 (Chevalier *et al*., 2002) which interacts with VP1 (Lombardo *et al*., 1999; Tacken *et al*., 2000) as well as with the viral dsRNA (Tacken *et al*., 2002) during encapsidation. The maturation process of pVP2 (residues 1–512) generates VP2 (residues 1–441) and four small peptides, of which at least three were shown to be associated with the viral particle (Da Costa *et al*., 2002). Excessive amounts of the precursor protein pVP2 can be found, together with a series of aberrant polypeptides, in "incomplete particles", formed under conditions of a high multiplicity of infection (m.o.i.), and which have the capacity to interfere with the replication of standard IBDV particles (Müller *et al*., 1986). The second ORF on segment A encodes the small, non-structural protein VP5 (Mundt *et al*., 1995), not essential for viral replication (Mundt *et al*., 1997; Yao *et al*., 1998), but considered to have a function in virus release (Lombardo *et al*., 2000).

Expression of VP2 induces apoptosis in a variety of mammalian cell lines, counteracted by the coexpression of the proto-oncogene *bcl*-2 (Fernandez-Arias *et al*., 1997). Yao and Vakharia (2001) revealed that both VP2 and VP5 are involved in the

induction of apoptosis in the chicken B-lymphocyte cell line RP9 as well as in chicken embryo fibroblast cells.

Genome segment B codes for VP1, a structural protein linked with the ends of both segments of the viral genome (Müller and Nitschke, 1987) and with multiple enzyme activities (Spies *et al*., 1987; Spies and Müller, 1990; Kibenge and Dhama, 1997). Birnavirus VP1 proteins form a distinct subgroup of RNA-dependent RNA polymerases lacking a GDD motif (Shwed *et al*., 2002). Remarkably, a phylogenetic analysis indicated that segment B nt sequences of the emerging vvIBDV strains formed a distinct cluster (Yamaguchi *et al*., 1997; Islam *et al*., 2001). It has been suggested, therefore, that these strains might have derived segment B from a hitherto unidentified source, possibly by segment reassortment (Muller *et al*., 2003).

This model suggests 780 copies of VP2, 600 copies of VP3, 60 copies of VP4 and is in accordance with the observed composition of 51% VP2, 40% of VP3, 6% VP 4 and 3%VP1(Dobos *et al*., 1979).

2.2.4Physicochemical properties of infectious bursal disease virus

The virus is non-enveloped and quite resistant to physical and chemical agents(Benton *et al.,* 1967a). Due to the stability and hardiness of the virus, it persists in poultry premises even after thorough cleaning and disinfection(Benton *et al.,* 1967a). The virus is inactivated at a pH of 12.0 but not at pH 2.0 (Benton *et al.,* 1967a). Benton *et al.* (1967a) reported that IBDV survived a temperature of 37°C for 90 min and 56°C for 5 hours. A marked reduction in infectivity of the virus was observed after treatment with 0.5% formalin for 6 hours. The virus remained unaffected by ether, choloroform, phenol, thiomaesal, Staphene and Hyamine 2389

treatments. The virus survived treatments with various concentrations of three disinfectants (an iodine complex, a phenolic derivative and a quaternary ammonium compound) for a period of 2 minutes at 23℃, only the iodine complex had any deleterious effects.

Cho and Edgar (1969) reported that the virus was inactivated by exposure for 1 hour to 1% formalin, 1% cresol and 1% phenol. It remained stable at 60℃ for 90 min and was still infectious at room temperature for approximately 25℃ for 21 days. Petek (1973) observed that IBDV was more resistant than Reovirus to heat, ultraviolet irradiation and photodynamic inactivation.

The hardiness of the virus makes it difficult to eradicate from poultry houses after outbreaks of IBD (Alexander and Chettle, 1998). Heat resistance of IBDV is an important factor to be considered in trade of poultry due to extensive international trade of processed and partially processed poultry meat (Shamaila, 2005).

Alexander and Chettle (1998) constructed the heat inactivation curves of classical IBDV at 70℃, 75℃ and 80℃. These biphasic multiple kinetic curves showed an initial rapid drop in infectivity followed by a more gradual decline. In the second phase it took 18.8 min at 70℃, 11.4 min at 75℃ and 3.0 min at 80℃ for reduction of the infectivity by 1 log 10. Lam (1991) showed that IBDV survived at 60℃ but did not survive at 70℃ for 30 min and 0.5% chloramines killed the virus after 10 min. Invert soaps with 0.05% sodium hydroxide either inactivated or had a strong inhibitory effect on the virus (Schnitzler *et al.,* 1993).

The virus is unusually resistant to inactivation by cooking so there is a risk of introduction to the backyard flocks through uncooked chicken meat products since viable virus might be present in meat from apparently healthy chickens. Drumsticks

and chicken patties experimentally injected with 107-109 TCID50 of virus and cooked to internal temperature of 71°C and 75°C, respectively in hot oil or steam in a flame grill still contained the infectious virus (Lukert and Davis, 1974)..

2.2.5Biochemistry of infectious bursal disease virus

The genome consists of two pieces of high Mr dsRNA that sediments at 14S components in sucrose gradients. The molecular weights of the two segments are 2.2 x 10^6 and 2.5 x 10^6. It was reported that the two segments migrated similarly when co-electrophoresed. The RNA segments from serotype 2 viruses migrated similarly, but differed from serotype 1 viruses when co-electrophoresed (Kibenge *et al*. 1988)

Buoyant density of 1.62 g/ml in cesium sulphate, melting point of 95.5^{0}C in the presence of RNase, pairing of Adenine and Uracil and Thymine and cytosine, precipitation from 4M but not from 2M LiCl, green staining with acridine orange, all point towards a dsRNA genome. Both genome segments contain 94 x 10^3 5′ genome-linked protein (VPg). There are no poly(A) tracts at the 3′ ends of the RNA segments. The defective particles banding at 1.30 g/cm^3 appear to have a truncated A segment (Kibenge *et al*. 1988).

The virion contains five polypeptides including VP1 (94 x 10^3) an RNA dependent RNA polymerase as well as VPg; pre-VP2 (62 x 10^3) and VP2 (54 x 10^3), the major capsid and type specific antigen VP3 (30 x10^3) and VP 4 (29 x10^3) a component of the virion. Negatively charged non-structural protein (16.5 x10^3) VP5 is encoded by an ORF proceeding to segment A encoded polypeptide. Although VP 1 can guanylylate itself, no guanylyl transferase activity has been detected and viral RNA made in the cells retains its 5′ VPg. Also, the viral RNA is not capped at the 5′end. No

N-linked glycosylation of any of the virion proteins has been detected (Kibenge *et al.* 1988).

2.2.6Viral proteins of infectious bursal disease virus

Five proteins have been identified in IBDV by SDS-PAGE analysis and they are generally referred to as VP1 (90Kd), VP2 (40 Kd), VP3 (35 Kd), VP4 (28 Kd) and VP5 (21 Kd). The VP2 is the most abundant polypeptide and makes up more than 50% of the virion protein (Kibenge and Dhama 1997). The VP3 is the second most abundant protein and makes up 40% of the virion protein. Both VP2 and VP3 are responsible for the structural integrity of the virion (Kibenge and Dhama 1997). The VP4 and the VP1 are minor proteins of the virion accounting for 6% and 3% respectively (Muller and Nitschke, 1987; Kibenge and Dhama 1997; Lombardo *et al.*, 1999; van den Berg, 2000).

2.2.7Replication of infectious bursal disease virus

A kinetic study using immunofluorescence (Muller *et al.*, 1979; Mahgoub, 2012) has shown that, 4 h after oral inoculation, the virus is found in the lymphoid tissues associated with the digestive tract, where the first cycle of viral replication occurs. The virus subsequently enters the general circulation via the hepatic portal vein. A phase of primary viraemia ensues, during which the virus reaches the bursa, 11 h after infection, and a major secondary replication cycle occurs (Lukert and Saif, 1997). A phase of secondary viraemia then occurs, and the other lymphoid organs become massively infected (Lukert and Saif, 1997; Murphy *et al.*, 1999; Okoye, 1984; van den Berg, 2000; Mahgoub, 2012).

A single replication cycle takes 6-8 hours for IBDV. The virus binding protein has been found at various types of chicken cells (Regenmortel, 2003). The cell receptor recognition site on the virus is not known (Lukert and Saif, 2003). Replication of IBDV is not restricted by the presence of specific receptor sites.

Strains of both IBDV serotypes bind to lymphoid cells isolated from bursa, thymus or spleen indicating that all these cells are susceptible and have the specific receptor site for the entry of the virus. The first report investigating the IBDV receptor was made by Ogawa *et al*. (1998). They described the protein as an N-glycosylated protein associated with the expression of B cell surface immunoglobulin (Ig) M^{+}. In a later study by Lin *et al*. (2007), chicken heat shock protein 90 was suggested to be part of the IBDV-receptor complex. Delgui *et al*. (2009) demonstrated the ability of α4β1 integrin to act as an IBDV receptor.

It has been suggested that cell tropism of the virus is determined by receptor-mediated entry step (Boot *et al*., 2007; Brandt *et al*., 2001). After entry into the host cell, the virion RNA dependent RNA polymerase becomes activated and produces two genome length (24 S) mRNA molecules from each of the 14S dsRNA genome segments (Regenmortel, 2003). These mRNA lack a cap and 3′ poly A tail and have a VPg attached to their 5′ ends (Regenmortel, 2003). Genome –linked proteins have been described indicating that the virus replicates by a strand displacement mechanism (Spies *et al*., 1987). The separate strands of the genomic ds RNA can be translated *in vitro* (Azad *et al*., 1987). Replicative intermediates have been described in the infected cells however, there are no reports on the minus strand synthesis (Regenmortel, 2003). The two mRNAs are synthesized in the same relative proportions throughout the replicative cycle (twice as many A as B mRNA molecules)

(Regenmortel, 2003). On a plus-strand IBDV segment A cRNA template, minus-strand synthesis occurred in such a way that a covalently linked double-stranded RNA product was generated by a 'copy-back' mechanism. Importantly, enzyme activity was observed only with templates that comprised the 3' non-coding region of plus-strand RNAs transcribed from IBDV segments A and B, indicating template specificity (Von Einem *et al.*, 2004).

2.2.8Antigenic properties of infectious bursal disease virus

Infectious bursal disease virus is endemic throughout the world but several different antigenic and pathogenic types exist in specific geographic locations. Two serotypes of IBDV occur in Europe and USA as recognized by the virus neutralization and cross protection test (van den Berg, 2000). These two serotypes are antigenically distinct (Macreadie*et al.,* 1990). Serotype 1 viruses are pathogenic to chickens and differ in their virulence (Winterfield and Thacker, 1978), they cause lesions in the bursa of Fabricius by lymphocytic depletion (Schroder, 2001), whereas serotype 2 viruses are avirulent to chickens and isolated mainly from turkeys (Ismail *et al.,* 1988; Kibenge *et al.,* 1999) and chickens.

Serotype 1 viruses can be broadly divided into classic, variant and very virulent IBDVs. Until 1987, the strains of virus were of low virulence and were controlled by vaccination. Emergence of variant viruses was first reported in USA in 1987. These viruses were reported to undergo an antigenic drift against which the classical IBD vaccines were not protective (Jackwood and Saif, 1987; Snyder *et al.,* 1992). Six antigenic subtypes of IBDV serotype 1 viruses have been identified by the virus neutralization test (Jackwood and Saif, 1987). Variant viruses occur in the USA and

Australia and are different from the classic viruses in terms of pathogenicity and immunogenicity. They overcome the immunity induced by classic serotype 1 viruses and cause rapid bursal atrophy with minimal or no inflammatory response (Jackwood and Saif, 1987).

Vaccination with one serotype 1 subtype did not ensure protection from challenge with another subtype (McFerran *et al.*, 1980; Ismail and Saif, 1984; Jackwood and Saif, 1987) suggesting that variant viruses are antigenically different from classical viruses. Variant viruses present in the USA and Australia are not closely related to each other (Sapats and Ignjatovic, 2000). Significant antigenic differences exist among serotype 1 strains as detected by virus neutralization and led to the grouping of the serotype 1 viruses into 6 subtypes (Jackwood and Saif, 1987). Virus neutralization test proved to be serotype specific and could distinguish between the two serotypes (McFerran *et al*., 1980; Jackwood *et al*., 1982;Jackwood *et al*., 1985;Jackwood and Saif, 1987).

Serotype 1 induced protection against challenge with variant viruses (Ismail and Saif, 1990). Serotype 2 viruses are immunologically distinct from serotype 1 viruses since vaccination with serotype 2 (OH) virus did not confer protection against serotype 1. Cross protection studies indicated that the variant viruses were different from other subtypes of serotype 1 IBDVs. Both serotype 1 and 2 viruses share common group antigens which could be detected by AGPT, Flourescent antibody test and ELISA (Chettle *et al.*, 1985;Jackwood *et al.*, 1985; Jackwood and Saif, 1987; Jackwood *et al*., 1987). Capsid proteins VP2 and VP3 contain epitopes that are responsible for group antigenicity. The VP2 carries the serotype specific antigens responsible for the induction of neutralizing protective antibodies (Azad *et al*., 1987; Becht, 1988).

Variation in IBDV antigenicity depends on amino acid changes in peak A and B. Serotype 2 strain 23/82, North American antigenic variants A, E, GLS-5 and DS 326 neutralization resistance escape mutants all exhibit amino acid changes in the hydrophilic peaks(Eterradossi *et al*., 1998). Only serotype 1 strains show changes in hydrophobic domain(Eterradossi *et al*., 1998). Two smaller hydrophilic areas of VP2 variable domain also influence IBDV antigenicity (Eterradossi *et al*., 1998).

Deletion mutagenesis studies with Australian strain 002/73 showed that virus neutralizing monoclonal antibody recognized a discontinuous epitope on VP2 (Azad *et al*., 1987). The antigenic region responsible for the production of neutralizing antibody is highly conformation dependent (Becht, 1988; Fahey *et al*., 1989) since the antibodies immunoprecipitate VP2 but did not react with the denatured protein in the immunoblot (Oppling *et al*., 1991). The VP2 carries the epitopes which elicit neutralizing antibodies and distinguish the two strains as well as those which elicit non-neutralizing antibodies and are common to both strains (Cruz-Coy *et al*., 1993). The antigenic region responsible for the production of neutralizing antibody is highly conformation dependent. Monoclonal antibodies have also defined a common sequence dependent antigenic site located at the VP2 (Becht *et al*., 1988). Since protective immunity against IBDV is dependent on the presence of neutralizing antibodies in susceptible birds, precise knowledge of antigenic sites responsible for the induction of neutralizing antibodies is of fundamental importance(Becht *et al*., 1988).

At least three distinct conformation dependent serotype 1 specific virus neutralizing antigenic sites have been identified on VP2 and one linear antigenic site on VP3 (Azad *et al*., 1987; Becht *et al*., 1988). Two conformational virus neutralizing

antigenic sites were localized in the central region of VP2 consisting of 156 amino acids residues while linear epitope was mapped to C-terminal 105 amino acid residues of VP3(Becht *et al*., 1988). Sequence specific epitope on VP2 are common to both serotypes (Becht *et al*., 1988). Of the two non-neutralizing monoclonal antibodies defined epitopes, one epitope is common to both serotypes whereas the second is distinct for serotype 1 and 2(Eterradossi *et al*., 1998).

Very virulent strains are not antigenically different from the classic strains unlike variant strains from USA and Australia (Sapats and Ignjantovic, 2000). It is not clear whether vvIBDV evolved from classical strains or pre-existed in nature with other avian species (Islam *et al*., 2001).

Nucleotide sequencing of the VP2 variable region of vvIBDV strains, which appeared at the same time in Europe, Africa and Asia, confirmed that they can be placed within the same group (Cao *et al*., 1998; Chen *et al*., 1998; Pitcovski *et al*., 1998; Zierenberg *et al*., 2000, 2001; Rudd *et al*., 2002) and that they are antigenically and genetically similar to each other (Eterradossi *et al*., 1999; Islam *et al*., 2001a).

Very limited antigenic variations have been reported in the French vv IBDVs (Fahey *et al*., 1991; Eterradossi, 1997). Fahey (1991) reported two non overlapping epitopes recognized by the virus neutralizing monoclonal antibodies on VP2. Four conformation dependent neutralization epitopes were recognized on VP2 (van den Berg, 1996). The VP2 sequencing results confirmed that neutralizing epitopes are clustered in the variable domain which is highly hydrophobic (van den Berg *et al*., 1996).

In the vvIBDV strain there is no evidence of antigenic variation from the classical strains (van den Berg *et al.,* 1991). Modified epitope has been described with the help

of neutralizing monoclonal antibodies corresponding to a mutation at amino acid position 222 in the first hydrophilic peak of VP2. No antigenic drift has been demonstrated by cross neutralization tests.

No genetic or antigenic absolute marker for virulence has yet been described. So the only way to demonstrate virulence is still by the *in vivo* inoculation (Eteraddossi, 2001).

2.2.9Pathotypic variation of infectious bursal disease virus

In addition to antigenic differences in serotypes and subtypes, the viral strains can also be classified according to their virulence (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 describe 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 bursa of Fabricius of vaccinated birds (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 specific pathogen free birds and not to any antigenic specificity.

-Apathogenic (Serotype 2) no mortality, no bursa lesions

-Pathogenic (Serotype 1)

- Mild — no mortality, increasing bursal lesions
- Intermediate
- Intermediate plus
- Classical — increasing mortality
- Variant
- Very or hypervirulent

Classification of IBDV strains as pathotypes. IBDV strains can be defined as apathogenic (serotype 2); mild, intermediate or ‘‘hot’’ (serotype 1 vaccines); classical virulent (IBDV), variant, or very virulent (serotype 1). Serotype 2 strains cause neither mortality nor bursal lesions in specified pathogen free birds. Serotype 1 vaccines cause no mortality but possess residual pathogenicity with bursal lesions varying from mild to moderate or even severe. Virulent serotype 1 strains induce both mortality and bursal lesions (van den Berg, 2000).

2.3Prevalence of Infectious Bursal Disease Virus

Classical IBDV have traditionally affected poultry worldwide ever since the first outbreak of disease was reported from Delaware, Maryland and Virginia (Delmarva) region(Cosgrove, 1962).By 1970, the disease had been reported from Canada (Ide and Stevenson, 1973), Mexico (Lucio *et al.*, 1972), Europe (Gukelberger, 1977; Landgraf *et al.,* 1972), Africa (Onunkwo, 1975; Onunkwo and Momoh, 1981), the Middle East (el-Zain *et al.*, 1974) and Asia (Mohantey *et al.*, 1971).

The infection is enzootic in nature and birds are constantly exposed to the virus (Lukert, 1986). Breeder flocks are vaccinated against the virus to provide maternal immunity to the off-springs so all chicken flocks are seropositive for the virus (Lukert, 1986; Abdu *et al.,* 1986). Infectious bursal disease is currently an international problem: 95% of the 65 countries that responded to a survey conducted by OIE in 1995 declared cases of infection (Eterradossi *et al.*, 1995), including New Zealand which had been free of disease until 1993 (Jones, 1986). These findings led to the adoption of a specific resolution of the International Committee of the OIE during the 63rd General Session in May 1995 (OIE, 1995).

2.4Transmission of Infectious Bursal Disease Virus

The IBDV is highly contagious and the disease is spread by direct contact between infected and susceptible flocks. Infected chickens shed IBDV one day after infection and can transmit the disease for at least 14 days (Vindevogel *et al.,* 1976) but not exceeding 16 days (Winterfield *et al.,* 1972). Indirect transmission of virus most probably occurs on fomites (feed, clothing and litter) or through airborne dissemination of virus-laden feathers and poultry house dust (Benton *et al.,* 1967a). Operation of multi-age broiler and pullet replacement farms, defects in biosecurity, or proximity of farms to roads used to transport poultry will contribute to a high prevalence of infection(Vindevogel *et al.,* 1976).

2.5Host Reservoirs of Infectious Bursal Disease Virus

Infectious bursal disease virus is highly host specific. The selected host for IBDV is the young chicken where a clinical disease occurs whilst in older birds the infection is essentially subclinical. Only chickens (*Gallus gallus domesticus*) develop IBD after infection by serotype 1 viruses and inoculation of IBDV in other avian species fails to induce disease. Turkeys (Meleagris gallopavo) and Peking ducks (Anas peking) may be asymptomatic carriers of serotype 1 viruses whose pathogenicity is ill defined (McFerran *et al.,* 1980). Experimental inoculation of game birds (quails, partridges, pheasants and guinea fowls) with serotype 1 viruses failed to induce any clinical sign or disease, even when a very virulent IBDV strain was used for challenge (van den Berg *et al.,* 2001). Varying susceptibility of different chicken breeds has been described with higher mortality rates in light than in heavier breeds (Bumstead *et al.,* 1993; Nielsen *et al.,* 1998). Studies with vvIBDV demonstrated the exacerbated

susceptibility of Leghorn-type SPF chickens (Rauw *et al.*, 2007). More recent studies have confirmed a significant influence of chicken's genetic background on IBD outcome and, more closely, its association with the early immune response during the acute phase after infection with vvIBDV (Aricibasi *et al*., 2010; Ruby *et al*., 2006). Furthermore, the differential immunopathogenesis of IBDV was investigated in different conventional layer and broiler type chickens in comparison to highly susceptible SPF layers often used for experimental studies. Layer-type chickens of all genetic backgrounds showed significantly higher IBDV antigen loads in the BF, clinical signs and death rate compared to broiler type birds (Tippenhauer *et al*., 2012).

Mortality from IBD is generally accepted to be higher in commercial hybrid Leghorn replacement pullets than in broiler strains (Benton *et al.*, 1967b; Winterfield and Thacker, 1978).

In Nigeria, local chickens are more susceptible to IBD than broilers and slightly more susceptible than pullets, explaining earlier reports that mortality due to IBD was higher in pullets or light breeds of chicken than in broilers or heavy breeds (Okoye and Aba-Adulugba, 1998). Outbreaks of IBD in local chickens in Nigeria was also reported by (Abdu and George, 1986; Okoye, 1987) and Sudan (Salman *et al*, 1983).

The virus has also been reported in Ostriches (Gouch *et al*., 1998), in Baltic ducks and herring Gulls (Hitchner, 1970), various raptor and passerine species in Japan (Nieper and Muler, 1996). Anti-IBDV antibodies have been reported from Antarctic penguins (Gardner *et al*., 19907). The virus has also been documented in the lesser mealworm (*Alphitobius sp*.) fed onIBDV contaminated feed. Experimental inoculation of pheasants, partridges, guinea fowls and quails showed no signs of disease (van den Berg *et al*., 2001). Japanese quails are refractory to IBDV infection (Grienfield *et al*.,

1983). They tested negative for virus replication, detection of precipitating or virus neutralizing antibodies and gross as well as microscopic changes in bursa of Fabricius associated with infection (Weisman and Hitchner, 1978). However, quail-chicken hybrids can be infected with IBDV (Greenfield *et al*., 1986).

Several studies have suggested that rats, mice, dogs, wild birds and mosquitoes are reservoirs of IBDV (Howie and Thorsen, 1981; Okoye and Uche, 1986; Park *et al*., 2008; Pagès-Manté *et al*., 2004; Jeon *et al*., 2008; Kasanga *et al*., 2008).

Neutralising or precipitating antibodies have been detected, *inter alia,* in various species of wild duck, goose, tern, puffin, crow and penguin, which may mean that wild birds act as reservoirs or vectors (Wilcox *et al*., 1983; Gardner, *et al*., 1997; Ogawa *et al*., 1998).

2.6Pathogenesis of infectious Bursal Disease

Under natural conditions, the most common mode of infection appears to be via the oral route(Muller *et al*., 1979). From the gut, the virus is transported to other tissues by phagocytic cells, most likely resident macrophages. Although viral antigen has been detected in liver and kidney within the first few hours of infection, extensive viral replication takes place primarily in the bursa of Fabricius (Muller *et al.,* 1979).

Following oral infection, the virus replicates in gut-associated macrophages and lymphoid cells and enters the portal circulation, leading to primary viraemia (Murphy *et al*., 1999, Mahgoub, 2012). The viral antigen is detectable in the macrophages and lymphoid cells of the caecum as early as 4 h postinfection (hpi) and reaches the liver by 5 hpi, and following primary viraemia, the virus reaches the BF by 11 hpi (Lukert

and Saif, 1997; Murphy *et al*., 1999; Sharma *et al*., 2000). Following IBDV replication in the BF, the virus enters the blood stream to cause secondary viraemia, which results in virus spread to other tissues (Mahgoub, 2012).

2.6.1Role of T-cells in the immunopathogenesis of infectious bursal disease virus

Appearance of viral antigen in bursa is accompanied by an infiltration of T cells while IgM + cells undergo a precipitous decrease and the immunoglobulin level remains the same (Lejal *et al*., 2000). Infiltrating T cells were first detected at 1st day post inoculation through flow-cytometry and were shown to persist till 12 weeks (Sharma *et al*., 2000). The ratio of CD4 and CD8 cells were the same during the 1st seven dpi but CD8 cells became predominant afterwards (Lejal *et al*., 2000).

Infectious bursal disease virus induced bursal T cells have increased surface expression of MHC-II and IL-2 receptors, elevated expression of cytokine genes like IFN-γ and IL-6 like factor (Sharma *et al*., 2000). T cells from the bursa of the recovered bird proliferate when exposed *in vitro* to purified IBDV. While the spleen cells from IBDV exposed – chicken produced nitric oxide stimulating factor when stimulated *in vitro* with purified IBDV (Sharma *et al*., 2000). Bursal T cells also suppressed the mitogenic proliferation of the spleen from normal, virus free chicken (Sharma *et al*., 2001).

T cell immunodeficiency can modulate pathogenicity of the virus since it has been shown that the TX birds have a higher viral burden in the bursa, lower inflammatory lesions in bursa, down regulated IFN-γ and IL-2 genes in bursal cells, have a lower incidence of apoptotic bursal cells (Sharma *et al*., 2000) and undergo a quick follicular recovery than T cell intact birds (Sharma *et al*., 2001).

2.6.2Effect of infectious bursal disease virus on innate immunity

Infectious bursal disease virus has been shown to modulate the macrophage function by altering the *in vitro* phagocytic activity (Lam *et al*., 1998). Macrophages from infected chicken have upregulated cytokine gene expression and produce increased levels of NO (Kim *et al*., 1998).There is growing evidence for a role of innate immunity, particularly proinflammatory mediators, in the pathogenesis of IBD (Ingrao *et al*., 2013) Indeed, during the acute phase of IBD and as early as 1 day postinfection (dpi), there is a dramatic infiltration of CD4 cells, $CD8^+$ cells and macrophages at and near the site of virus replication, mainly in the BF (Sharma *et al*., 2000; Withers *et al*., 2005). Bursal T cells are activated and exhibit up-regulation of gene transcription of pro-inflammatory cytokines, e.g., ChIL-1β, ChIL-6, CXCi2 and ChIFN-γ (Eldaghayes *et al*., 2006). High levels of systemic ChIFNγ and ChIL-γ were also observed during the acute phase following vvIBDV challenge demonstrating the role of an exacerbated innate immune response in the acute phase of the disease, leading to a so-called ‘‘cytokine storm’’ (Rauw *et al*., 2007). The ChIFN-γ up-regulation was correlated with production of IL12a, an increased level of IL18 mRNA in splenic macrophages and pro-inflammatory factors including ChIL-1β, ChIL-6, and inducible nitric oxide synthetase (iNOS) (Palmquist *et al*., 2006) that may promote cellular dysregulation and accentuate tissue destruction (Digby and Lowenthal, 1995; Karaca *et al*., 1996; Kim *et al*., 1998). Moreover, macrophages and monocytes infected by IBDV are directly activated producing high levels of mediators such as proinflammatory cytokines, interleukin-1 (IL-1) and IL-6, chemokines (IL-8 and MIP-α and nitric oxide (NO) (Kim *et al*., 1998; van den Berg, 2000; Khatri *et al*., 2005; Rauf *et al*., 2011a). The signal transduction pathways involved in macrophage activation have also been examined (Khatri and Sharma, 2006). The role of mitogen-

activated protein kinases (MAPKs) and NF-κB was tested by using specific pharmacological inhibitors. The addition of p38 MAPK inhibitor, SB-203580 and NF-jB inhibitor Bay 11-7082, suppressed IBDV-induced NO production and mRNA expression of iNOS, IL-8 and COX-2 (Khatri and Sharma, 2006). These results suggest that IBDV uses cellular signal transduction machinery, in particular the p38 MAPK and NF-κB pathways, to elicit macrophage activation. The increased production of NO, IL-8 and COX-2 by macrophages may contribute to bursa inflammatory responses commonly seen during the acute phase of IBDV infection (Khatri *et al.*, 2005). This was confirmed in a more recent study (Rauf *et al*., 2011b), where the overexpression of chemokines genes, IL- 8 and MIP-α was also higher in IBDV-infected chickens during the early phase of infection (chicken IL-8 acts as a chemoattractant for heterophils and monocytes). In summary, IBDV appears to trigger both direct (T cell activation) and indirect (macrophage activation) pathways to induce a ‘‘cytokine storm’’ during the acute phase of the disease and the individual susceptibility must be related to the variable intensity of the innate immune response (van den Berg, 2000; Rauw *et al*., 2007; Rauf *et al*., 2011b). In a study, an Agilent microarray was used to investigate different transcriptional profiles of the TLR pathway and related genes of chicken bursa at 48 h after infection with IBDV, compared with simulated infection. Expression of 58 genes changed significantly (Guo *et al.,* 2012). Forty-six genes associated with chicken bursa proinflammatory effects, chemotactic effects, and T-cell stimulation were upregulated, which meant enhancement of these features (Guo *et al.*, 2012). Twelve genes that are related to proliferation and differentiation of bursal cells were downregulated, implying suppression of these features. These results revealed that genes of the TLR pathway play an important role in the pathogenicity of IBDV infection (Guo *et al*., 2012).

2.6.3Role of cytokine in the pathogenesis of infectious bursal disease virus

Infectious bursal disease virus modulates the T cell functions (Sharma *et al*., 2001). During the acute phase of the disease septic shock-like symptoms have been observed(Hack *et al*., 1997). In septic shock syndrome, there is an up-regulation of the cytokine gene resulting in an excessive immune response and increased levels of IFN-γ and TNF-α (Hack *et al*., 1997). The TNF -α is a macrophage produced cytokine involved in inflammation and septic shock. Chicken IFN-γ can activate macrophages and enhance their anti-microbial activity (Kim *et al*., 1998).

The ChIFN-γ and TNF-α levels in serum were measured by capture ELISA and cytotoxic bioassay respectively. The increase in the levels of cytokines like ChIFN-γ and TNF-α corelated with the acute phase of the disease. Levels of circulating ChIFN-γ and TNF-α increased as the disease progresses and were highest in the birds that died of infection. The TNF-α levels lasted longer than the ChIFN-γ levels (Sharma *et al*., 2001).

2.7 Immunology of Inffectious Bursal Disease Virus

Infectious bursal disease virus is ubiquitous in commercial chickens environment and chickens acquire the infection orally or by inhalation. The virus is transferred from the gut to the other tissues by phagocytic cells like macrophages. In macrophages of the gut associated tissues it could be detected as early as 4 hours after oral inoculation using immunoflorescence (Muller *et al*., 1979). The virus then reaches the bursa via the blood where the most extensive virus replication occurs. By 13 hours post inoculation (p.i) most follicles are positive for virus and by 16 hours p.i a second and

pronounced viremia occurs accompanied by secondary replication in other organs resulting in disease and death (van den Berg *et al*., 2000).

The IgM^+ cells are the target organs for the virus. During the acute phase of the disease the bursa undergoes atrophy as the bursal follicles get depleted of B cells. Virus replication causes extensive damage to lymphoid cells in medullary and cortical regions of the follicle. Apoptosis of the neighboring B cells augments the destruction of the bursal morphology. By this time an ample amount of viral antigen can be detected in other immune organs(Giambrone, 1977; Kim *et al*., 1999).

T cells are resistant to infection by IBDV (Hirai *et al*., 1979). During the acute phase of the disease lesions appear in the thymus which is quickly overcome within a few days (Sharma *et al*., 2000). A dramatic influx of T cells is reported in and around the site of virus replication. The infiltrated T cells could be detected from $1^{st}$to 12^{th}weeks post inoculation although the viral antigen disappears by the 3^{rd}week. The IBDV induced cytotoxic T cell limit the spread of the virus by destroying the cells expressing the viral antigen and thus can initiate the recovery process. At the same time IBDV-induced T cells might enhance the viral lesions by producing inflammatory cytokines. T helper cells produce inflammatory cytokines like IFN-γ which activates the macrophages to produce nitric oxide (NO) (Sharma *et al*., 2000). Both humoral and cellular arms of the immune system are compromised during the IBDV infection due to lysis of the B cells and altered antigen-presenting cells (Ingrao *et al.,* 2013).

The IBDV causes a transient inhibition of *in vitro* proliferative activity of T cells to mitogens. The virus stimulates the macrophages to produce T cell cytokine like IFN-γ to produce nitric oxide (NO) and other cytokines with anti-proliferative activity.

Infectious bursal disease does not affect natural killer cells levels in chickens (Sharma *et al*., 2000).The NO production after IBD virus infection exerts antiviral effect since the immune-suppressed chickens that failed to induce NO had more severe disease and higher degree of virus replication, but does not seem to correlate with the hemorrhagic lesions which result from the reaction of host-factors and the determinants responsible for virus virulence and virus clearance (Poonia *et al*., 2005).

The IBDV induced damage to humoral immunity is reversible. Antibody production correlates with the morphologic restoration of the bursal follicles. Mitogenic response of T cells returned to the normal levels. During the course of mitogenic inhibition, T cells of infected chicken also failed to secrete IL-2 upon *in vitro* stimulation (Sharma *et al* 1987; Kim *et al*., 1996).

Intra bursal T cells and T-cell-mediated responses play a significant role in viral clearance and promoting recovery from infection. They defend the host cell by reducing the viral burden but at the same time produce inflammatory cytokines and nitric oxide inducing factor that enhance tissues destruction and also delay the recovery process (Sharma *et al*., 2001).

Intrabursal T cells were activated by *in vitro* stimulation with IBDV. The activated cells had increased surface expression of chicken MHC class II molecule, Ia and IL-2 receptor CD25. In addition, these cells have an up regulated IFN-γ gene (Kim *et al*., 2000). Splenocytes exposed to IBDV produced nitric oxide inducing factor (IFN-γ) (Sharma *et al*., 2001). Intrabursal T cells inhibited the mitogenic response of normal splenocytes by 90%. This bursal T cell –induced mitogen inhibition was found to be dose-dependent and not MHC-restricted (Kim and Sharma 2000). In contrast to the bursal T cells, the splenocytes from IBDV exposed chickens did not have suppressive

activity(Kim and Sharma 2000). Mitogenic inhibition by bursal T cells is mediated by soluble factors, the nature of which is still unknown (Sharma *et al*., 2000). Chickens that survive the disease clear the virus and recover from its pathologic effects (Sharma *et al*., 2000). It has been shown that the more virulent the virus the stronger is the suppression of the humoral and cell mediated immunity (Shamaila, 2005). Virulent virus also produced a detectable NO production in serum (Shamaila, 2005).

2.7.1Target organsfor infectious bursal disease virus

The target organ for IBDV is the bursa of Fabricius (BF) at its maximum development, which is a specific source of mature B-lymphocytes in avian species (Cheville 1967; Okoye and Uzoukwu 1984; Saif, 1998; van den Berg *et al.,* 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi 2011; Mahgoub, 2012; Ingrao *et al.*, 2013). Bursectomy can prevent illness in chicks infected with virulent virus (Okoye and Uzuokwu, 1990; Hiraga *et al*., 1994). The severity of the disease is directly related to the number of susceptible cells present in the BF; therefore, the highest age susceptibility is between 3 and 6 weeks, when the BF is at its maximum level of development (Muller *et al*., 2003; Mbuko *et al*., 2010). This age susceptibility is extended in the case of vvIBDV infection (Nunoya *et al*., 1992; van den Berg *et al*., 1991and van den Berg, 2000). Depletion of lymphoid B cells in the Bursa of Fabricius after IBDV infection is due to both necrosis and apoptosis. Actively dividing, surface immunoglobulin M-bearing B cells are lysed by IBDV infection (Hirai and Calnek, 1979; Hirai *et al*., 1981; Rodenberg *et al*., 1994). Apoptosis, characterized by nuclear fragmentation and cellular breakdown into apoptotic vesicles also plays an important role in IBDV pathogenesis. A high level of apoptosis can be evidenced in peripheral

blood lymphocytes of chickens infected with serotype 1 IBDV (Vasconcelos and Lam, 1994).

Chickens infected with IBDV immediately after hatching develop a subclinical infection with atrophy of BF and B cell depletion (Hudson *et al*., 1975 and Winterfield *et al*., 1972). Chickens infected with IBDV when older than 12 weeks do not show clinical signs (Becht, 1980). The bursectomized chicken survives the IBDV infections lethal for normal chicken (Kaufer and Weiss, 1980).

High concentrations of antigens and high infectivity titres were found in BF of infected chickens, whereas only traces of antigen and low virus titres were detected in the thymus, spleen (Kaufer and Weiss, 1980) and peripheral blood (Muller, 1986; Burkhardt and Muller, 1987). *In vitro* infection studies have shown that IBDV replicates in the population of proliferating B cells (Muller, 1986 and Nieper; Muller, 1996) but not in very immature lymphobalsts (Beug *et al.*, 1979) or competent B cells (Becht, 1980).

Apathogenic serotype 2 strains do not replicate in lymphoid bursal cells or in other lymphoid cells (Nieper and Muller, 1996). Treatment of chicken with cyclophosphamide (CY) or surgical bursectomy at 4 weeks of age is known to prevent IBDV infection (Kaufer and Weiss, 1980). Bursectomized chickens did not show the disease and had transient lesions in the lymphatic tissues (Okoye and Uzoukwo, 1990). However, these chickens contained virus and produced antibodies against it. The virus concentrations in the bursectomized chickens were about 1000 times lower than the non-bursectomized chickens (Kaufer and Weiss, 1980; Okoye and Uzoukwo, 1990). The availability of a large number of bursal cells is an essential factor in the development of IBD (Ismail *et al*., 1987).

2.7.2Immunosuppression

Immunosuppression has been defined as a ‘‘state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease’’ (Dohms and Saif, 1984) and often a suboptimal antibody response (Lutticken, 1997). However, this definition considers more the consequences (increased disease incidence) than the causes, of which mechanisms, beyond the destruction of a specific cell type, are still not fully understood (Ingrao *et al*., 2013). The destruction of the BF by IBDV creates an immunosuppression, which will be more serious in the younger, and infected bird. In addition to its zootechnical impact and its role in the development of secondary infections, it may affect the immune response of the chicken to subsequent vaccinations, essential in all types of intensive farming (Ingrao *et al*., 2013). The immunosuppression has been most often evident using experimental models based on the measurement of humoral responses induced by different antigens such as Brucella abortus, sheep red blood cells, or Newcastle disease vaccines (Allan *et al*., 1972; Giambrone *et al*., 1976; Giambrone, 1979). The best assessment is clearly the measurement of vaccinal protection against a challenge infection by the Newcastle virus as described by OIE (2012). However, OIE only give a partial picture of the immunosuppression as, according to the clonal nature of immunity, it will depend on the number of NDV-specific clones that will be destroyed (OIE, 2012). Using OIE diagnostic tests, the most serious and long-lasting immunosuppression was described when day-old chicks were infected by cIBDV (Allan *et al*., 1972; Faragher *et al*., 1974) with duration up to the age of 6 weeks (Giambrone *et al*., 1976). In field conditions, chickens tend to become infected toward the age of 2– 3 weeks, when

maternal antibodies decline(Guittet *et al*., 1992). Unfortunately, these techniques are time-consuming, laborious, costly, and contrary to animal welfare(Guittet *et al*., 1992). Thus, they are usually confined to the evaluation of safety in the IBDV vaccine registration procedures (Guittet *et al*., 1992). Developments in the field of avian immunology should allow moving in the future from a purely descriptive definition of immunosuppression to the analysis of the immunocompetence with a closer relationship between structure and function of the implicated immune cells and organs(Giambrone *et al*., 1976). In any case, recovery from IBD or subclinical infection will be followed by immunosuppression with more serious consequences if the strain is very virulent and infection occurs early in life. Although the immunosuppression caused by IBDV is principally directed towards B-lymphocytes, an indirect effect on cell-mediated immunity (CMI) has also been demonstrated (Cloud *et al*., 1992a,b; Sharma and Fredericksen, 1987; Sharma *et al*., 1989; Rauw *et al*., 2007) therefore increasing the impact of IBDV on the immunocompetence of the chicken. The infiltrated T cells constituting the majority of the bursal population after IBDV infection are unresponsive to mitogen activation at days 4 and 9 dpi (McNeilly *et al*., 1999; Rauw *et al*., 2007). Moreover splenocytes from IBDV-infected chickens were also shown to be deficient in secretion of ChIL-2 (Kim *et al*., 1998; Sharma and Fredericksen, 1987; Ingrao *et al.*, 2013). It is now well accepted that macrophages are the central effector cells of the innate immune system and influence the nature of the adaptive immune response. In a study performed at 3 and 5 dpi, spleens of virus-exposed chickens had fewer macrophages than those of virus-free controls; the robust expression of proinflammatory cytokine transcripts, along with a decrease in macrophage numbers, suggest that IBDV activates and may lead to a reduction of

resident macrophages in vivo (Palmquist *et al*., 2006). However, it remains unclear how these changes play a role in immunosuppression (Schat and Skinner, 2008).

2.7.3Effect of infectious bursal disease virus on humoral immunity

Infectious bursal disease virus has a predilection for the immature (Sivanandan and Maheswaran, 1980) actively dividing B lymphocytes and causes lytic infection of IgM bearing B cells resulting in decrease in circulating IgM^+ cells. Infected chicken produce less level of antibodies against the antigen (Kim *et al*., 1999). Only primary antibody responses are affected. Secondary responses remain unaltered (Sharma *et al*., 1989; Rodenberg *et al*., 1994). Infectious bursal disease virus induced humoral deficiency is reversible and overlaps with the restoration of bursal morphology (Sharma *et al*., 2000).

Chickens infected with IBDV at 1 day of age were found to be completely deficient in serum immunoglobulin G and produced only a monomeric immunoglobulin M (IgM) (Ivanyi, 1975; Ivanyi and Morris, 1976). IgG levels varied depending on the age at the time of infection (Hirai and Shimakura, 1979). The number of B cells in peripheral blood was reduced after infection with IBDV but T cells were not appreciably affected (Hirai and Shimakura, 1979). The adverse effect on antibody responses is due to the damage to the B cells in the bursa and the blood. Since the virus has a predilection for actively dividing B cells as compared to the mature B cells (Sivanandan and Maheswaran, 1980).

2.7.4Effect of infectious bursal disease on cellular immunity

The extent of which the cellular immune response is affected is not well understood(Shamaila, 2005). However, it is known that the effect of IBDV on CMI is transient and less pronounced than the effect on humoral response (Shamaila, 2005). Infected chickens show a poor cellular response to certain antigens and show increased susceptibility of disease that are under the control of cellular immune defence (Anderson, 1977). The thymic lesions were transient and appeared within the first week of infection peaked at 3-4 days post inoculation (PI) and then subsided (Okoye and Uzoukwo, 1990). The presence of thymic lesions were not associated with active viral replication of the virus in the thymic cells as shown by the immunoflorescence (IF) and antigen capture ELISA(Confer *et al*., 1981). In addition, T cells from infected chickens during the early stages of virus infection fail to respond optimally to mitogens *in vitro* (Confer *et al*., 1981).

Maximum depression in the cellular immunity was shown to occur at 6 weeks post-infection by using the lymphoblast transformation assay(Confer *et al*., 1981). The reason for the delay in this response is not clear considering that the virus persists in the host for approximately 3 weeks(Confer *et al*., 1981). It was speculated that this depression is the overall depression of T-cell function during the virus infection (Sivanandan and Maheswaran, 1981). The effect of IBDV on two CMI functions i.e. natural killer cell cytotoxicity and mitogenic response had been studied. It was reported that IBDV had an inconsistent effect on the natural killer cell cytotoxicity but caused a transient early depression of the blastogenic response of spleen cells to phytohaemaglutinin (Sharma and Lee, 1983). *In vitro* mitogen hypo-responsiveness of T cells is mediated by the suppressor cells in the spleens of the infected chicken; the mechanism of reduced *in vivo* cellular immunocompetence is not known (Lam, 1991).

Infectious bursal disease infected chickens were shown to have a normal natural killer cell levels, mononuclear phagocytic activity and delayed –type hyper sensitivity reaction (Giambrone, 1977; Hudson *et al*., 1975). Neither did virus infection alter the normal proportions of CD4 and CD8 subsets of T cells in the circulation and spleen (Sharma *et al*., 1973).

It was reported that variant A strain of IBD had a significantly higher effect on CMI as compared to the standard Edgar strain when given to 1-day-old chicken which lingered on till 5 weeks(Sharma *et al*., 1973). A similar effect was reported in chicken infected at 3 weeks of age (Craft *et al*., 1980).

Harderian gland is another component of immune system associated with the local immune system of the oculonasal duct and respiratory tract. IBDV infection of 1-5 day-old chickens produced a dramatic decrease in plasma cell content of the harderian gland that lasted till 7 weeks (Dohms *et al*., 1981). Broilers infected with IBDV at 3 weeks of age had reduced antibody titres to Brucella abortus (T cell independent antigen) and sheep red blood cells (SRBC, a T cell dependent antigen) in extracts from harderian gland and serum(Craft *et al*., 1980). Decreasing antibody responses to B. abortus were evident at a later time as compared to SRBC antibody response (Craft *et al*., 1980).

2.7.5Mechanism of immunosuppression

Reduction in the number of B cells in the BF due to viral infection is the major cause of immunosuppression (Abdu, 1985; van den Berg, 2000). Suppression of B cell function might be caused by damage to helper T cells or other cells involved in generating the immune responses (Sharma and Metz, 1989). Chickens infected with

IBDV have suppressor cells in the spleen, which cause *in vitro* mitogenic hypo responsiveness to concavalin A(Sharma and Metz, 1989). These cells prevent normal spleen cells from responding to the mitogen (Sharma and Fredericksen, 1987). The impairment of T cells and development of suppressor cells (Sharma and Fredericksen, 1987) was demonstrated *in vitro* by using proliferation tests (Confer *et al.*, 1981;Confer and MacWiliams, 1982; Ingrao *et al.*, 2013) or by measuring the cytokine release after mitogen activation of T cells (Lambrecht *et al.*, 2000).

2.7.6Apoptosis

Apoptosis, or programmed cell death, is a process where, in response to specific stimuli, cells die in a controlled, programmed manner(Shamaila, 2005). Besides lymphocyte lysis, apoptosis also plays a role in immunosuppression (Vasconselos and Lam, 1994; Ojeda *et al*., 1997; Tanimura an Sharma, 1998). Apoptosis could occur in a variety of organs (Allan *et al*., 1997) like thymus (Inoue, 1994) BF and spleen (Vasconselos and Lam, 1995; Lam, 1997). The CEF and Vero cells infected with IBDV show the biochemical features of apoptosis (Tham and Moon, 1996). In addition to causing necrosis, IBDV can also induce apoptosis in avian lymphocytes *in vitro* (Vasconselos and Lam, 1994). Viral proteins like VP2 and VP5 have been implicated in the induction of apoptosis (Fernandez-Arias *et al*., 1997; Yao *et al*., 1998). Expression of VP243 polyprotein in transiently transfected DT40 B lymphocyte culture suppresses cell growth and proliferative responses to mitogen stimulation indicating that IBDV polyprotein is a mediator of immunosuppression (Peter and Wu, 2004). Apoptotic cells have also been observed in antigen negative

bursal cells indicating that immunological mediators like cytokines might be involved in the process (Tanimura an Sharma, 1998).

Many different cell species can undergo apoptosis but immature B and T cells are particularly susceptible to apoptotic cell death. Only 20% of the lymphoid cells in the BF contain replicating IBDV (Shamaila, 2005). The severe damage to the bursa can be ascribed to apoptosis (Muller, 1986; Burkhardt and Muller, 1987). In addition to necrosis, marked atrophy of the BF occurs without eliciting an inflammatory response that is a characteristic sign of the apoptotic process (Burkhardt and Muller, 1987). Replication of the virus in bursa of Fabricius results in secondary viraemia thus spreading the virus to other tissues. The IBDV infection of a susceptible chicken has been shown to induce apoptosis in the bursa as well as thymus (Vasconcelos and Lam, 1995; Ojeda *et al*., 1997; Tanimura and Sharma, 1997; Tanimura and Sharma, 1998).

Morphological and biochemical features of apoptosis were also observed after *in vitro* infection of IBDV in chicken peripheral blood lymphocytes (Vaconcelos and Lam, 1994) and chicken embryo fibroblasts (Than and Moon, 1996). Apoptosis occurs in lymphocytes of various organs like thymus (Inoue, 1994) bursa and spleen (Lam, 1997). Some researchers believed that apoptosis induced by IBDV in cell cultures following *in vitro* infection was an early genetic response of the host cells and was independent of virus replication while others showed that appearance of CPE coincided with virus replication (Than and Moon, 1996). Jungmann *et al*. (2001) however, showed that proportion of apoptotic cells increased from 5.8 % at 4 hrs post infection (p.i) to 64.5% at 48 hrs p.i. in CE cells after infection with IBDV strain Cu-1 (Jungmann *et al*., 2001). However, treatment of CE cell cultures with UV inactivated IBDV did not induce apoptosis.

Whether apoptosis is triggered via virus receptor activation is not yet known (Jungmann *et al*., 2001). Double labelling technique revealed that during the course of early infection maximum number of antigen- expressing cells were not apoptotic. It was only later in the infection that the double-labelled cells appeared (Jungmann *et al*., 2001). Double labelling technique determined the distribution of both apoptotic cells and cells containing viral antigen in the same section of BF (Jungmann *et al*., 2001).

Double labelling studies for apoptotic or antigen positive cells revealed that apoptosis in the bursa of Fabricius occurs both in IBDV positive and IBDV negative cells (Tanimura and Sharma, 1998) whereas apoptosis in the thymus occurs in the antigen negative cells only (Tanimura and Sharma, 1998). It was concluded that IBDV induced apoptosis indirectly in non-bursal organs(Tanimura and Sharma, 1998). It has been postulated that IBDV impairs the withdrawal of apoptotic cells and therefore results in the increased number of the apoptotic cells (Ojeda *et al*., 1997). Apoptotic cells were located mostly in an area between the cortex and medulla whereas majority of cells positive for viral antigens were found in the medulla(Tanimura and Sharma, 1998).

Indirect mechanisms might also be involved in the induction of apoptosis and could have induced apoptosis in vivo resulting in rapid depletion of cells in BF (Jungmann *et al*., 2001). In the infected follicles large numbers of cells were apoptotic but very few contained the viral antigen (Tanimura and Sharma, 1998; Jungmann *et al*., 2001). Interferon production occurs after IBDV infection and is thought to be the major apoptosis–inducing factor in the neighbouring cells along with TNF-α (Jungmann *et al*., 2001).

Viral protein VP2 and VP5 have been implicated to play a role in apoptosis. The VP2 induced apoptosis in mammalian cells but not in CE cells (Fernandez-Arias *et al*., 1997). The VP5 deletion mutant of IBDV induced less apoptotic cells in infected CE cells and replicated slower than the parental strain (Yao and Vakharia, 1998). A correlation exists between virus replication and apoptosis in cells of BF (Nieper *et al*., 1999).

2.8Persistence of infectious bursal disease virus in organs

The bursa and spleen were reported to have substantially higher concentration of the virus as compared to any other tissue(Winterfield *et al*., 1972). The bursa harvested from chickens at 72 hours pi yielded high virus titres followed by the spleen and kidneys. No virus was detected beyond 10 dpi(Winterfield *et al*., 1972). IBDV was reported to persist in the chicken for a few days but the lesions could be seen for at least 10 weeks, the longest interval evaluated in that study (Winterfield *et al*., 1972).

The IBDV was re-isolated most consistently from the bursa and less frequently from thymus, liver, kidneys, lung and spleen(Mackenzie and Spradbrow, 1981). No virus was isolated from the pancreas (Mackenzie and Spradbrow, 1981). The virus was not detected beyond 11 dpi in commercial chickens when inoculated at 1,7 or 14 days of age(Mackenzie and Spradbrow, 1981). In birds inoculated at 21 days of age, IBDV was re-isolated till 8 days only(Mackenzie and Spradbrow, 1981). The precipitating antigen was detected only in the bursa and only at 3rd, 4th and 5th dpi but was not detected in any organ of chicken infected at 21 days of age (Mackenzie and Spradbrow, 1981).

When chickens were inoculated with an attenuated cell culture adapted virus at one day of age, the virus could be detected in cell culture inoculated with homogenate of BF, spleen, thymus, liver, kidney and lung for up to 14 dpi in one experiment and 10 dpi in another (Skeeles and Lukert 1979). No virus was detected after the levels of neutralizing antibodies became significantly high (Skeeles and Lukert 1979). Also, the virus was not detected in tissues from birds given the virus at 3 weeks and low virus neutralizing antibody titres were detected, indicating an age resistance to CEF virus or insufficient dose (Skeeles and Lukert, 1979).

When SPF chickens were inoculated with IBDV at 3 weeks of age, the viral RNA was detected by RT/PCR up to 21 dpi, but attempts to isolate infectious virus from bursal homogenates failed. Infectious virus was detected by embryo inoculation up to 7 dpi in the bursa of SPF chickens inoculated at 2 or 3 wk of age, whereas the viral RNA was detected by RT/PCR for up to 28 dpi(Abdel-Alim and Saif, 2001). In SPF chickens inoculated at 1 day of age, the bursa-derived virus or its RNA was detected at 7 and 14 dpi when inoculated at a high dose of 10^{4} EID_{50}/bird or at a low dose of $10^{2.5}$ EID_{50}/bird(Abdel-Alim and Saif, 2001).

In commercial 1-day-old broiler chickens, the bursa-derived virus was detected at 7 and 14 dpi when inoculated at a high dose (10^{4} EID50/bird), whereas the virus was detected only at 14 dpi when inoculated at a low dose ($10^{2.5}$ EID50/bird). In SPF and commercial chickens, vaccinated with a modified live IBDV vaccine, the virus is known to persist in the bursa of SPF chickens till 3weeks but maternal antibodies in the commercial chickens rapidly eliminate it from the bursa since no live vaccine

virus nor its RNA was detected in commercial broilers vaccinated at 1 day or 2 wk of age (Abdel-Alim and Saif, 2001).

2.9Pathology of Infectious Bursal Disease

2.9.1Gross pathology

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the virus (Rosenberger and Cloud, 1986; Tanimura *et al.,* 1995). Infected birds are dehydrated and have darkened discoloration of pectoral muscles. Individuals that die or are sacrificed approximately four days after infection show a doubling in size of the bursa due to oedema (Tanimura *et al.,* 1995). A straw-coloured viscous transudate may surround the organ, which is pale yellow in colour and shows striations (Ley *et al.,* 1979). On section, the bursa is hyperaemic and intrafollicular haemorrhages may be present (Helmboldt and Garner, 1964). From the fifth day after infection the organ decreases in size and within eight days may be only one-third of the weight of the bursae of unaffected chickens (Lukert and Saif, 1991; 2003). Haemorrhages occur in thigh and pectoral muscles and are also reported from the mucosa at the proventriculus-ventriculus junction and on the serosal surface and plica of the bursa (Hanson, 1962). There is increased mucus in the intestine and renal changes are observed in diseased birds, that is due to dehydration (Lukert and Saif, 2003).

The bursa of Fabricius is the target organ for the replication of IBDV and hence the most severely affected (Cheville 1967; Okoye and Uzoukwu 1984; Abdu *et al.,* 1986; Saif, 1998; van den Berg *et al.,* 2000; Lukert and Saif, 2003; Mac Lachlan and Dubovi 2011; Mahgoub, 2012).

In a detailed study using the Edgar strain of the virus, Cheville recorded the bursal weights for 12 days post inoculation (Mahgoub, 2012). The bursa began to increase in size and weight due to oedema and hyperaemia on 3 dpi and by day 4 it doubled in size(Cheville, 1967; Lukert and Saif, 2003; Mahgoub, 2012). By day 5, the bursa returned to its normal weight and from day 8 it atrophied further and became one-third its original weight (Cheville, 1967; Lukert and Saif, 2003; Mahgoub, 2012).

By day 2 or 3 post-infection, the bursa had a gelatinous yellowish transudate covering the serosal surface. Longitudinal striations became prominent and the colour changed from white to creamy. The transudate disappeared as the bursa returned to its normal size and the organs turned gray during the period of atrophy (Lukert and Saif, 2003).

The Delaware variant strains of type 1 IBDV do not cause acute bursal enlargement but infection results in rapid and profound atrophy (Rosenberger and Cloud, 1986; Rosenberger *et al.*, 1987). Bursa1 size is usually quantified as a ratio of bursal (numerator) to body mass (denominator) (Lucio and Hitchner, 1979). Various authors quote ratios based on different formulae. Most papers incorporate a factor (10^2 or 10^3) to compensate for the numerical difference between body and bursal mass expressed in grams (Lasher and Shane, 1994). General comparisons cannot be made among publications unless the same factor is applied, but bursal atrophy can be characterized in a specific trial using a common formula to calculate bursal to body ratio (Lasher and Shane, 1994). In a trial reported by van den Berg *et al.*(1991) a bursa:body weight ratio of 1.3 was determined in specific pathogen free Leghorn pullets 10 days after challenge with vvIBDV, strain 849VB. In contrast, non-infected, unvaccinated male and female controls yielded a bursa: body ratio of 5.9. Similar results were shown by Mazariegos *et al.* (1990) who infected l-day-old specific pathogen free chicks with

Edgar strain IBDV which resulted in a bursa1: body weight ratio of 2.3 at 14 days compared to controls with a value of 10.

Pathologic changes in the spleen and thymus were less prominent than those of the bursa (Cosgrove, 1964; Inoue *et al*., 1994; Lukert and Saif, 2003 and Okoye, 2005). The spleen might be slightly enlarged and usually had small gray foci uniformly dispersed on the surface (Lukert and Saif, 2003). Lesions in these organs were noticed at the same time as the changes occurred in the bursa and resolved within 1 or 2 days of appearance (Helmboldt and Garner, 1964).

Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules, visible as white flecks beneath the capsule (Cosgrove, 1962; Hitchner, 1978; van den Berg *et al.*,2000). The renal lesions were prominent in early outbreaks which were described in the USA (van den Berg *et al.,* 2000). This is attributed to the fact that cases of IBD were coinfected with nephropathogenic strains of avian infectious bronchitis (Hitchner, 1963). Renal changes which are observed in carcasses and in chickens sacrificed *in extremis* represent water deprivation associated with recumbency (Lukert and Saif, 1991).

The very virulent infections are characterized by severe clinical signs, high mortality, and a sharp death curve followed by rapid recovery (van den Berg *et al.,* 2000). The vvIBDV strains have the same clinical signs and incubation period of 4 days as classical viruses but the acute phase is exacerbated (van den Berg, 2000). The vvIBDV strains cause more severe lesions in the caecal tonsils, thymus, spleen and bone marrow and a greater decrease in thymic weight index as compared to the moderately pathogenic strains but bursal lesions are similar (van den Berg *et al.,* 2000). It has been shown that the pathogenicity of field strains of IBDV correlate with

lesion production in non-bursal lymphoid organs(Tanimura *et al*., 1995). The results also suggest that pathogenicity of IBDV may be associated with virus antigen distribution in non-bursal lymphoid organs (Tanimura *et al*., 1995).

Splenic enlargement was documented by Morales and Boclair (1993) who showed highly significant differences in bursa:spleen weight ratio of 2.4 for controls compared with 0.9 in chicks seven days after challenge. 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 (van den Berg, 2000).

2.9.2Histopathology of infectious bursal disease

Histopathologic lesions occur in the bursa, spleen, thymus, harderian gland and caecal tonsils. The first signs of infection occur in the bursa and it is the most severely affected organ. Degeneration and necrosis of individual lymphocytes in the medullary region of the bursa occur as early as 1dpi (Lukert and Saif, 2003). Lymphocyte degeneration is accompanied by nuclear pyknosis and formation of lipid droplets in the cytoplasm (Cheiville, 1967). Degenerating lymphocytes are surrounded by macrophages. Lymphocytes are soon replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial cells(Cheiville, 1967). By day 3 or 4 postinfection, all of the lymphoid follicles are affected. Severe oedema, hyperaemia, and marked accumulation of heterophils are evident, which cause the increased bursal weight (Lukert and Saif, 2003; Mahgoub, 2012). Cystic cavities develop in the follicular medulla. These cystic cavities are caused by necrosis and phagocytosis by heterophils

and macrophages. During the stage of bursal atrophy, fibroplasia of the bursal tissue becomes evident(Mahgoub, 2012). In addition, the bursal epithelium becomes proliferative, forming a glandular-like structure, which consists of the bursal columnar epithelium containing globules of mucin(Mahgoub, 2012). In the late stages, scattered lymphocyte foci appear without the ability to form functional follicles (Mahgoub, 2012). Permanent loss of lymphocytes in the bursa leads to atrophy, premature involution of the organ and immunosuppression which may be transient or permanent in birds that recovered from IBD (Okoye, 1983; Okoye and Shoyinka, 1983; Okoye, 2005).

The thymus exhibited some cellular reaction in the lymphoid tissues. Evidence of lymphocyte necrosis in the inner cortex appears at 2dpi in 4-day-old and 2-week-old chickens infected with IBD (Inoue *et al.*,1994). Many altered lymphocytes where detected throughout the entire cortex and lymphocyte depletion was prominent in areas of the cortex at 3 and 4dpi(Inoue *et al.,* 1994). Lymphocyte depletion was observed at 5 dpi in the whole cortex. At 7 dpi, cortical atrophy was greatest but visible lymphocytes remained focally and diffusely in the outer cortex(Inoue *et al.,* 1994). At 14 dpi, the cortex showed apparent repopulation and recovery. In the medulla, macrophage and plasma cells infiltration and haemorrhage were prominent from 5 to 7 dpi (Mahgoub, 2012).

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 the spleen and thymus unlike in the bursa of Fabricius (Ley *et al.*, 1983; Okoye, 1984; Lukert and Saif, 2003).

The harderian gland is reported to be severely affected by the virus in 1 day old chickens (Skeeles and Lukert, 1979). Normally, the gland is populated with plasma cells as the chicken ages but the infection prevents this infiltration(Skeeles and Lukert, 1979). Harderian gland of the chickens infected at 1 day of age has 5-10 folds fewer plasma cells than those of uninfected chickens from 1-7 weeks of age (Dohms *et al*., 1981). However, lymphoid follicles and heterophil populations in the harderian gland are not affected by IBDV infection, nor could necrotic or degenerative changes be found in the acini or excretory ducts(Skeeles and Lukert, 1979).

In contrast, the broilers infected at 3 weeks of age have a 51 % reduction in plasma cell content at 5-14 dpi. (Helmboldt, 1964; Dohms *et al*., 1988). Plasma cell numbers reduction was temporary and levels became normal after 14 days. Histologic lesions appearing in the kidneys were nonspecific and resulted from dehydration (Helmboldt, 1964). The liver had some slight perivascular infiltration of monocytes (Parkhurst, 1964).

Indeed, using various immunostaining methods, a higher frequency of antigen-positive cells could be demonstrated after infection of birds with vvIBDV than with other strains, in the thymus (Nunoya *et al*., 1992; Sharma *et al*., 1993; Inoue *et al*., 1994), the spleen and the bone marrow (Tanimura *et al*., 1995; Tsukamoto *et al*., 1995; Inoue *et al*., 1999;). In particular, atrophy of the thymus has been associated with the acute phase of the disease and might be indicative of the virulence of the isolate, although it is not associated with extensive viral replication in thymic cells (Sharma *et al*., 1993).

The ability to cause histological lesions in the non-bursal lymphoid organs, such as the thymus (Inoue *et al.,* 1994), the spleen or bone marrow (Inoue *et al.,*1999), has been reported as a potential characteristic of hypervirulent IBDVstrains. The histological approach has the advantage of allowing for diagnosis of both the acute and chronic or subclinical forms of the disease (van den Berg, 2000).

Interstitial haemorrhages and perivascular accumulations of lymphoid cells, oedema, tubular necrosis and glomerular nephrosis in the kidney of IBDV-infected birds have been reported (Mandelli *et al.,* 1966; Del Bono *et al.,* 1968; Ley *et al.,* 1983). Homogenous materials sometimes infiltrated by heterophils and cellular debris have also been observed in the tubules and ureters of such birds (Helmboldt and Garner 1964; Del Bono *et al.,* 1968; Ley *et al*., 1983).

Blood lymphocytes of chickens infected with IBDV by subconjunctival route were found to possess fewer nucleoli than those of uninfected (Klucinski *et al.,* 1984). As from the 3rd day after infection, the percentage of lymphocytes without lysosomes increased whereas those with large single and large multiple lysosomes decreased (Klucinski *et al.,* 1984). Microscopic changes observed in smears of tissue and blood following ocular application of IBDV were, nuclear vacuolation and chromatolysis associated with cytoplasmolysis in lymphocytes, macrophages and reticular cells of the BF and spleen, lymphocytes and macrophages of the blood; and cytoplasmic vacuolation in the kidney. No changes were detected in the liver (Bayoumi *et al.,* 1984).

2.9.3Ultrastructural Changes

Sequential morphologic changes in surface epithelium of the bursa were observed by scanning electron microscope after oral inoculation of 1-day-old chickens with IBD virus. A reduction in the number and size of microvilli occurred at 48 hours post inoculation. There was a gradual loss of button follicles at the surface and by 72 hours PI most have involuted. By 96 hours PI many epithelial cells showed erosion. The surface was intact by day 9 PI, but follicles were involuted, leaving deep pits (Naqi and Miller, 1979).

2.10Diagnosis of Infectious Bursal Disease

2.10.1Clinical and differential diagnosis

The clinical diagnosis of the acute forms of IBD is based on disease evolution (a mortality peak followed by recovery in five to seven days), and relies on the observation of the clinical sings and post-mortem examination of the pathognomonic lesions, in particular of the bursa of Fabricius (Lukert and Saif, 2003). The conditions most likely to be clinically mistaken for IBD are avian coccidiosis, Newcastle disease in some visceral forms, stunting syndrome, chicken infectious anaemia, mycotoxicoses and nephropathogenic forms of infectious bronchitis, inclusion body hepatitis and lymphoid leukosis(Barron *et al.*, 1966; Faragher, 1972; Okeke, 1984). In all acute cases, the presence of bursal lesions allows for a diagnosis of IBD (Lukert and Saif, 2003). In subclinical cases, an atrophy of the bursa may be confused with other diseases such as Marek's disease or infectious anaemia. A histological examination of the bursa will allow differentiation between these diseases (Lukert and Saif, 1997).

Sudden onset, morbidity, ruffled feathers and droopy appearance of the birds in initial disease outbreaks are suggestive of acute outbreaks of coccidiosis(Lukert and Saif, 2003). In some cases there is blood in the dropping that would lead one to suspect coccidiosis(Lukert and Saif, 2003). The muscular haemorrhages and enlarged oedematous or haemorrhagic bursa of Fabricius would however, suggest IBD (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(Jakowski *et al.*, 1969). Visceral form of Newcastle disease has been observed to cause severe degeneration of lymphocytes in the medullary region of the bursa of Fabricius (Okoye, 2000; Lukert and Saif, 2003) but necrosis and later replacement of lymphocytes by heterophils are additional features of IBD.

2.10.2Histological Diagnosis

Histological diagnosis is based on the detection of modifications occurring in the bursa and bursal lymphoid organs (van den Berg, 2000). The ability to cause histological lesions in the non-bursal lymphoid organs, such as the thymus (Inoue *et al*., 1994), the spleen or bone marrow (Inoue *et al.,* 199), has been reported as a potential characteristic of hypervirulent IBDV strains(Inoue *et al*., 1994). The histological approach has the advantage of allowing for diagnosis of both the acute and chronic or subclinical forms of the disease (van den Berg, 2000).

2.10.3Virus Detection

The virus can be found in other organs such as the thymus, liver and bone marrow but in significantly low quantities than in the bursa (Cheville, 1967 and Solano *et al*.,

1985). The inoculum for virus isolation is prepared by homogenizing the tissue sample in antibiotic containing buffer that is centrifuged to remove larger tissue particles and is used for inoculating embryonated eggs and tissue culture (Lukert, 1986).

The chorioallantoic route of inoculation in 9-11 days old embryos is the most sensitive route for the isolation of the virus(Hitchner, 1970 and Lambrecht, 2000). Classic viruses usually kill the embryos in 3-5 days and produce lesions of vascular congestion and subcutaneous hemorrhages in the embryos (Hitchner, 1970 and Lambrecht, 2000). Variant viruses however, do not kill the embryos but cause embryo stunting, discoloration, splenomegaly and hepatic necrosis (Lukert, 1986).

Primary cell cultures of chicken embryo fibroblasts (CEF), bursa (CEB) and kidney (CEK) have been used to propagate the virus. Macreadie *et al.* (1990) reported that 3 out of 7 chicken isolates failed to grow in chicken embryo fibroblast (CEF) cells but propagated well in embryonating eggs. Some strains grow well in embryos but are not readily adapted to grow in CEF or CEK (Lasher, 1997).

Nowadays, reverse transcription-polymerase chain reaction (RT-PCR) is a molecular tool frequently applied in IBDV diagnosis. RT-PCR in combination with restriction enzyme analysis allows for rapid identification of vvIBDV (Lin *et al*., 1993; Jackwood and Jackwood, 1994; Zierenberg *et al*., 2001). Restriction fragment length polymorphism (RFLP) has also been used to form six different molecular groups of IBDV (Ture *et al*., 1998; Jackwood and Sommer, 1999). Nucleotide sequencing of RT-PCR products is widely used for further characterization of IBDV strains (Sapats and Ignjatovic, 2000; Zierenberg *et al*., 2000; Islam *et al*., 2001a; Liu *et al*., 2002; Viswas *et al*., 2002). Most RT-PCR protocols are based on VP2 nt sequences. Only

recently, protocols based on the VP1 gene (Raue and Mazaheri, 2003; Tiwari *et al*., 2003) as well as real-time RT-PCR protocols were published (Moody *et al*., 2000; Raue and Mazaheri, 2003). Real-time RT-PCR has been applied to identify IBDV quasispecies using fluorescence resonance energy transfer (FRET) in a two probe system (Jackwood and Sommer, 2002). An in situ RT-PCR was developed to investigate early stages of infection in the IBDV-infected BF (Zhang *et al*., 2002).

2.10.4Serological test

Serological tests generally used for the detection of IBDV are ELISA, VN and AGP. The ELISA is the most commonly used test for the detection of antibodies to IBDV(Lukert, 1986). It is economical, simple, quick and tests a large number of samples at the same time and is adaptive to automation to computer software (Lukert, 1986).

The ELISA allows the quantification of antibodies to IBDV and is therefore used for monitoring the immune status of the chicken flocks (Lukert and Saif, 2003), to check response to vaccination, natural field exposure and decay of maternal antibody titre (Lasher and Shane, 1994 and Lukert and Saif 2003). However, ELISA cannot differentiate between the antibodies specific to the two serotypes (Ismail and Saif, 1990; Lukert and Saif, 2003). Therefore, while using ELISA for monitoring the chicken flocks for antibodies to IBDV, careful consideration should be given to the fact that the serotype 2 viruses are widespread in commercial chickens and could result in erroneous impression of antibody levels of the flock (Abdel-Alim and Saif, 2001).

There are several commercially available ELISA kits for the detection of antibodies in the serum. Sub-clinical infections and any outbreak of the disease in the large poultry production units could have significant economic impact; therefore the flocks are regularly monitored for any change in the baseline titre(Lukert, 1986).

The VN titres accurately reflect the relative protection of chickens to IBDV (Mcferran *et al*., 1980; Jakwood *et al*., 1985; Jackwood and Saif, 1987; Ismail and Saif, 1990). It is essential to use appropriate indicator viruses in VN tests to avoid artificially low titres due to the existence of several antigenic variants (Ismail and Saif, 1990). Most chicken sera have high levels of neutralizing antibodies to a broad spectrum of antigenically diverse viruses due to vaccine and field exposure (Jackwood and Saif, 1987). However, VN is laborious and time consuming and therefore its use is limited to research applications. Although *in vitro* VN tests can be used for detection of antigenic differences between the virus strains, *in vivo* cross protection studies are essential for determining immunogenicity of the virus and complete evaluation of host response (Jackwood and Saif, 1987).

Another method used to detect antibodies to IBDV is the agar gel immunodiffusion (AGID) test(Cullen an Wyeth, 1975). This test has been adapted to the quantitative format (Cullen an Wyeth, 1975). It is rapid but insensitive. It does not detect serotypic differences and measures primarily group-specific soluble antigens (Lukert, 1986).

2.10.5Virological Isolation

Infectious bursal disease virus may be detected in the bursa of Fabricius of chicks in the acute phase of infection, ideally within the first three days following the appearance of clinical signs (van den Berg, 2000).

Isolation of the virus is achieved when filtered homogenate of the bursa of Fabricius is inoculated in nine- to eleven-day-old embryonated eggs originating from hens free of anti-IBDV antibodies (Lukert and Saif, 1997). The most sensitive route of inoculation is the CAM; the yolk sac route is also practicable, and the intra-allantoic route is the least sensitive (van den Berg, 2000). The specificity of the lesions observed must be demonstrated by neutralising the effect of the virus with a monospecific anti-IBDV serum. Isolation in embryonated eggs does not require adaptation of the virus by serial passages, and is suitable for vvIBDVs. In the absence of lesions, the embryos from the first passage should be homogenised in sterile conditions and clarified, and two additional serial passages should be performed (Hitchner, 1970; Lukert and Saif, 1997 and Rosenberger, 1989).

The viral antigens specific to IBDV may be detected by direct and indirect immunofluorescence (Allan *et al*., 1984; Meulemans *et al*., 1977) or by immunoperoxidase staining (Cho *et al*., 1987) in the bursal follicles of infected chickens between the fourth and sixth day after inoculation. No viral antigen is detectable from the tenth day (Vindevogel *et al*., 1976). However, the virus can be isolated from bursae sampled from the second to the tenth day, with a maximum infectious titre after four days (Winterfield *et al*., 1972; Vindevogel *et al*., 1976). The use of monoclonal antibodies for detection of the virus enhances the specificity of the test (Cho *et al*., 1987).

2.10Economic Significance of Infectious Bursal Disease Virus

Uncomplicated IBDV infection with original type 1 isolates results in up to 90% flock morbidity and 20% mortality in susceptible 3-8-week-old hybrid Leghorn

replacement pullets (van den Berg *et al*., 1991; Nonaya *et al.*, 1992; Lasher and Shane, 1994 and Okoye, 2005). In broilers aged 3-6 weeks lower flock morbidity (50%) occurs following infection and primary mortality from IBD seldom exceeds 3% (van den Berg *et al*., 1991; Nounaoya *et al.*, 1992; van den Berg *et al*., 2000; Okoye, 2005). Exposure of flocks younger than 2 weeks of age is generally asymptomatic (Okoye and Shoyinka, 1983; Abdu, 1985; Nounaoya *et al.*, 1992; van den Berg *et al*., 2000; Okoye, 2005). Currently, widespread application of preventive vaccination of chickens and the transfer of maternal antibody from parents to progeny have reduced the impact of primary IBD infection in broiler and replacement layer flocks.

Until the emergence of vvIBDV in 1986 the significance of IBD was confined to the immunosuppressive effect of the virus (Allan *et al.*, 1972; Hirai *et al.*, 1974; Rosenberger; Gelb, 1976; Abdu, 1985). Both broiler and pullet flocks may become non-responsive to live attenuated vaccines against respiratory infections such as Newcastle disease and avian infectious bronchitis (Rosenberger; Gelb, 1978; Snyder *et al.*, 1986; van den Berg, 2000). In broilers, a high prevalence of viral respiratory infections and elevated mortality due to airsacculitis and colisepticaemia during the terminal third of the 6-8 week growing cycle are denoted by immunosuppression (Lasher and Shane, 1994). In addition, the plant condemnation rate due to septicaemia-toxaemia syndrome may be increased tenfold from 0.5% to over 5% in affected flocks (Lasher and Shane, 1994).

A 14% depression in financial return from broiler flocks with subclinical IBD compared with unaffected flocks surveyed in Ireland was documented by McIloy *et al.*(1989). Lasher and Shane, (1994) documented an 11% depression in net income in

flocks which showed serological evidence of IBD during a mean 42-day growing period, in comparison to non-exposed broilers. The 10% reduction in profit for the 991 flocks in the study affected with IBDV was attributed to relative depression in body mass and feed conversion efficiency, but not in liveability, compared with non-exposed flocks. Cold winter temperature exacerbated the difference in performance between IBDV infected and clinically unaffected flocks. The appearance of an IBDV strain of moderate pathogenicity on the Island of Mauritius in 1992 resulted in a 4% depression in broiler live mass. A 2-5% elevation in mortality was recorded, divided evenly between acute 18-25 day death from IBD and subsequent losses due to septicaemia during weeks 5-6 of the growing cycle (Lasher and Shane, 1994).

Generally, mortality from IBD is accepted to be higher in commercial hybrid Leghorn replacement pullets than in broiler strains (Benton *et al.*, 1967b; Winterfield and Thacker, 1978).In the early to mid-1960s, 20% losses were recorded following infection of susceptible flocks before vaccination became a routine industry practice (Lasher and Shane, 1994).

Exposure to IBDV impairs the response to vaccines administered after IBD-induced immunosuppression(Faragher et *al.*, 1974). Increased susceptibility to respiratory viruses, including Newcastle disease (Faragher et *al.*, 1974) and avian infectious bronchitis (Pejkovski *et al.*, 1979), leads to depression in egg production and deterioration in egg shell and internal quality in commercial laying flocks. Immunosuppressed breeder flocks may undergo a decline in egg production and hatchability following exposure to viral pathogens. Chick yield per breeder is diminished by reduced egg numbers and hatchability(Lucio and Hitchner, 1979). Performance of broiler progeny from immunosuppressed parent flocks is adversely

affected by relatively low maternal antibody transfer (Lucio and Hitchner, 1979). Infection with IBDV may exert a profound impact on the profitability of an integrated broiler operation by reducing efficiency and return from both parent and commercial generations (Lasher and Shane, 1994).

The advent of IBDV infection has imposed additional costs on the poultry industry through enhanced decontamination and preventive procedures(Lasher and Shane, 1994). Immunization programmes dictate the purchase, storage, and administration of both live and inactivated vaccines. Monitoring vaccination efficiency requires collection of blood samples and laboratory determination of antibody titre(Lasher and Shane, 1994). The capital investment in analytical equipment and computer software and in reagents, commercial kits, and laboratory personnel has become a significant but necessary component of veterinary costs (Lasher and Shane, 1994).

The emergence of highly pathogenic (very virulent) strains of IBDV (vvIBDV) in Europe during 1988 (Chettle *et al.*, 1989; van den Berg *et al.*, 1991) increased the financial impact of the disease on producers. Outbreaks of vvIBD resulted in up to 30% mortality in infected broiler flocks, especially in areas with a high density of poultry. In studies on wIBD van den Berg and Meulemans (1991) showed that infection of 38-day-old hybrid Leghorn pullets with 100 chicken LD50 of the 849 VB isolate resulted in 60% losses. In contrast, broiler chicks infected at the same age showed 17% mortality (Lasher and Shane, 1994).

In developing countries, the presence of IBD imposes a serious restraint to profitable production due to mortality and secondary losses due to immunosuppression. In areas with emerging industries, deficiencies in diagnostic facilities and non-availability of vaccine impose higher costs (Chulan, 1993).

In Nigeria, outbreaks of IBD in vaccinated flocks are frequent (Abdu, 1985; Abdu *et al.,* 2001; Okoye, 2005; Adamu *et al.*, 2013). These outbreaks have rendered investment in poultry farming fearful and unrealistic to individuals and organizations (Okoye, 1983; Abdu, 1986; Lukert andSaif, 1997; Musa *et al.*, 2010; Musa *et al.,* 2012). Unfortunately, the control of infectious bursal disease in Nigeria presents a complex problem because of its becoming highly endemic and ubiquitous (Nawathe and Lamorde, 1982). An estimated economic loss of over three billion naira was reported during a three year studies (2009-2011) by Musa *et al.* (2012).

2.11Prevention and Control of Infectious Bursal Disease

2.11.1Biosecurity and eradication

An important characteristic of IBDV is its high stability in the environment, even after disinfection(Benton *et al*., 1967). Indeed, the virus can persist in installations for 54–122 days (Benton *et al*., 1967). Due to the stable nature of the virus and the large amounts excreted following infection, it is practically impossible to remove all sources of infection once a rearing site has been contaminated. There is evidence, however, that thorough cleaning and disinfection of houses between flocks and the practice of all-in all-out farming methods, cleaning and disinfection of premises, and observance of a 'down time' (a period of rest between depopulation and restocking) (Heine and Boyle, 1993) can reduce infection rates. Given the very contagious nature of the disease and the resistance of the virus, certain essential steps in the cleaning/disinfection process should be adhered to(Benton *et al*., 1967). Prior to cleaning, all insects and pests (e.g. rats and mice) must be eliminated as soon as the farm premises are empty. Old bedding and dung must be eliminated and composted.

All farm equipment must be disassembled and stored in cleaning rooms located outside the farm buildings. The buildings, immediate surroundings and farm equipment must be dry-cleaned first, in order to eliminate all dust, and then hose down using hot water (60℃) with a detergent, at a pressure of 80 bar to 150 bar(Benton *et al*., 1967). A second disinfection of the full premises must be performed before the introduction of the chicks. Feed silos must be emptied completely and cleaned inside and outside. Under no circumstances may feed remains from previous flocks be reused. Disinfection is to be undertaken only after all the buildings have been cleaned. All disinfectants are more active at a temperature above 20℃; however, chlorinated and iodinated disinfectants cannot be heated above 43℃(Higashihara, 1991). The quantity of disinfectant solution to be used is approximately 4 litres per $15m^2$ (Higashihara, 1991) reduces the challenge virus. It may also delay challenge thus allowing more time for vaccines to induce immunity (Ingrao *et al*., 2013). Rigorous biosecurity measures have to be implemented in order to stop the spread of virus from one flock to the next(Higashihara, 1991). The virus is environmentally stable and resistant to many physical and chemical agents. Integrated nature of commercial poultry operations and vectors like lesser mealworm, mosquitoes and rats pose extra problems for the control of this infection (McAllister *et al.,* 1995; Howie and Thorsen, 1981; Okoye and Uche, 1986).

2.11.2Vaccination

In practice, control of IBD is greatly dependent upon the use of vaccines. Taking all the previous considerations into account, a satisfactory vaccine should protect against the disease, especially the acute phase, and the consequences of the disease, namely

immunosuppression(Ingrao *et al*., 2013). Humoral immunity plays a decisive role in protection against IBD (Ingrao *et al*., 2013). There is indeed a close correlation between titres in neutralizing antibodies on one hand and protection on the other hand(Ingrao *et al*., 2013). This is borne out by the excellent passive protection provided by maternal antibodies against mortality, lesions of the bursa and immunosuppression, respectively. The half-life of the passive antibodies, depending on blood volume, varies between 3 (for broilers) and 5 days (for laying hens)(Ingrao *et al*., 2013). However, vaccination can be disturbed by the interference of maternal-derived antibodies (MDA) (Block *et al*., 2007; Mahgoub, 2012 and Ingrao *et al*., 2013). Indeed, MDA are transmitted from the mother to her offspring through the yolk and protect the chicks until the development of the adaptive immune response (Davison, 2008). Thus, if one knows the antibody titre of a chick at hatch, one can determine the time of maximum susceptibility to the vaccine. This determination is very important when establishing vaccination programs (Abdu, 1985; De Herdt *et al*., 2005). Neutralizing antibodies are protective and these can be provided by immunizing chickens with live attenuated vaccines given in the drinking water(Yeh *et al*., 2002). In addition, a live vaccine will induce a strong CMI response, the role of which is still unclear but important (Ingrao, 2013). Protection in the absence of virus-specific antibodies and studies with T cell compromised chickens have indicated that functional T cells are needed to control IBDV replication during the acute phase of infection (Yeh *et al*., 2002; Rautenschlein *et al*., 2002). It has been recently shown that a dominant fragment of VP2 can induce humoral and cellular immunity against IBD and elicits a protective immune response in chickens better than the available attenuated viral strains and thus could be used as or in a vaccine (Pradhan *et al*., 2012).To obtain high levels of MDA in the progeny, parent stock are vaccinated

between 4 and 10 weeks of age with live vaccine and again at approximately 16-18 weeks with inactivated oil-adjuvanted vaccine. In the progeny, the MDA levels wane with time, but may protect against virulent challenge up to between 4 to 7, 2 to 5 and 3 to 5 weeks of age (Abdu, 1985; van den Berg, 2000; Mahgoub, 2012; Ingrao *et al*., 2013). Mild vaccine strains that cause no bursal lesions cannot be used effectively in chicks with MDA until about 4 weeks of age as they are neutralized. Moderately virulent vaccine ('intermediate') strains that are less affected by MDA can be given with some success as early as 2–3 weeks of age, depending upon MDA titres (Gardin *et al.,* 2014). As MDA levels may vary within a flock and between flocks repeated vaccination is practiced by some in order to ensure that chicks are actively immunized as soon as the MDA levels have waned to a level at which they do not neutralize the vaccine (Gardin *et al*., 2014). Intermediate and hot vaccine strains can induce bursal lesions and cause immunosuppression (Mazariegos *et al*., 1990). The intensive use of this kind of vaccines increased preoccupations about residual pathogenicity and about the decreasing effectiveness of vaccines against other diseases(Mazariegos *et al*., 1990).

Most commercially available conventional live IBDV vaccines are based on classical virulent strains(Mazariegos *et al*., 1990). Those classified as ''mild'' vaccines exhibit only poor efficacy in the presence of certain levels of maternally derived antibodies and against vvIBDV. 'Intermediate' and 'intermediate plus' or 'hot' vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions and, thus, cause corresponding levels of immunosuppression (Mazariegos *et al*., 1990; Tsukamoto *et al*., 1995; Kumar *et al*., 2000; Rautenschlein *et al*., 2005). They may not fully protect chickens against infection by the vvIBDV strains (Rautenschlein *et al*., 2005) or by

antigenic variants. Safety and efficacy of this type of vaccine still remain a major concern(Rautenschlein *et al*., 2005). In addition, it should be kept in mind that the practical onfarm administration of the conventional live vaccines to a large number of animals is also a technically demanding process, with difficulties inherent to farm-to-farm variability (variable chicks, variable farming conditions, variable skills in vaccination crews, variable vaccine efficacy and potencyetc.) that should not be underestimated when assessing the results of vaccination programmes (Muller *et al.*, 2012).

Vaccination in the presence of IBDV antibody levels above the breakthrough titre of the vaccine will lead to a significant delay of IBDV replication and the induction of immunity, as shown by laboratory investigations (McCarty *et al.*, 2005; Rautenschlein *et al.*, 2005). Other experimental studies have shown that IBD vaccine virus may even be completely neutralized by maternally derived antibodies (Abdu, 1985; van den Berg and Meulemans, 1991; Tsukamoto *et al.*, 1995; Alam *et al.*, 2002; Hair-Bejo *et al.*, 2004; Moraes *et al.*, 2005; Okoye, 2005). In order to have chickens protected against IBDV field challenge, it is crucial to determine the optimal timing for IBD vaccine delivery (Tsukamoto *et al*., 1995). The optimal timing is often predicted based on serological data following detection of IBDV MDA by an ELISA system during the first week post hatch (Kouwenhoven and van den Bos, 1992; 1994).

Three types of method have been developed to determine, as accurately as possible, the vaccination date based on the type of production and the level of antibodies usually present on day one:

1. Mathematical formulae: such as Kouwenhoven's formula and Deventer's formula, used to calculate the vaccination age for broilers or pullets based on

the level of antibodies at 1, 2 or 3 days of age, the vaccine used and the rate of decrease of the maternal antibodies (Pattison *et al.*, 1998; de Wit 1998; de Wit, 1999; Gardin 1994; de Wit, 2001).

2. Calculation based on the half-lives: the vaccination age is calculated based on the level of antibodies on a given date, and on the rate of decrease of these antibodies. This technique is applied particularly when blood samples are taken from birds between 6 and 10 days old (Ceva, 2005).
3. Central vaccination date (CVD) for pullets: A table has been compiled based on the serological monitoring of young pullets intended to become layers or breeders, and can be used to determine the vaccination ages as a function of the ELISA serological titre determined on day one. Pullets intended to become layers or breeders must be vaccinated twice due to the slow decline in maternal antibodies. The first vaccination is given three days before the calculated central vaccination date and the second vaccination is given three days after the CVD. The first vaccination immunises those birds which had less than average maternal antibodies on day one, and which consequently are receptive to the vaccine earlier than the other birds. The second vaccination immunises those birds which had an above average level of antibodies on day one and which are receptive later (Ceva,2005).

The “Deventer formula” was developed to estimate the optimal vaccination time point based on the half life time of the MDA, the age of the chicken at sampling, genetic background, breakthrough titre of the vaccine, and the requested percentage of the flock having antibody levels below the breakthrough titre of the vaccine at the time of administration (de Wit, 1998, 2001).

Vaccination age = {(log2 IBDV antibody ELISA titre of bird (%) - log2 breakthrough titre of the vaccine) x t 0.5} + age at sampling + correcting value 0 to 4.

Where; bird (%) is the titre of the bird (at sampling) that represents a certain percentage of the flock (in this study, 75%) that is desired to be susceptible to the vaccine at the time of the application; breakthrough isthe breakthrough (ELISA) titre of the vaccine to be used (for example Cevac® IBD L vaccine in the ELISA system used, a titre of 500);$t_{0.5}$ is the half-life timeof the antibodies (ELISA titre) in the type of chickens that were sampled (cockerels, 5.5 days); and correcting value 0 to 4 is the extra dayswhen the sampling was done at 0 to 4 days post hatch (de Wit, 2001).

When estimating the optimal age for vaccinating against IBD, a few important factors for the Deventer formula need to be considered as follows (de Wit, 2001):

(1) Number of samples per flock. A minimum of 18 samples per house is required to obtain a representative sample of the flock. Estimations based on less than 18 samples are less reliable. It is false economy to try and save money by taking only 10-15 samples per flock. If the progeny of two houses originate from 1 breeder flock, sampling of 1 house will be sufficient(de Wit, 2001).

(2) Quality of chickens used for sampling. High quality chicks should be selected for sampling. Samples should not be taken from poor quality, ill or stressed chicks, as these do not represent the flock as a whole(de Wit, 2001).

If these conditions are not met, estimate of the optimal date of vaccination will be unreliable (de Wit, 2001).

The ideal IBDV vaccine should thus be safe and capable of priming an immune response after a single inoculation in ovo or at hatching in the presence of MDA

(Ingrao, 2013). Other approaches are still in experimental phase and have not been brought to practice so far (Mahgoub, 2012; Muller *et al*., 2012).

An "immune complex" vaccine has been developed, in which the vaccine virus is complexed in vitro with an optimum amount of antibodies (Whitfill *et al*., 1995; Gardin *et al*., 2014) and is used for *in ovo* vaccination. The exact working mechanism of the "immune complex" vaccine is not yet clear, however, it has been suggested that the immune complex is taken up by follicular dendritic cells (macrophages) where the virus resides until the drop of maternal antibody (Jeurissen *et al*., 1998). A number of experimental recombinant IBD vaccines have been developed which used fowl poxvirus (Bayliss *et al*., 1991; Shaw and Davison, 2000), herpesvirus of turkey (Darteil *et al*., 1995), fowl adenovirus (Sheppard *et al*., 1998; Francois *et al*., 2001), Marek's disease virus (Tsukamoto *et al*., 1999, 2000, 2002) and Semliki Forest virus (Phenix *et al*., 2001) as the vector. *In vitro* expressed VP2 (Vakharia *et al*., 1993, 1994; Pitcovski *et al*., 1996; Dybing and Jackwood, 1998;Wang *et al*., 2000; Yehuda *et al*., 2000) or *in vitro* generated virus-like particles (VLP) of IBDV (Hu *et al*., 1999; Kibenge *et al*., 1999) have been found to be immunogenic. DNA vaccines also have been developed for IBDV (Fodor *et al*., 1999; Chang *et al*., 2001, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1Experimental Birds

Two hundred and seventy (270) Dominant black hybrid commercial cockerels were obtained from Terudee Chicks Farm, Ibadan at day old. They were housed in the poultry pen of the Avian Medicine Unit of Ahmadu Bello University, Zaria, and were fed with chick mash (Hybrid feeds®) and water was administered*ad libitum* for the period of the experiment (5 weeks). The breeder flocks were vaccinated against IBD with an intermediate live IBD vaccine at 4 and10 weeks and inactivated IBD vaccine at 16-18 weeks.

3.2Vaccines

Four (4) commercially available IBD vaccines MB® (MB Intermediate strain, Abic, Israel), Cevac® IBDL (Intermediate Plus strain, Winterfield 2512 G 61,Ceva sante Animale, Hungary), Bursine 2® (Intermediate Lukert stock, Fort Dodge, USA) and Infectious Bursal Disease Vaccine® (Georgia, NVRI, Nigeria) were used in the study. The vaccines were obtained from vaccine retailers to simulate the common practice by farmers.

3.3Challenge virus (Field Isolate)

Infectious bursal disease vaccine virus candidate was obtained from the Virology Laboratory of the Department of Veterinary Microbiology, ABU, Zaria and served as the challenge virus.

3.4Experimental Design

The 270 cockerels were randomly divided into six groups (Table 3.1) consisting of 45 birds each. Birds were vaccinatedafter determining the level of maternally antibody titre (MDA). The optimal vaccination time (OVT) was estimated using the ‘‘Deventer formula’’, a formula based on the half life time of MDA level of antibodies on a given date, and on the rate of decrease of these antibodies as described by CEVA, (2005). The vaccines were administered as directed by the manufacturers, while 0.05ml of the wild field virus was administered orally on the 17^{th} day post hatch (Dph).

Table 3.1: Experimental Design.

		Days post vaccination/challenge (Dpv/c)										
Group of Birds	Vaccination/challenge age	1	2	3	4	5	7	10	14	21	Total	Organs
A	17 Days post hatch (Dph)	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
B	17 Dph	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
C	17 Dph	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
D	17 Dph	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
E	17 Dph	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
F	17 Dph	5	5	5	5	5	5	5	5	5	45	BF, Spleen & Thymus
TOTAL		30	30	30	30	30	30	30	30	30	270	

Keys:BF= Bursa of Fabricius

Group A= Vaccinated with MB® at the indicated time intervals

B= Vaccinated with Bursine®-2at the indicated time intervals

C= Vaccinated with Cevac® IBD Lat the indicated time intervals

D= Vaccinated with IBD-Vac®at the indicated time intervals

E= Not Vaccinated; F= Control

3.5BLOOD SAMPLING

Two millilitres of blood were collected from each bird by cardiac punctureand transferred to plain universal bottles. The collected blood samples were allowed to clot at room temperature to obtain sera. Serum samples were transferred into eppendorf tubes and stored at -20°c until they were assayed.

3.6Enzyme Linked Immunosorbent Assay (ELISA)

Infectious bursal disease antibody test kit for the detection of IBDV antibodies was sourced from IDEXX Laboratories, Incorporate, Westbrook, Maine 04092, in the United States. The reagents preparation and the test procedure were strictly carried out according to the manufacturer's instructions.Serum samples were tested for IBDV antibodies by the ELISA test. Serum samples with the S:P ratio (S = serum sample, P = positive control) less than or equal to 0.2 were considered negative. Ratios above 0.2 (titres higher than 396) were considered positive (as indicated in the IDEXX ELISA Kit manual).

3.7Bursametry

Bursa of Fabricius (BF) diameter was measured with a ruler called bursameter (Fort Dodge Animal Health). In the bursameter, eight orifices are graded as follows:

1=3.17mm, 2=6.35mm, 3=9.25mm, 4=12.70mm,

5=15.87mm, 6=19.05mm, 7=22.22mm and 8=25.40mm.

3.8Relative weight of the Bursa of Fabricius and Spleen

The BF and spleen were removed and weighed before being fixed for histopathology. Bursa and spleen/body weight ratios were calculated for each bird and expressed as arithmetic means in each group of birds by the following formula: organ weight in grams divided by body weight of individual bird in grams multiplied by 1000 (Lucio and Hitchner, 1979).

3.9Bursa Body Index

This is defined as follows: BB index = BB ratio of infected (or vaccinated) birds / BB ratio of the controls (Cazaban *et al.*, 2012). The standard of BB index as given by Cazaban *et al.*(2012) was used in comparing the BB indices across the group.

The standard BB index used to classify IBD viruses or conventional live IBD vaccines follows;

>0.7	Physiological variability = no atrophy, Mild vaccine strain
0.3-0.7	Relative and transient atrophy= Intermediate and Intermediate plusvaccine
<0.2	Strong atrophy= hot vaccine.

3.10Histopathology

During necropsy,bursa of Fabricius, spleen and thymus were collected at post mortem and each piece of tissue was trimmed, fixed in 10% phosphate-buffered formalin, paraffin embedded, cutat 5 microns (μ) in thickness and stained with haematoxylin and eosin(Luna., 1968).

3.11Histopathological Observations

The slides were studied under a light microscope at various magnifications. The bursal lesions were recorded and scored on the basis of the following criteria of Williams and Davison (2010): 0, normal architecture; 1, minor lymphocyte depletion from isolated follicles; 2, lymphocyte depletion from most follicles, heterophilia and hyperaemia; 3, cystic cavity formation in most follicles, necrotic foci, vacuolation and frequent pyknotic nuclei; 4, severe lymphocytic depletion from all follicles, hyperaemia and severe heterophilia, disruption of plical membranes; 5, complete loss of bursal architecture, fibroplasia.

A shorter scoring system that incorporated the severity of disease progression was devised for the spleen: 0, normal structure; 1, hyperaemia with heterophilia; 2, severe hyperaemia and heterophilia, minor lymphocyte depletion and pyknotic nuclei; 3, normal architecture lost with heterophilia, oedema and necrotic foci throughout. For the thymus, scoring was as follows: 0, normal; 1, hyperaemia and heterophilia with few necrotic foci in cortex; 2, severe hyperaemia and heterophilia with necrotic foci and frequent pyknotic nuclei; 3, large necrotic foci, numerous pyknotic nuclei and lymphocyte depletion (Wiliams and Davison, 2005).

3.12Statistical Analysis

Data obtained was expressed as mean ± SEM and analysed by one way ANOVA using GraphPad prism Version 5.0. Tukeys post hoc test was employed to determine differences between groups. Values of $p<0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.1 Serology

4.1.1 Estimation of the optimal vaccination time.

Twenty (20) serum samples were investigated for IBDV MDA by ELISA 6days post hatch based on the Deventer formula obtained from HIPRA IBD online optimal vaccination time (OVT) calculator. The optimal vaccination time for the cockerels in this experiment was between 27, 38 and 40 days post hatch for 'intermediate plus/hot' (referred to as 'Strong'), 'intermediate' and 'mild' vaccine strain of the IBDV respectively (Appendix I).

Although 27^{th} and 38^{th} days post hatch are the two days given for optimal vaccination time as indicated in figures 2 and 3 (Appendix I), the cockerels were vaccinated on the 17^{th} day post hatch considering the endemic status of IBDV virus in the study area.

4.1.2 Antibody titre post vaccination and challenge

Antibody levels in between the groups were not different ($p>0.05$) from the levels in the challenge group and the control group. Although antibody titres across the groups was high, the values were not significant ($P>0.05$) (Table 4.1).

Table 4.1: Mean ELISA Antibody Titre of Cockerels in Different Vaccinated/Challengeand Control Groups.

TIME (Dpv/c)	MB®	BURSINE-2®	CEVAC IBD L®	IBD-Vac®	CNTRL	CHAL
1Dpv/c	4.173±0.06	3.996±0.10	3.972±0.09	4.102±0.18	3.892±0.08	3.776±0.16
2Dpv/c	4.038±0.06	4.012±0.06	3.700±0.09	3.786±0.16	3.668±0.19	3.616±0.08
3Dpv/c	3.628±0.12	3.704±0.12	3.598±0.13	3.850±0.18	2.876±0.19	3.540±0.24
4Dpv/c	3.880±0.06	3.836±0.08	3.654±0.09	3.550±0.16	3.834±0.13	3.852±0.05
5Dpv/c	3.618±0.16	3.664±0.11	3.620±0.15	3.460±0.10	3.27±0.13	4.436±0.03
7Dpv/c	3.898±0.08	N.S.T.	3.642±0.10	4.246±0.25	3.844±0.17	3.476±0.22
10Dpv/c	3.880±0.22	N.S.T.	4.480±0.03	4.036±0.36	3.836±0.17	4.476±0.03
14Dpv/c	3.492±0.72	N.S.T.	3.914±0.22	4.420±0.00	4.410±0.03	4.384±0.02
21Dpv/c	3.778±0.25	3.578±0.22	4.154±0.21	4.342±0.02	4.092±0.21	4.370±0.03

Keys; N.S.T= No Sample Taken

P>0.05

CNTRL= CONTROL

CHAL= CHALLENGE

4.2 Clinical Signs and Mortality

At 1Dpc, depression appeared in the challenge group. The number of affected birds with signs rose from 2 to 5with signs of depression and ruffled feather at 2 Dpc. Severe depression, ruffled feathers (PlatesIa and Ib), anorexia and diarrhoea, characterized by greenish yellow colour, were observed in chickens in the challenge group 2 Dpc. Mortality began at 3 Dpc, peaked at 4 Dpc, and declined at 5 Dpc. At 6 Dpc, survivors appeared apparently normal.

Clinical signs were observed 9Dpv in the Bursine 2® group. Mortality began on day 10pv with 2 mortalities and 6 mortalities 11dpv. The mortality declined on 12Dpv to 3 mortalities and maintained same (3 mortalities) for 2days (13Dpv and 14Dpv).

The control group, and the IBD-Vac® exhibited depression, ruffled feathers and anorexia on 14Dpv (29days post hatch). The mortality pattern in the two groups was similar to that observed in the Bursine 2® group (Plates Ic and Id).

Plate Ia: Challenge birds exhibiting depression, anorexia and ruffled feathers (yellow arrows); dead birds (red arrows).

Plate Ib: Challenge birds exhibiting depression, anorexia and ruffled feathers (yellow arrows); a dead bird (red arrow) and greenish yellowish diarrhoea (green arrow).

Plate Ic: IBD-Vac®treated group exhibiting depression, anorexia and ruffled feathers (yellow arrow); dead bird (red arrow).

Plate Id: Non-vaccinated nonchallenged (control) group exhibiting depression, anorexia and ruffled feathers (yellow arrow); dead bird (red arrow).

4.3 Pathology

4.3.1 Gross pathology

Gross lesions were observed as early as 1 Dpc in the challenge group and 3 Dpv in three (MB®, Cevac IBD L® and IBD-Vac®) out of the four vaccinated groups. The observed lesions were slight oedema, hyperaemia and gelatinous yellowish transudate covering the serosa surface. On the mucosal surfaces, the bursa as well as the thigh and breast muscles showed mild petechial haemorrhages.The thymuses were also congested at 1 Dpc and 3 Dpv in the challenge group and three out of the four vaccinated groups(Plate IIa and b). At 3 Dpc, bursal haemorrhage became extensive in the dead sacrificed birds (plate IIIa and b): in these birds, both the serosal surfaces and mucosa plicae of the bursa showed significant congestion and haemorrhages. There were petechial and ecchymotic haemorrhages in the thigh (plate IVa and b), and breast muscles of the sacrificed birds as well as the dead ones from challenge group.

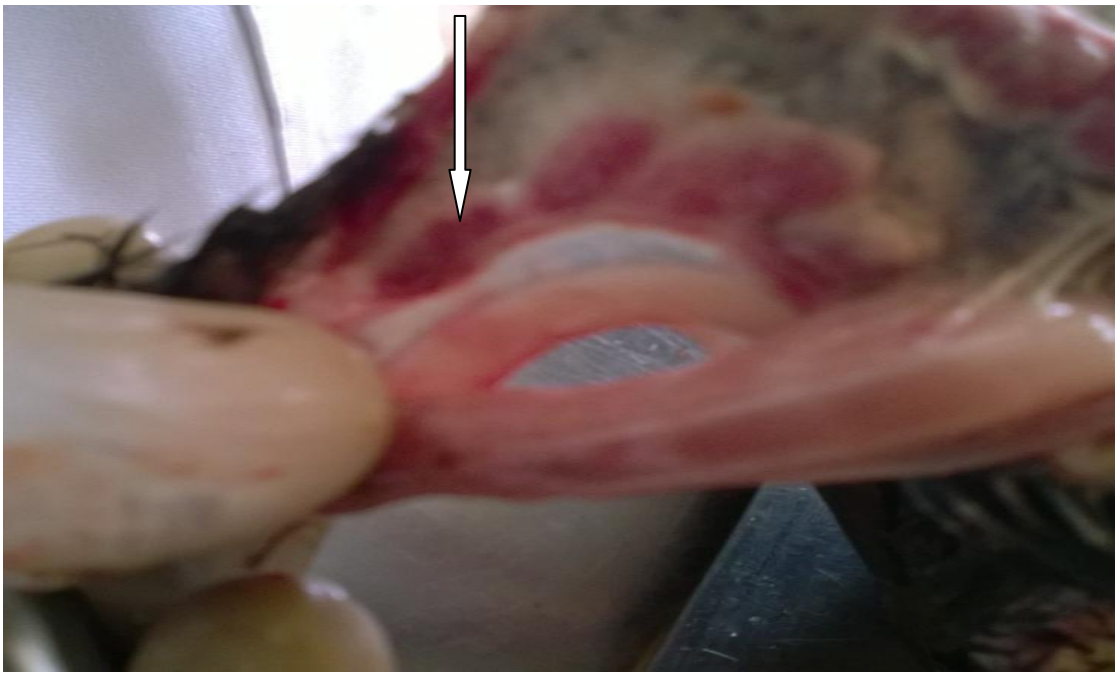

Plate IIa: Haemorrhagic thymic lobes (white arrow) observed at 1 Dpv/c-3 Dpv in MB®.

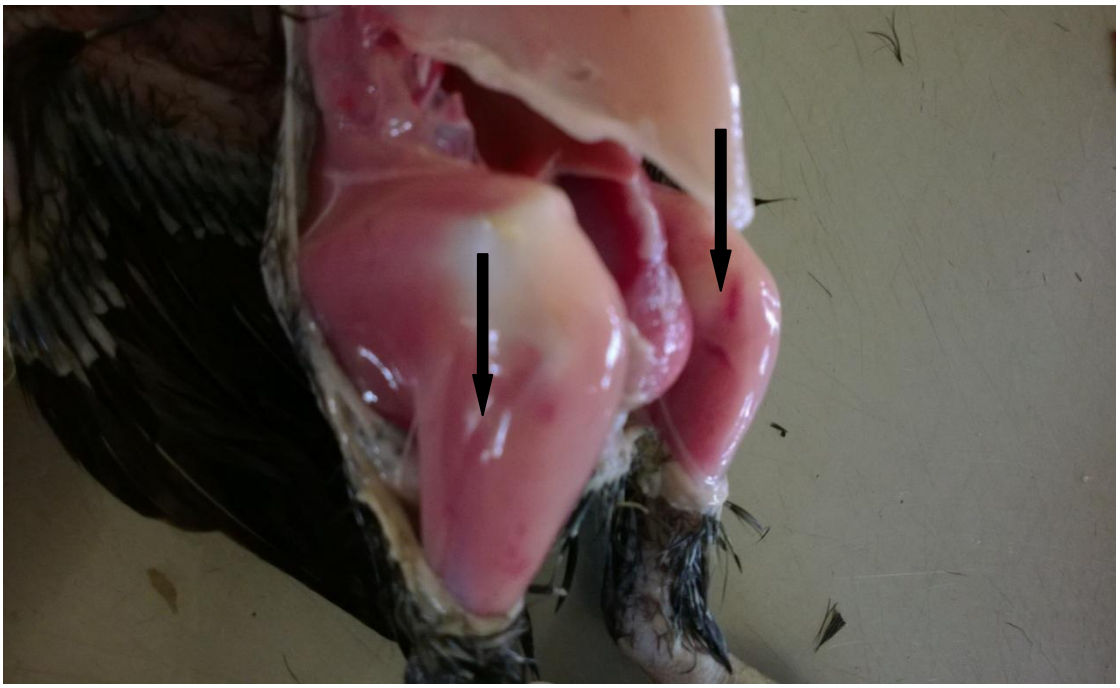

Plate IIb: Mild petechial haemorrhages on the thigh muscles (black arrows) observed at 1 Dpv/c- 3 Dpc in challenged group.

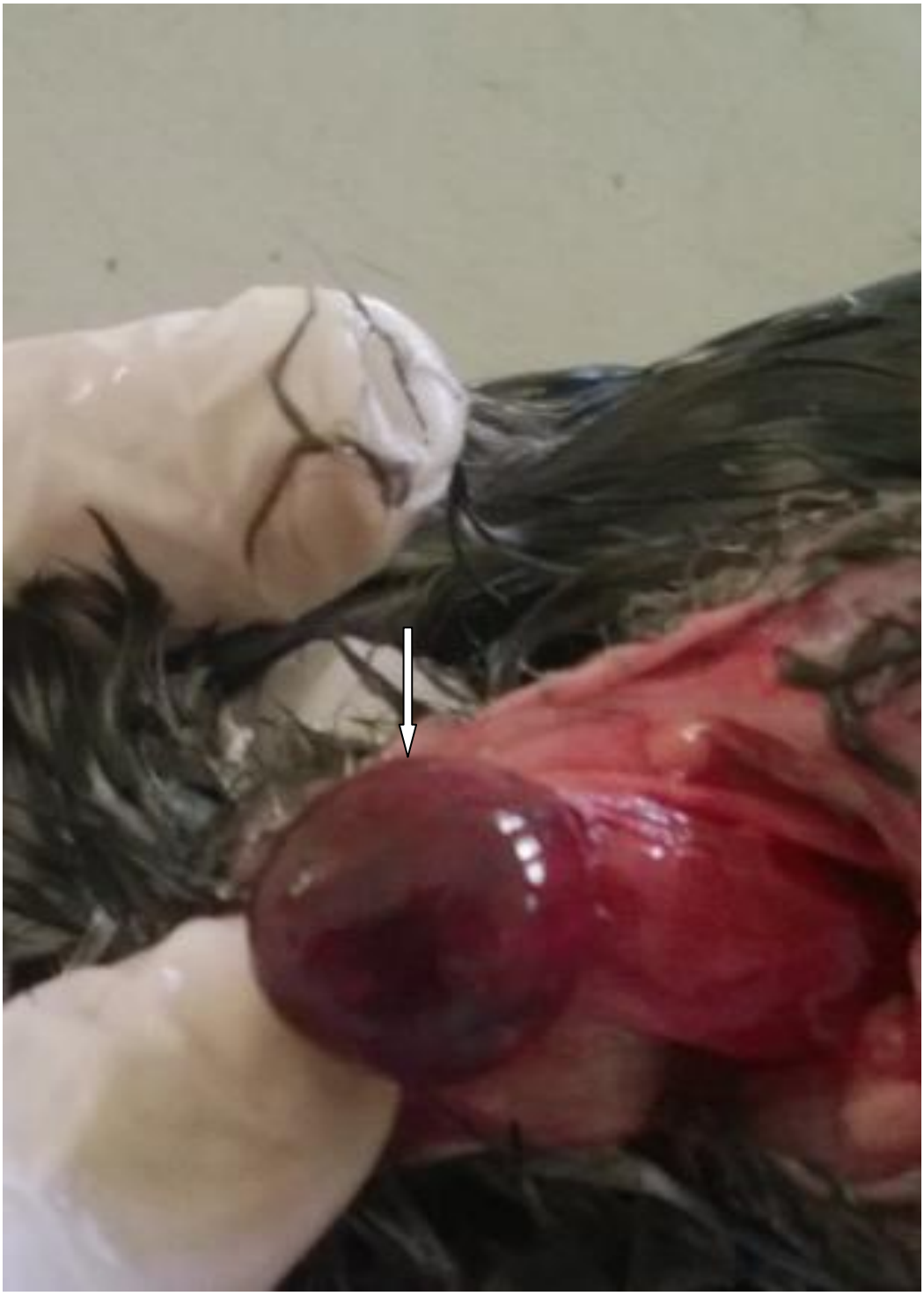

Plate IIIa: Severe bursalhaemorrhage (white arrow)3 Dpv/c in the challenge group

Plate IIIb: Severe congestion and haemorrhages in the bursa plicae (white arrow)3 Dpv/c in the challenge group

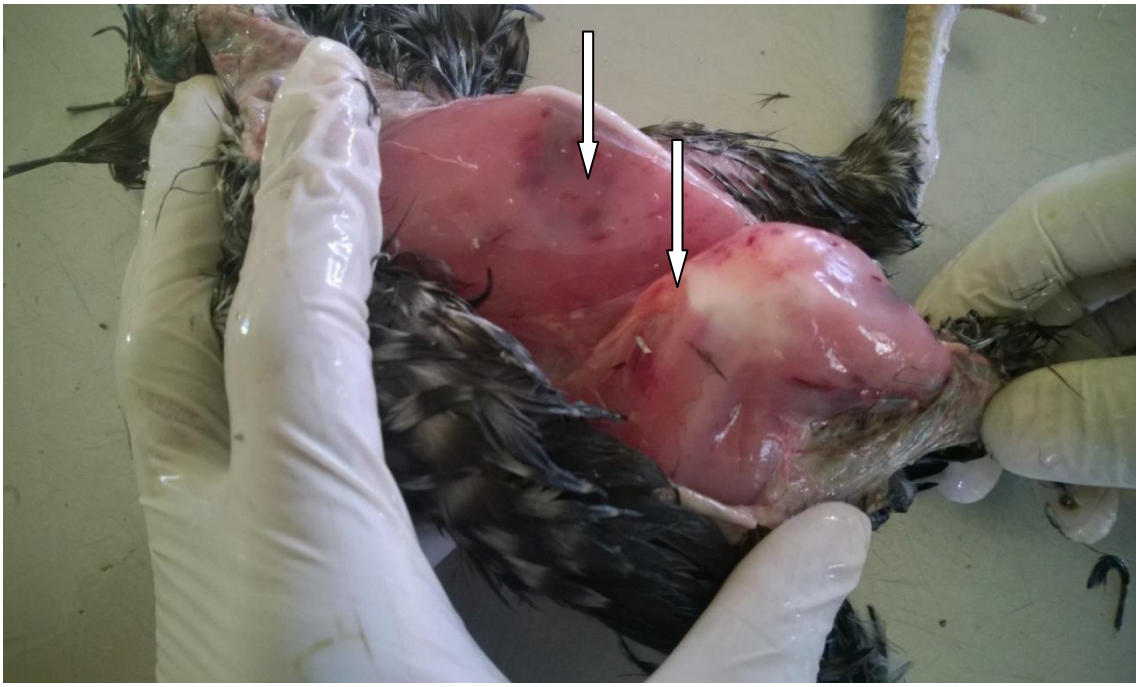

Plate IV: Severe petechial and ecchymotic haemorrhages on the breast and thigh muscle (white arrows)3 Dpv/c in the challenge group

By 4 Dpc, while the bursae were still enlarged, haemorrhages were even severe in both sacrificed and dead birds in the challenge groups. Variable degrees of haemorrhage were present in the bursae, breast and thigh muscles, with the highest proportion being in dead birds when compared with sacrificed birds. Cheesy materials in the bursae of all chickens examined. The thymus and spleen were congested.

At 5Dpc, mild haemorrhages were observed in the bursae and thigh of both sacrificed and dead birds, the spleen was also enlarged (plate V). The bursae have atrophied to about $^{1}/3$ of its size when compared with the control (plate VI). No relevant gross pathology was observed in birds in the control group.

Plate V: Enlarged spleen (right) 3 Dpv/c compared to atrophied spleen (left) 5 Dpv observed inIBD-Vac® group.

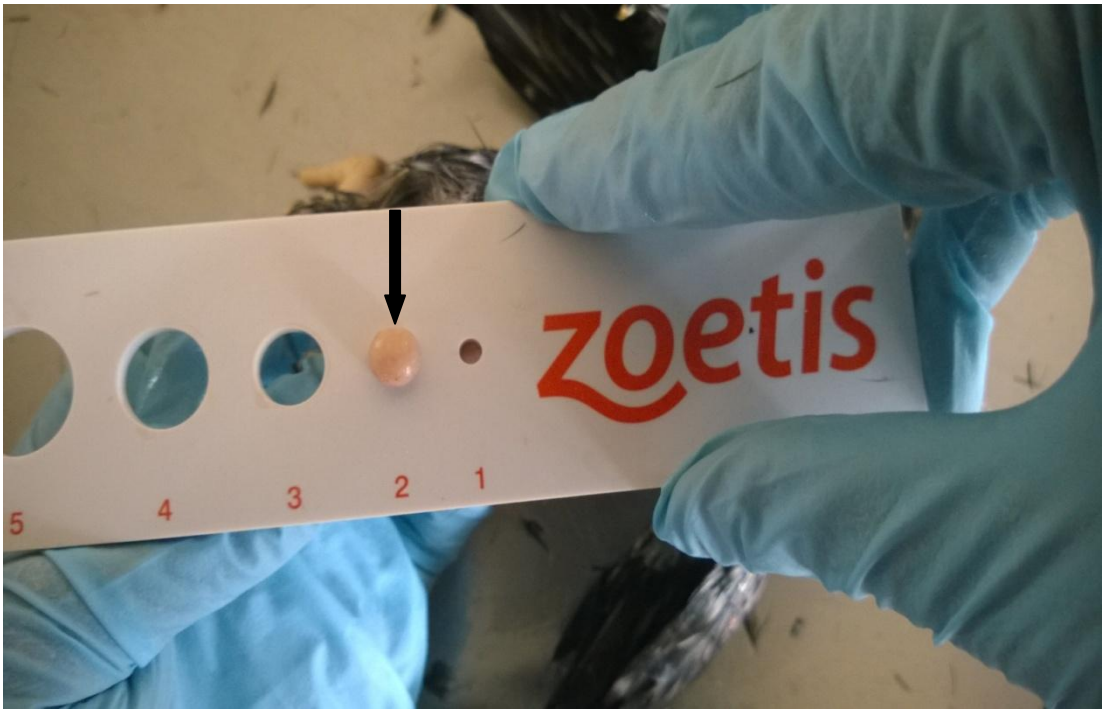

Plate VI: Bursal atrophy (black arrow) measured using a bursameter observed 7 Dpcin the challenge group.

4.3.2 Bursa of Fabricius relative weight

The vaccine group were not able to reduce bursa size significantly when compared to the challenge and control group ($p>0.05$). However, the challenge group showed significant ($p<0.05$) reduction in bursa of Fabricius weightwhen compared to the control group (Table 4.2). The MB® and Cevac® IBD L groups presented the same weight on 10, 14 and 21D'spv/i.

4.3.3 Bursa of Fabricius diameter

There were no significant differences($p>0.05$) in the diameter of the bursa between the groups. The challenged group presented a decrease in the bursal diameter after an initial increase on days 5 and 7 pc (Table 4.3).

4.3.4 Bursa body weight ratio (B:BW)

There was no significant difference($p>0.05$) in the B:BW ratios between the groups (Table 4.4). However, at 21dpv all groups of chickens revealed lower B:BW, with the challenge group exhibiting an early reduction in the bursal weight on day 3 to 21 pc.

4.3.5 Bursal body index (BB Index)

There was no statistical difference($p>0.05$) observed between the groups, but values recorded on the 7^{th} dpv/c indicated and confirmed the precision of BB index as a useful tool in an experimental protocol. With an index value of <0.3 on day 7 pc in the challenged group, it affirmed that, the field virus used in this study was a very virulent strain of the IBDV (Table 4.5).

4.3.6 Bursa lesion scores

A statistical significant difference($p<0.05$) was observed between the groups. A lesion score of 0 was recorded throughout the period of study in the control group, with the challenge recording as high as 4 on 21 Dpc (Table 4.6).

Table 4.2: Mean bursa of Fabricius relative weight (BFRW) of Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBD L	IBD-Vac®	CONTROL	CHALLENGE
1Dpv/c	0.90±0.10	0.93±0.09	1.03±0.09	0.80±0.15	0.87±0.17[a]	1.10±0.06[b]
2Dpv/c	1.27±0.09	0.90±0.12	0.97±0.07	1.10±0.06	1.10±0.12[a]	1.13±0.12[b]
3Dpv/c	1.33±0.19	1.37±0.28	1.07±0.20	0.87±0.12	1.20±0.10[a]	0.50±0.06[b]
4Dpv/c	0.97±0.15	1.17±0.13	1.27±0.13	0.90±0.06	1.30±0.25[a]	0.63±0.12[b]
5Dpv/c	1.60±0.60	1.53±0.12	1.33±0.20	1.53±0.23	1.50±0.10[a]	0.43±0.09[b]
7Dpv/c	0.60±0.06	1.63±0.12	1.10±0.06	1.57±0.18	1.50±0.17[a]	0.30±0.00[b]
10Dpv/c	0.57±0.09	N.S.T	0.73±0.34	1.67±0.52	1.33±0.18[a]	0.27±0.09[b]
14Dpv/c	0.50±0.10	N.S.T	0.43±0.13	1.93±0.07	2.07±0.26[a]	0.47±0.07[b]
21Dpv/c	0.60±0.12	0.47±0.07	0.67±0.13	0.37±0.03	0.57±0.03[a]	0.67±0.09[b]

Values in rows with superscript[a.b] differ significantly (P<0.05)

N.S.T. = No Sample Taken

Table 4.3: Mean Bursa of Fabricius Diameterof Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac®IBDL®	IBD-Vac®	CONTROL	CHALLENGE
1Dpv/c	5.00±0.00	5.00±0.00	5.00±0.00	4.00±0.00	5.00±0.00	4.00±0.00
2Dpv/c	5.00±0.00	4.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	4.00±0.00
3Dpv/c	5.00±0.00	4.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	4.00±0.00
4Dpv/c	5.00±0.00	4.00±0.00	5.00±0.00	4.00±0.00	5.00±0.00	4.00±0.00
5Dpv/c	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	3.00±0.00
7Dpv/c	5.00±0.00	5.00±0.00	4.00±0.00	5.00±0.00	4.00±0.00	2.00±0.00
10Dpv/c	3.00±0.00	N.S.T.	3.00±0.00	4.00±0.00	5.00±0.00	3.00±0.00
14Dpv/c	3.00±0.00	N.S.T.	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
21Dpv/c	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00

N.S.T. = No Sample Taken.

Table 4.4:Mean Bursa Body Weight Ratioof Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBD L	IBD-Vac®	CONTROL	CHALLENGE
1Dpv/c	4.89±0.53	5.08±0.50	5.62±1.48	4.35±0.83	4.71±1.38	5.98±0.31
2Dpv/c	6.85±0.48	4.49±0.52	5.22±1.36	5.80±0.22	6.06±1.68	6.23±0.91
3Dpv/c	6.65±0.93	6.80±1.39	5.31±1.48	4.31±0.57	5.98±1.55	2.49±0.28
4Dpv/c	4.69±0.80	5.63±0.68	6.08±1.56	4.37±0.46	1.27±0.18	1.27±0.13
5Dpv/c	6.70±0.22	6.44±0.58	5.60±1.47	6.45±1.06	6.28±1.53	1.82±0.37
7Dpv/c	2.34±0.08	6.45±0.07	4.37±1.09	6.19±0.79	5.90±1.47	1.18±0.09
10Dpv/c	1.93±0.38	N.S.T	2.49±0.96	5.68±1.86	4.48±1.06	0.86±0.24
14Dpv/c	1.44±0.22	N.S.T	1.25±0.34	5.73±0.53	6.20±1.51	1.36±0.15
21Dpv/c	1.36±0.16	1.03±0.05	1.25±0.67	1.13±0.29	1.42±0.28	1.39±0.24

N.S.T. = No Sample Taken

Table 4.5: Mean Bursa Body Indexof Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBD L	IBD-Vac®	CHALLENGE
1Dpv/c	1.04±0.60	1.08±0.62	1.19±0.69	0.92±0.53	1.27±0.73
2Dpv/c	1.13±0.65	0.74±0.43	0.86±0.50	0.96±0.55	1.03±0.59
3Dpv/c	1.11±0.64	1.14±0.66	0.89±0.51	0.72±0.42	0.42±0.24
4Dpv/c	3.69±2.13	4.43±2.56	4.79±2.76	3.44±1.99	1.00±0.58
5Dpv/c	1.07±0.62	1.03±0.59	0.89±0.52	1.03±0.59	0.29±0.17
7Dpv/c	0.40±0.23	1.09±0.63	0.74±0.43	1.05±0.61	0.20±0.12
10Dpv/c	0.43±0.25	N.S.T	0.55±0.32	1.27±0.73	0.19±0.11
14Dpv/c	0.23±0.13	N.S.T	0.20±0.12	0.92±0.53	0.22±0.13
21Dpv/c	0.96±0.55	0.73±0.42	1.61±0.93	0.79±0.46	0.98±0.56

N.S.T.= No Sample Taken

>0.7= Physiological variability; no atrophy; mild vaccine

0.3-0.7= Relative transient atrophy; intermediate and intermediate plus vaccine

<0.3= Strong atrophy; Hot vaccines

Table 4.6: Mean Bursa Lesion Scores of Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBD L	IBD-Vac®	CONTROL	CHALLENGE
1Dpv/c	0.3±0.0 ^{b}	0.3±0.0 ^{bd}	0.3±0.0^{a}	0.0±0.0 ^{c}	0.0±0.0$^{e\ abc}$	0.7±0.0 ^{acde}
2Dpv/c	1.0±0.0 ^{b}	0.7±0.0 ^{bd}	0.7±0.0 ^{a}	0.7±0.0 ^{c}	0.0±0.0$^{e\ abc}$	1.3±0.0 ^{acde}
3Dpv/c	1.7±0.0 ^{b}	0.7±0.0 ^{bd}	0.3±0.0 ^{a}	0.3±0.0 ^{c}	0.0±0.0$^{e\ abc}$	1.7±0.0 ^{acde}
4Dpv/c	2.0±0.0 ^{b}	1.3±0.0 ^{bd}	1.0±0.0 ^{a}	1.0±0.0 ^{c}	0.0±0.0$^{e\ abc}$	2.0±0.0 ^{acde}
5 Dpv/c	2.0±0.0 ^{b}	1.3±0.0 ^{bd}	1.3±0.0 ^{a}	0.7±0.0 ^{c}	0.0±0.0$^{e\ abc}$	2.3±0.0 ^{acde}
7Dpv/c	2.0±0.0 ^{b}	1.3±0.0 ^{bd}	1.3±0.0 ^{a}	1.0±0.3 ^{c}	0.3±0.0$^{e\ abc}$	2.3±0.0 ^{acde}
10Dpv/c	1.7±0.0 ^{b}	N.S.T	1.7±0.0 ^{a}	1.7±0.0 ^{c}	0.3±0.0$^{e\ abc}$	2.7±0.0 ^{acde}
14Dpv/c	1.7±0.0 ^{b}	N.S.T	1.3±0.0 ^{a}	1.7±0.0 ^{c}	0.3±0.0$^{e\ abc}$	3.0±0.0 ^{acde}
21Dpv/c	2.0±0.0 ^{b}	1.3±0.0 ^{bd}	1.7±0.0 ^{a}	1.3±0.0 ^{c}	0.3±0.0$^{e\ abc}$	3.7±0.0 ^{acde}

N.S.T. = No Sample Taken
Values in rows with different superscripts^{a,b,c,d,e} differ significantly (P<0.05)

4.3.7 Spleen Relative Weight

A statistical significant difference ($p<0.05$) was observed between Cevac® IBD L and the challenge group (Table 4.6).

4.3.8 Spleen Body Weight Ratio (S:BW)

A statistically significant variation($P<0.05$) was observed in birds vaccinated with Bursine®-2 against Cevac® IBD L group; and a significant difference($p<0.05$) between Cevac® IBD L and challenge group (Table 4.6).

4.3.9 Spleen Lesion Scores

A statistical significant difference($p<0.05$) was observed between the groups (Table 4.7).

4.3.10 Thymus Lesion Scores

A statistical significant difference($p<0.05$) was observed between the groups (Table 4.8)

Table 4.7: Mean Spleen Weight of Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBDL	IBD-Vac®	CONTROL	CHALLENGE
1Dpv/c	0.70±0.06	0.47±0.09	0.70±0.06[a]	0.60±0.06	0.40±0.06	0.77±0.09[b]
2Dpv/c	0.77±0.09	0.50±0.06	0.70±0.06[a]	0.53±0.09	0.50±0.12	0.53±0.09[b]
3Dpv/c	0.80±0.06	0.63±0.12	0.70±0.12[a]	0.50±0.06	0.40±0.00	0.37±0.09[b]
4Dpv/c	0.60±0.06	0.67±0.03	0.67±0.15[a]	0.40±0.06	0.50±0.10	0.30±0.00[b]
5Dpv/c	0.67±0.90	0.67±0.09	0.63±0.12[a]	0.60±0.12	0.43±0.12	0.30±0.06[b]
7Dpv/c	0.47±0.07	0.33±0.09	0.57±0.15[a]	0.40±0.06	0.47±0.06	0.20±0.00[b]
10Dpv/c	0.50±0.06	N.S.T.	0.93±0.18[a]	0.43±0.09	0.40±0.06	0.10±0.00[b]
14Dpv/c	0.53±0.09	N.S.T	1.23±0.12[a]	0.93±0.12	1.30±0.32	0.56±0.12[b]
21Dpv/c	0.53±0.07	0.90±0.26	1.00±0.00[a]	0.93±0.54	0.87±0.17	0.29±0.09[b]

N.S.T. = No Sample Taken

Values in rows with different superscripts[a,b] differ significantly (P<0.05)

Table 4.8: Mean Spleen Body Weight Ratioof Different Treatment and Control Groups.

TIME	MB®	BURSINE2®	Cevac® IBD L	IBD-Vac®	CONTROL	CHALLENGE
24Hpv/c	3.78±0.31	2.53±0.47[a]	3.80±1.00[b]	3.26±0.30	2.17±0.59	4.17±0.49[a]
48Hpv/c	4.14±0.48	2.51±0.33[a]	3.78±0.99[b]	2.81±0.43	2.81±0.89	2.88±0.38[a]
3 Dpv/c	3.98±0.26	3.15±0.58[a]	3.48±0.95[b]	2.49±0.27	1.99±0.50	1.82±0.43[a]
4 Dpv/c	2.91±0.36	3.24±0.29[a]	3.28±0.98[b]	1.91±0.20	1.27±0.26	1.27±0.13[a]
5 Dpv/c	2.79±0.36	2.79±0.35[a]	2.65±0.72[b]	2.52±0.51	1.83±0.55	1.26±0.25[a]
7Dpv/c	1.83±0.24	1.27±0.26[a]	2.31±0.73[b]	1.55±0.16	1.85±1.85	0.79±0.06[a]
10Dpv/c	1.71±0.28	N.S.T	3.23±0.88[b]	1.42±0.18	1.35±0.33	1.36±0.10[a]
14Dpv/c	1.55±0.18	N.S.T	3.66±0.82[b]	2.72±0.21	3.72±0.92	2.02±0.51[a]
21Dpv/c	1.24±0.18	1.95±0.42[a]	3.29±0.79[b]	2.34±1.04	2.21±0.55	1.30±0.18[a]

N.S.T. = No Sample Taken

Values in rows with different superscripts[a,b] differ significantly (P<0.05)

Table 4.9: Spleen Lesion Scores of Different Treatment and Control Groups

TIME	MB®	Bursine®- 2	Cevac® IBD L	IBD-VAC®	Control	Challenge
24Hpv/c	0.0±0.0^{e}	0.0±0.0^{c}	0.0±0.0^{a}	0.0±0.0^{d}	0.0±0.0$^{b\ e}$	0.3±0.0^{abcd}
48Hpv/c	0.0±0.0^{e}	0.0±0.0^{c}	0.0±0.0 ^{a}	0.0±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.0±0.0^{abcd}
72Hpv/c	0.3±0.0^{e}	0.3±0.0^{c}	0.3±0.0 ^{a}	0.3±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.3±0.0^{abcd}
96Hpv/c	0.7±0.0^{e}	0.7±0.0^{c}	0.7±0.0 ^{a}	0.7±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.3±0.0^{abcd}
120Hpv/c	0.7±0.0^{e}	0.7±0.0^{c}	0.7±0.0 ^{a}	0.7±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.7±0.0^{abcd}
7Dpv/c	1.3±0.0^{e}	1.0±0.0^{c}	0.7±0.0 ^{a}	0.7±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.0±0.0^{abcd}
10Dpv/c	1.0±0.0^{e}	0.0±0.0^{c}	0.3±0.0 ^{a}	0.3±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.3±0.0^{abcd}
14Dpv/c	0.7±0.0^{e}	0.0±0.0^{c}	0.0±0.0 ^{a}	0.0±0.0 ^{d}	0.0±0.0$^{b\ e}$	1.0±0.0^{abcd}
21Dpv/c	1.0±0.0^{e}	1.0±0.0^{c}	0.7±0.0 ^{a}	0.7±0.0 ^{d}	0.0±0.0$^{b\ e}$	0.3±0.0^{abcd}

Values in rows with different superscripts $^{a, b, c, d, e}$differ significantly (P<0.05)

Table 4.10: Thymus Lesion Scores of Different Treatment and Control Groups

TIME	MB®	Bursine®- 2	Cevac® IBD L	IBD-VAC®	Control	Challenge
24Hpv/c	0.0±0.0	0.0±0.0	0.0±0.0^{c}	0.0±0.0 ^{d}	0.0±0.0^{acd}	0.0±0.0 ^{ab}
48Hpv/c	0.3±0.0	0.3±0.0	0.3±0.0 ^{c}	0.3±0.0 ^{d}	0.0±0.0 ^{acd}	0.7±0.0 ^{ab}
72Hpv/c	0.3±0.0	0.3±0.0	0.3±0.0 ^{c}	0.3±0.0 ^{d}	0.0±0.0 ^{acd}	0.3±0.0 ^{ab}
96Hpv/c	0.7±0.0	0.7±0.0	0.7±0.0 ^{c}	0.7±0.0 ^{d}	0.0±0.0 ^{acd}	1.0±0.0 ^{ab}
120Hpv/c	1.0±0.0	1.0±0.0	1.0±0.0 ^{c}	1.0±0.0 ^{d}	0.0±0.0 ^{acd}	1.0±0.0 ^{ab}
7Dpv/c	0.3±0.0	0.0±0.0	0.7±0.0^{c}	0.7±0.0 ^{d}	0.0±0.0 ^{acd}	0.3±0.0 ^{ab}
10Dpv/c	0.3±0.0	0.0±0.0	0.3±0.0 ^{c}	0.3±0.0^{d}	0.0±0.0 ^{acd}	0.3±0.0 ^{ab}
14Dpv/c	0.3±0.0	0.0±0.0	0.0±0.0 ^{c}	0.0±0.0 ^{d}	0.0±0.0 ^{acd}	1.0±0.0 ^{ab}
21Dpv/c	0.3±0.0	0.0±0.0	0.7±0.0 ^{c}	0.7±0.0 ^{d}	0.0±0.0 ^{acd}	0.7±0.0 ^{ab}

Values in rows with different superscripts ^{a,b,c,d} differ significantly (P<0.05)

4.4 Histopathology

4.4.1 Bursa of Fabricius

The Observable histological lesions in the bursa of Fabricius of the challenge group were mild lymphocytic depletion and haemorrhagic inter-follicular interstitium in the medullary areas of the bursal follicles 1 Dpv/c (Plate VII and VIII); haemorrhagic follicles were also noticed in the Cevac® IBD L group, with a few empty spaces within the follicles. At 3 Dpv/c, necrosis and depletion of lymphocytes had become more pronounced in the follicular medulla and foci of lymphocyte necrosis have spread to the cortical areas in the MB®, IBD-Vac® and the challengegroups; the inter-follicular space was alsowidened (Plate IXa). By 4 Dpv/c, the lymphoid follicles were only sparsely populated with lymphoid cells. The undifferentiated epithelial layer between the cortex and medulla was clearly visible only noticed in the challenge group. Lymphoid necrosis was also noticed in the challenge group (Plate IXa). At 5 Dpv/c, lymphoid necrosis progressed and areas of coagulative necrosis were seen within the follicles forming cystic areas. At 7^{th}, 10^{th} and 14^{th}Dpv/c, necrosis and depletion of lymphoid cells, plicated glandular epithelium of the epithelial lining and marked interstitial oedema were observed not only in the lymphoid follicles but also in the interfollicular space (Plate X). At 7Dpv/c and 10Dpv/c moderate to marked follicular atrophy was noticed in MB® and challenge groups, Cevac® IBD L presented a mild to absence of atrophy. Depletion of lymphoid cells created marked vacuoles which were seen within the follicles and in the interfollicular connective tissues (Plate XI) by21 Dpcin the challenge group.

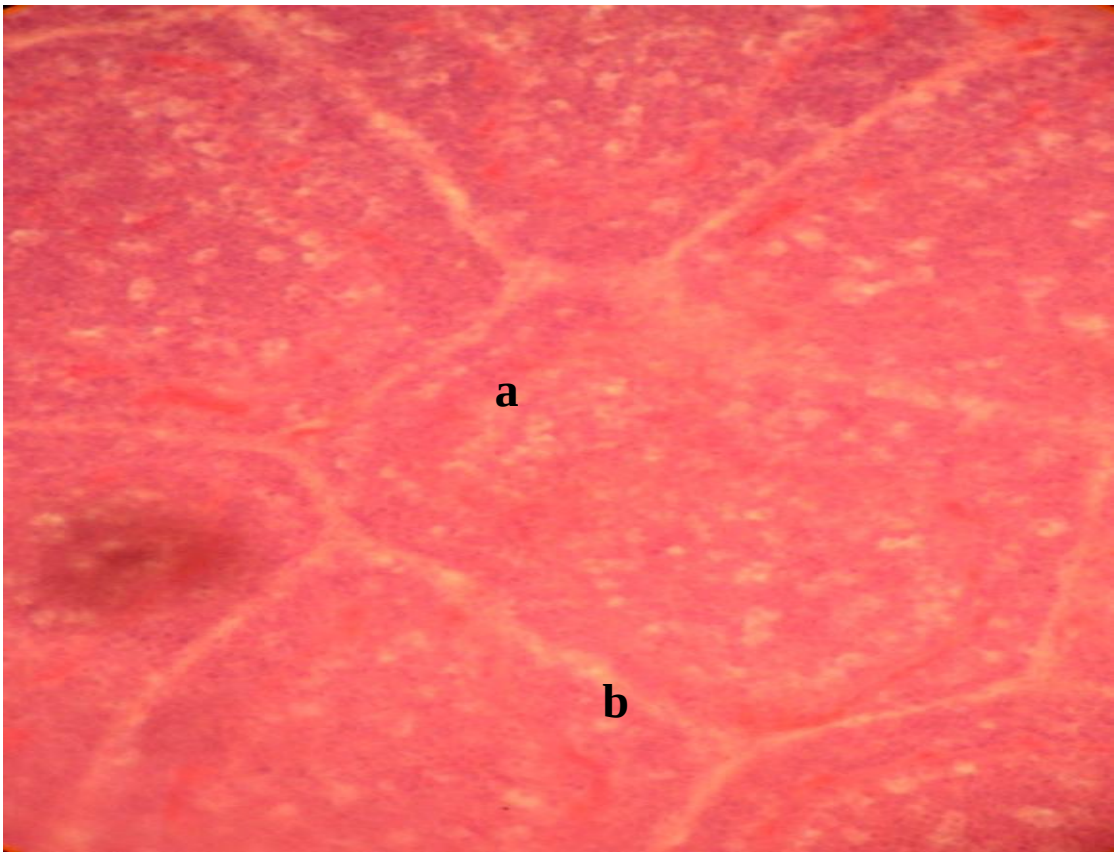

Plate VII: Bursa of Fabricius from Challenge group 1 Dpc;Perifollicular haemorrhages (a); Depletion of lymphocytes from the cortical area (b). H&E stain x400

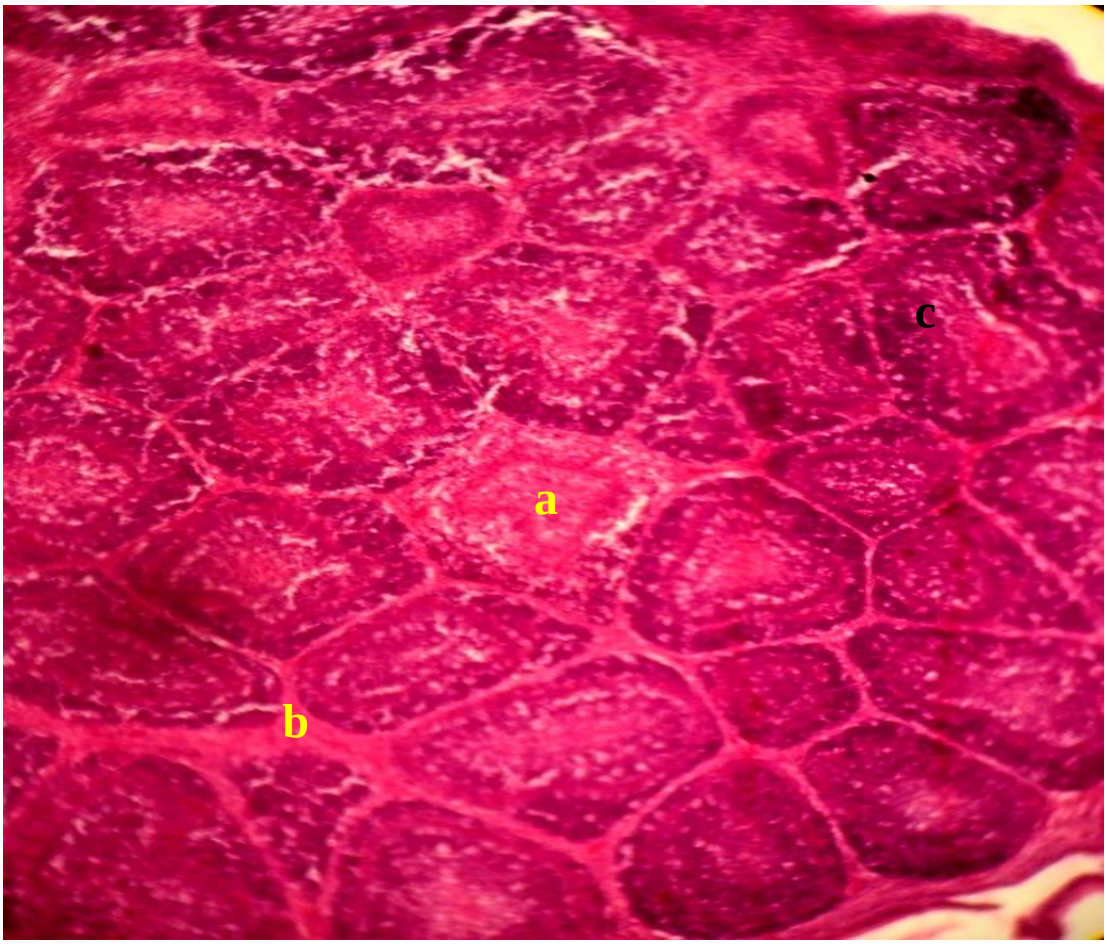

Plate VIII: Bursa of Fabricius fromChallenge group3 Dpv/c. Marked depletion of lymphoid cells in the medullary areas (a); Fibroplasia of the interfollicular tissues (b). H&Estain x200

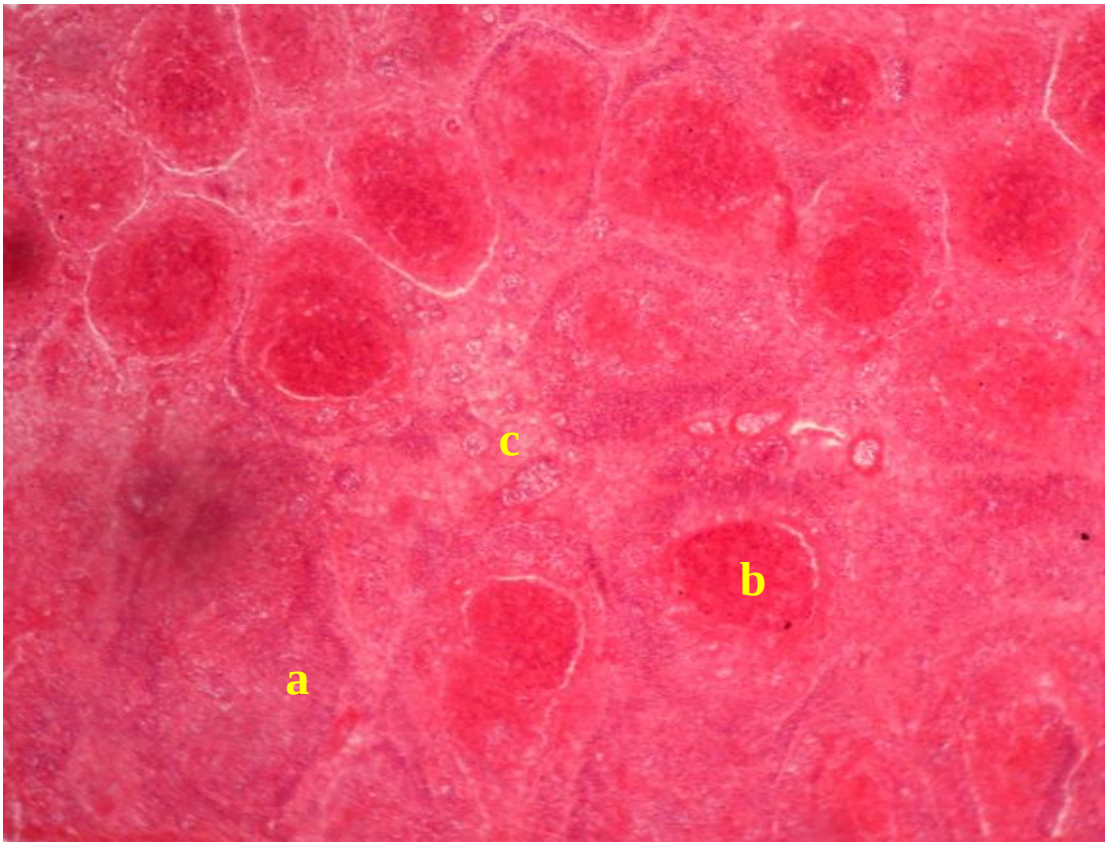

Plate IXa: Bursa of Fabricius fromChallenge group4 Dpv/c; Lymphoid follicular necrosis (a); Follicular focal haemorrhages (b); Marked oedema in the interstitial connective tissue (c). H&E stain x200

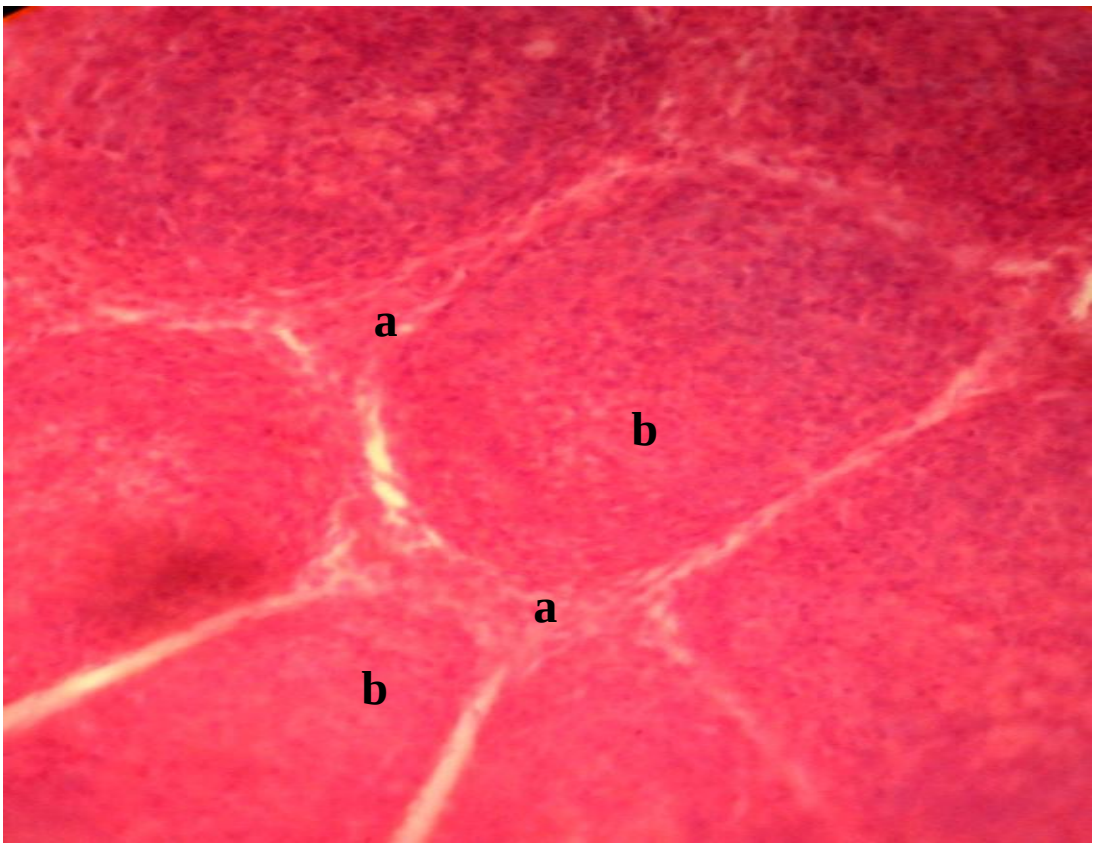

Plate IXb: Bursa of Fabricius from MB® groups 3 Dpv/c. Interfollicular hyperplasia (a);Marked follicular lymphoid cells depletion (b). H&E stain x400.

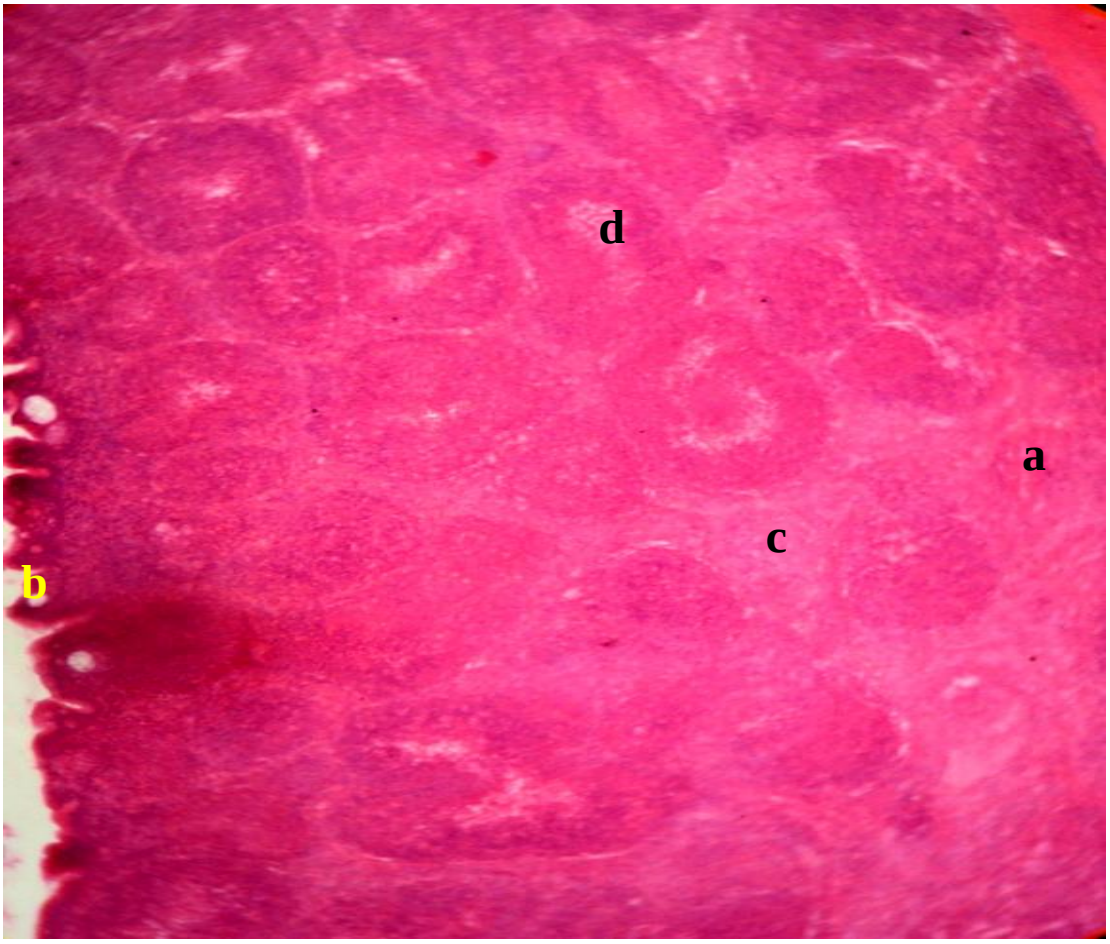

Plate X: Bursa of Fabricius from IBD-Vac® group 10Dpv;Follicular atrophy (a); Plicated glandular epithelium of the epithelial lining (b); Marked interstitial oedema (c); mild vacoulation (d). H&E stains x200.

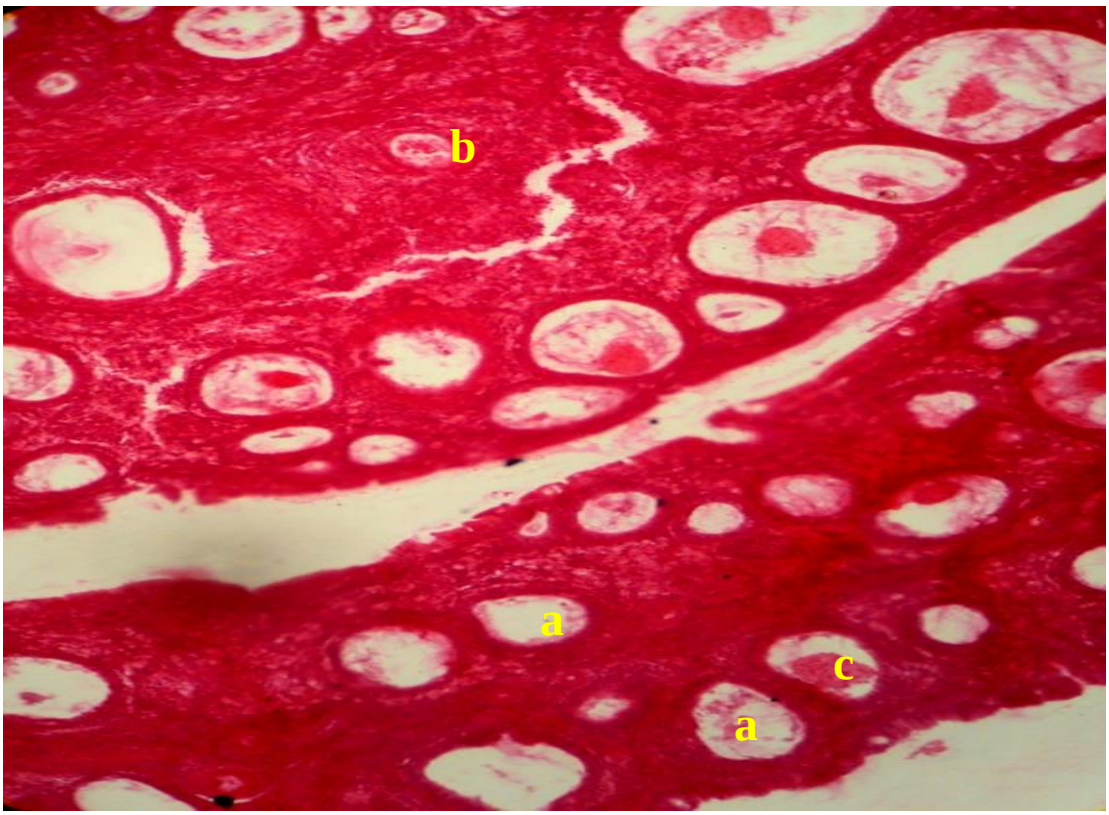

Plate XI:Bursa of Fabricius from MB® group 21Dpv/c:Vacuolar degeneration of the epithelium (a); Follicular atrophy and vacuolar degeneration of the medullary area (b); Follicular necrosis (c). H&E stain x 400

4.4.2 Spleen

As early as 1 Dpv/c congestion and depletion of lymphoid cells in the germinal centres was noticed (Plate XI) in the challenge group and MB®. At 3 Dpv/c moderate depletion of lymphoid cells was observed across the groups excluding the control group. Lymphoid cell necrosis and depletion was observed in both the germinal follicles and the periarteriolar lymphoid sheaths (PALS) in the MB®, Cevac® IBD L and Challenge groups 4 Dpv/c (plate XIV). Beyond this period, the spleen appeared to have regenerated as no significant changes were observed.

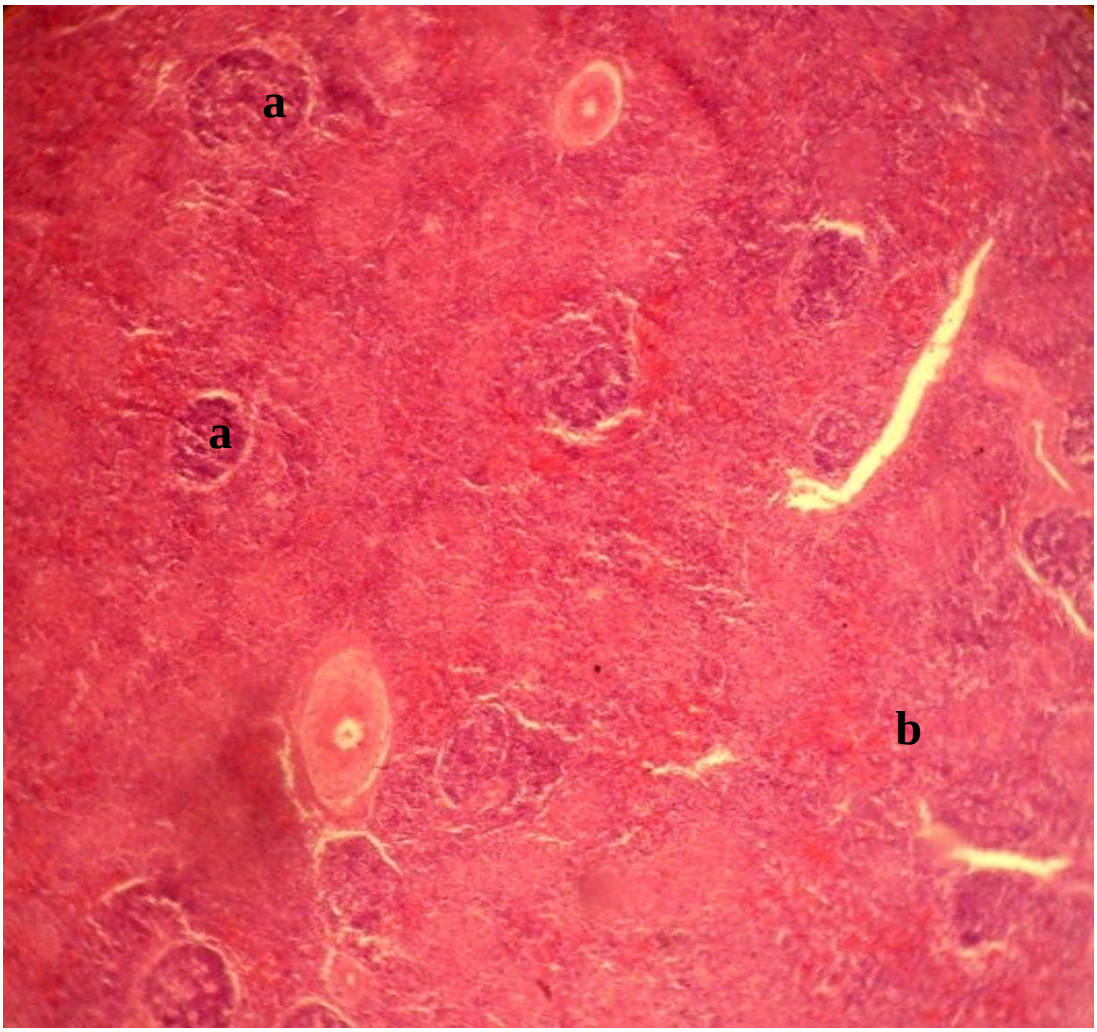

Plate XII: Spleen 1 Dpv from Cevac® IBDL group; Focal areas of active lymphocyte proliferation (a); Haemorrhages (b). H&E Stain x200

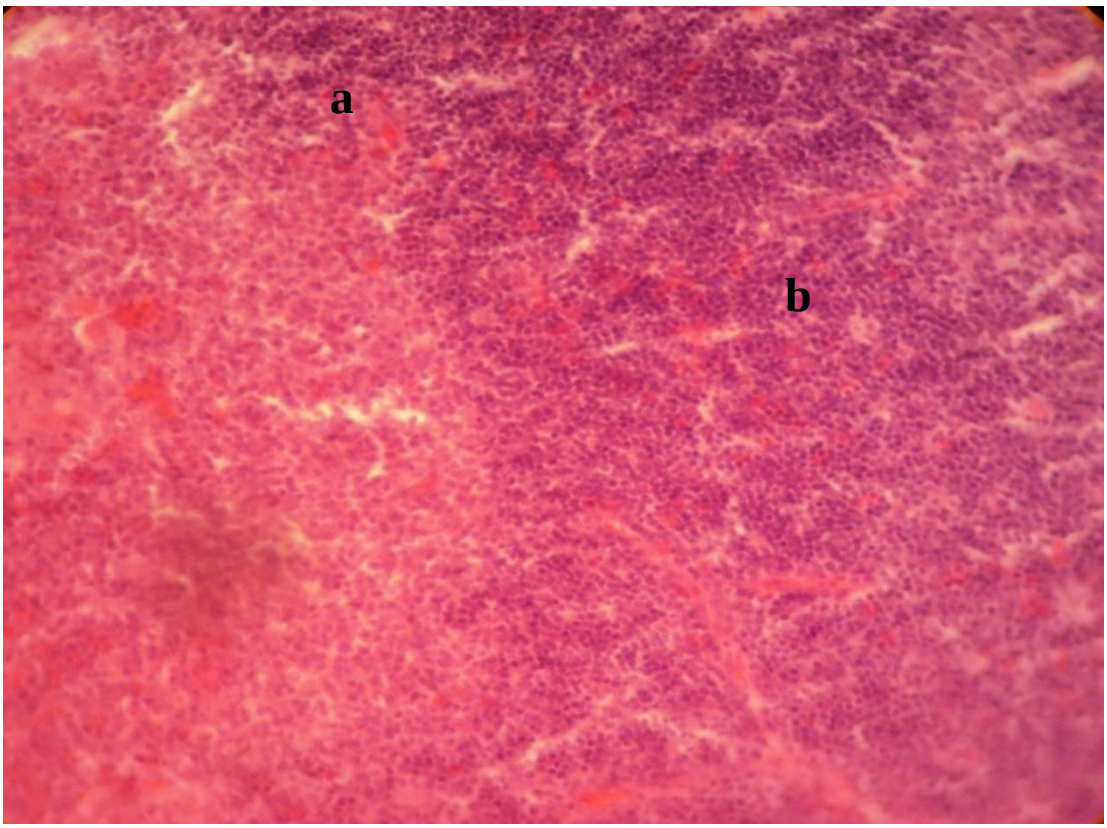

Plate XIII: Spleen from Bursine®-2 group 1Dpv/c; Lymphocytic depletion (a); haemorrhages (b). H&E stain x 200.

4.4.3 Thymus

Sections of the thymus, lymphocytic depletion, heterophil infiltration and haemorrhages in the medulla 24Hpv/c in Cevac® IBD L and challenge groups (Plate XIV). At 2 Dpv/c, lymphocytic necrosis increased in number more than that of control chickens. The width of the cortex and medulla decreased. At 72 to 21Dspv/c the lymphocyte depletion and haemorrhages was noticed across the group (Plate XV).

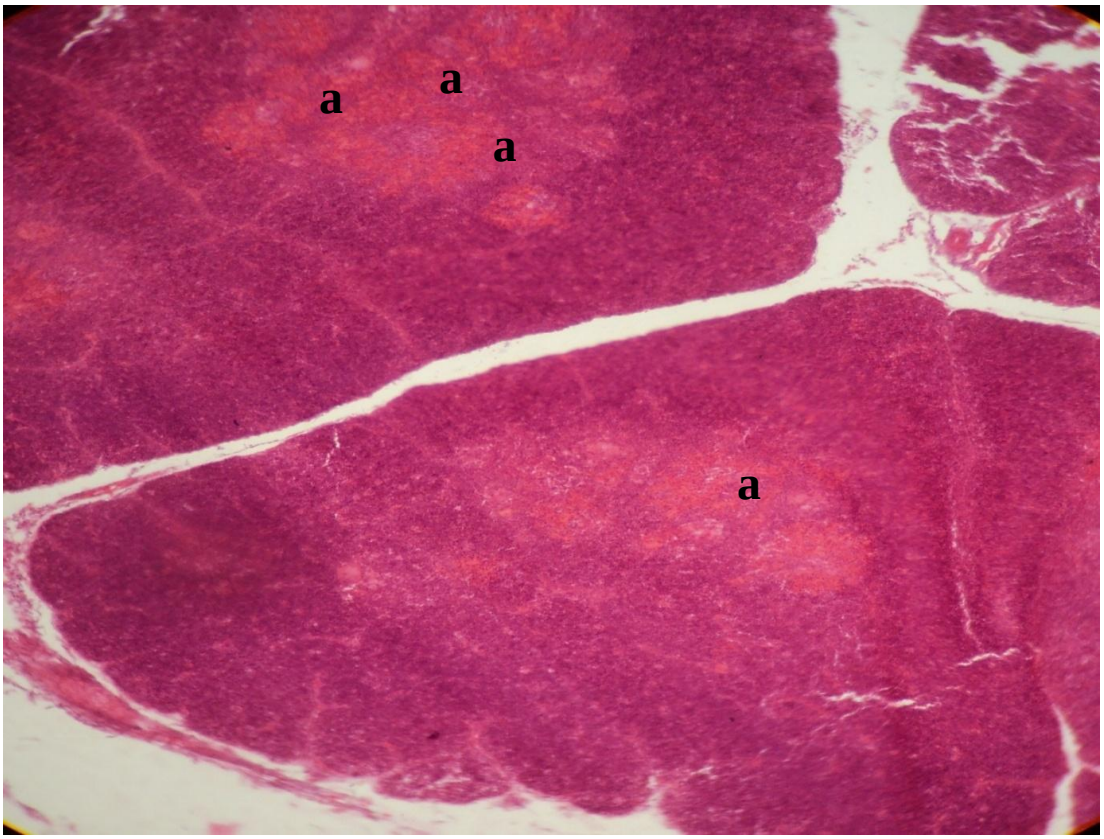

Plate XIV:Thymus from Cevac® IBD L group 1Dpv: Haemorrhages (a). H&E stain x 200.

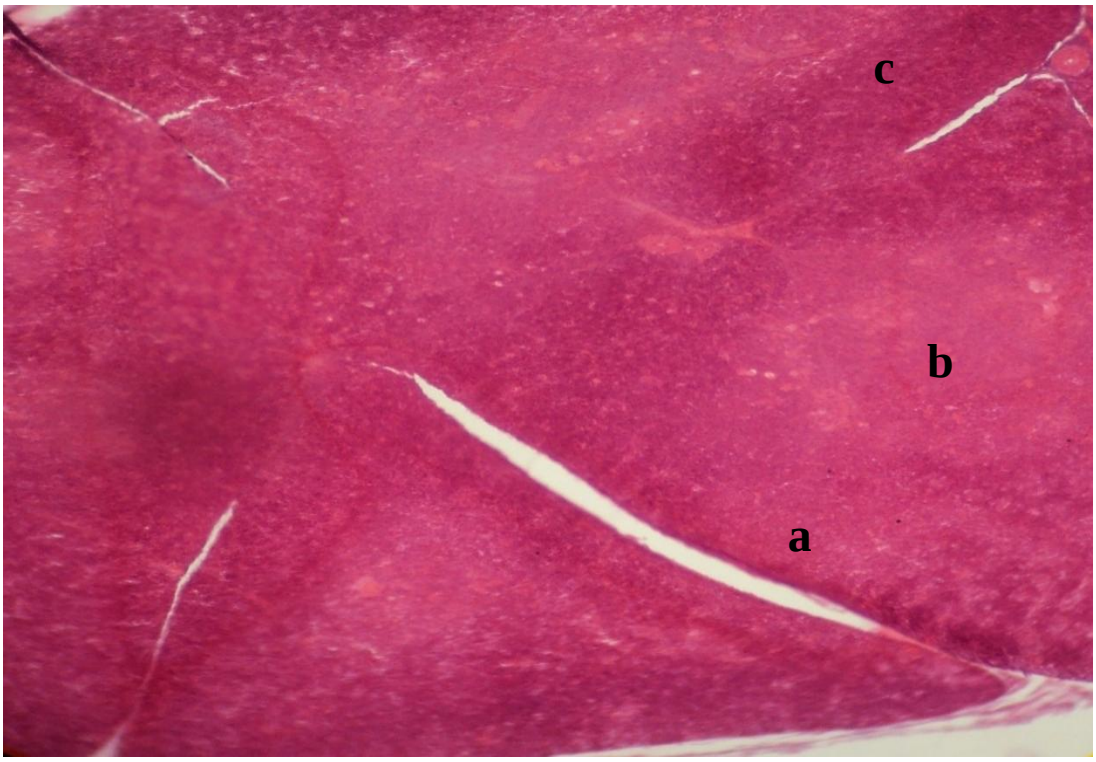

Plate XV: Thymus fromMB®group 1 Dpv:Mild to moderate cortical atrophy (a); Moderate lymphocytic depletion (b). H&E stain x 200

CHAPTER FIVE

DISCUSSION

In this study, the optimal vaccination time was investigated in the cockerels vaccinated at the optimal vaccination time estimated with the Deventer formula (de Wit, 1998, 2001), which presented a protective MDA for the period of 4-5 weeks. The protective cover of the MDA conforms with previous observations (Abdu, 1985; van den Berg, 2000; Mahgoub, 2012; Ingrao *et al*., 2013; Gardin *et.al.*, 2014).

This study also revealed that the clinical manifestations, gross and histopathological changes observed in the different groups were typical of experimental infections/vaccination and field outbreaks of IBD. The clinical signs observed in the infected chickens were however a replica of the field case from which the virus was isolated. This severity was also exhibited by some of the vaccines used in this experiment. This finding disagrees with earlier reports by Helmbolt and Garner (1964), Chineme and Cho (1984) as well as Oladele *et al*. (2010) who stated that the clinical and pathological changes in experimental IBDV infection of chickens were with rare exceptions milder than in spontaneous disease. The reason behind this disagreement could be as a result of variation in the virulence of theIBDV strain.

The clinical signs exhibited by the cockerels in this experiment correspond with the clinical manifestations exhibited by chickens infected with the very virulent IBDV. At 1 Dpv/c, clinical signs and gross lesions were observed in the challenge group, and at 2 Dpv/c, mortalities were recorded. These manifestations were in agreement with findings of previous works(Kaufer and Weis, 1976; 1980; van den Berg, 2000; Mahgoub, 2012). The disease peaked, declined and maintained a plateau of mortality on the 4^{th} and 5^{th}Dpv/c conforming to the field infection of the disease. Gross lesions

which include, petechial haemorrhages were observed in three out of the four vaccinated groups (MB®, Cevac® IBD L and IBD-Vac®) on the thigh, pectoral muscle, bursa of Fabricius, spleen and thymus; petechial haemorrhages, mild oedema were also noticed on the bursa of Fabricius. These lesions were observed when an 'Intermediate' and 'intermediate plus' or 'hot' vaccines are used.In this study, Cevac® IBD L vaccine is an intermediate plus vaccine; MB®, Bursine® -2 and IBD-Vac®are intermediate vaccines. These vaccines have a better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions, and thus, cause corresponding levels of immunosuppression (Mazariegos *et al*., 1990; Tsukamoto *et al*., 1995; Kumar *et al*., 2000; Rautenschlein *et al*., 2005). Mazariegos *et al*. (1990) showed that intermediate vaccines varied in their pathogenicity and they divided intermediate vaccines into 3 pathogenic categories 'highly pathogenic, moderately pathogenic and low pathogenic or mild'. This study observed that MB® was highly pathogenic, while Bursine® -2 and IBD-Vac® were moderately pathogenic.

Grossly, a yellowish transudate covered the BF at 1 Dpv/c and the BF began to increase in size at weight at 3 Dpv/c, although the bursae in this study did double in size by the 4Dpv/c the weight and sizes (diameter) were maintained till the 6Dpv/c when they began to decrease in size. The transudate disappeared on the 7Dpv/c and atrophy started to set in. The initial stage of this gross pathology in the challenged group, agrees with the findings of previous authors (Chettle *et al.,* 1989; van den Berg *et al.,* 1991; Nunoya *et al.,* 1992; Tsukamoto,*et al.,* 1992; van den Berg, 2000; Mahgoub, 2012). The petechial and ecchymotic haemorrhages observed in the challenged group are similar to those reported by (Lukert and Saif, 2003; Zeryehun *et al.,* 2012) while those in the bursa and paintbrush haemorrhages in the muscles of

breast, thigh and legs are similar to was reported by (Okoye,2005). The frequency of haemorrhage in the bursa, skeletal muscles and the proventricular-gizzard mucosa was found to be higher in the dead birds of the challenge group.

Bursal, spleenic and thymic enlargemnt observed early in the vaccinated and challenge chickens is believed to be due to inflammatory response to tissue damage by the IBDV in the bursa of Fabricius of such birds. The oedema and hyperaemia observed grossly and the widening of the inter-follicular 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 (Cheville, 1967; Ley *et al*., 1983; Chineme and Cho, 1984; Oladele *et al*., 2010; Gardin *et al*., 2014).

The results of this study showed that bursal diameters varied from 2 to 5suggesting that there were similar histological injuries between the vaccinated and challenge birds, irrespective of the source of the vaccine,thus corroborating results previously reported by Pereira (2002); and Moraes *et al.* (2012). In a study conducted in broilers vaccinated with different intermediate IBD vaccines, injury indexes could not be attributed to disease or vaccination based solely on bursa diameter (Pereira, 2002). Based on the presnt observations, it can be suggested that BF diameter measurement should not be used as a single parameter for disease diagnosis or in decisions related to the evaluation of vaccination programs. The measurement of BF diameter must be evaluated with caution and used only as an auxiliary method, rather than a definitive one.

As a result of inadequacies in the B:BW ratio and BFRW, the BB indices of birds in this study gave a similar standard BB index recorded by Cazaban *et al.,* (2012) (Appendix IV).

Histopathologic examination of the BF, spleen and thymus from the chickens vaccinated, and infected with IBDV showed that the predominant lesions were in the bursa of Fabricius followed by the spleen and thymus. This suggests that the target cells for IBDV are concentrated in the bursa of Fabricius and to affirm earlier speculations by Ley *et al.* (1983), and recent assertion by Mahgoub, (2012) and Gardin *et.al.,* (2014)that the unique sensitivity of bursal lymphoid cells to IBDV suggests the possibility of specific viral receptors on a B-cell sub-population that is most abundant in the bursa but may also occur in other lymphoid organs. Mac Lachlan and Dubovi, (2011) and Ingrao *et al.*(2013) 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.

A distinctive difference was noticed between the vaccinated and the challenge groups. The MB® and Cevac®IBDL, and the vaccine virus candidate which is a very virulent strain caused medullary lymphoid cell depletion. Lymphocyte depletion observed in this study could be the result of phagocytosis of the necrotic lymphocytes by macrophages in the follicles. Also the formation of cystic cavities in the bursa by 5 Dpc following the necrosis and clearance by phagocytic cells and their eventual contraction could have contributed to the bursal atrophy which has been reported in most clinical cases of IBD, beginning at about 5 days pi (Cheville, 1964; Okoye and Uzoukwu, 1984; Mahgoub, 2012). In this study, bursal atrophy was noticed as early as 4 days(96h) pc, and this could be attributed to the virulence of the virus strain used.

Although bursal regeneration was not observed in this study, Abdu *et al.,* (1988), reported the ability of BF regenerating completely at 7 weeks pi,this may be due to the duration of study which was only for 3 weeksThe actual duration of the recovery process mainly depends upon the virulence of the virus strain, the quantity of virus invading the BF, and the age at colonization. In other words, an attenuated strain of IBDV (e.g. a vaccine strain) will colonize the bursa as well.

In this study thymic lesions were characterized by medullary lymphoid condensation and depletion, and haemorrhages. However, the very virulent wild field virus used in this study (challenge) caused a slight cortical lymphoid condensation and depletion in the thymus but did not result in well-pronounced cortical atrophy. These findings agree with the observations made by Inoue *et al.* (1994), who reported the depletion in lymphoid cells of highly virulent HPS-2 strain and a mild cortical atrophy in a virulent reference GBF-1 strain. Damage to thymic lymphatic tissue was also reported by Sharma *et al.*(1995).

Microscopic assessment of the spleen which revealed congestion, mild lymphoid cell depletion and absence of significant lymphoid necrosis in this study did not necessarily preclude viral presence in the organ. Spleen was reported to have substantially higher concentration of IBDV after BF as compared to any other tissue. Studiesconducted by Inoue *et al.,* (1991) and Jackwood *et al.* (2009), had shown lympholysis and lymphocytic depletion in sections of the spleen seen in present investigation.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1Conclusions

From this work it is concluded that:

i. The cockerels administered different infectious bursal disease vaccines presented ELISA antibody titres similar to those infected with field IBDV isolate.

ii. Although gross and histopathologic lesions varied between the vaccinated birds, the challenge group presented more severe lesions.

iii. The MB ® and Cevac® IBD L vaccines conferred a better protection against IBD with minimal bursal lesions.

6.2 Recommendations

It was recommended that,

- Farmers and clinicians should emphasise on routine MDA determination of chicks before designing vaccination schedule for poultry farm.
- Intermediate strains of IBDV should be used in vaccinating birds against IBD.
- Poultry clinicians should try and establish a data base for recording BB index.
- Additional studies be carried out to better describe and update the bursa size, bursa, spleen and thymic lesion scores dynamics according to the current standards of rearing chickens.

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APPENDICES

Appendix I: Calculation of optimal vaccination (OVT).

CALCULATE OPTIMAL DAY FOR VACCINATION
Deventer formula

ELISA values

1. 4908
2. 5117
3. 7986
4. 8773
5. 9312
6. 9532
7. 9702
8. 9853
9. 10278
10. 11683
11. 12027
12. 12112
13. 13989
14. 14128
15. 14146
16. 14492
17. 15215
18. 15938
19. 19931
20. 20922

Type of vaccine: \n\n\n

Intermediate *

Type of poultry: \n\n

Layers *

Age of chicken at time of blood extraction: * 6

Date of blood extraction: 18/06/2014

dd/mm/aaaa

ACCEPT

RESULT
Age of vaccination (Intermediate strain): 38days
Date of vaccination: 20/07/2014

CALCULATE OPTIMAL DAY FOR VACCINATION
Deventer formula

ELISA values

1. 4908
2. 5117
3. 7986
4. 8773
5. 9312
6. 9532
7. 9702
8. 9853
9. 10278
10. 11683
11. 12027
12. 12112
13. 13989
14. 14128
15. 14146
16. 14492
17. 15215
18. 15938
19. 19931
20. 20922

Type of vaccine: \n\n\n

Strong *

Type of poultry: \n\n

Layers *

Age of chicken at time of blood extraction: * 6

Date of blood extraction: 18/06/2014

dd/mm/aaaa

ACCEPT

RESULT
Age of vaccination (Intermediate plus/hot): 27days
Date of vaccination: 09/07/2014

CALCULATE OPTIMAL DAY FOR VACCINATION
Deventer formula

ELISA values

1.	4908
2.	5117
3.	7986
4.	8773
5.	9312
6.	9532
7.	9702
8.	9853
9.	10278
10.	11683
11.	12027
12.	12112
13.	13989
14.	14128
15.	14146
16.	14492
17.	15215
18.	15938
19.	19931
20.	20922

Type of vaccine: \n\n\n

Mild *

Type of poultry: \n\n

Layers *

Age of chicken at time of blood extraction: 6 *

Date of blood extraction: 18/06/2014
dd/mm/aaaa

ACCEPT

RESULT
Age of vaccination (Mild strain): 40days
Date of vaccination: 22/07/2014

The formula should provide an estimation of the vaccination time but we should seek advice before implementing vaccination. It should be discussed with a veterinarian, who is aware of the epidemiological status and production particularities of the farm.

http://www.hipra.com/wps/portal/web/inicio/serviciosAlCliente/formuladeDeventerEN

Appendix II: Antibody titre of Different Treatment and Control Groups Recorded at Various Time Intervals

MB® Antibody titre								
	Wells	*O.D.*	*NCx*	*SM -NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
MB®								
24Hpv/c	A1	1.183	0.094	1.089	0.166	6.56	4.25	17,801
	A2	1.096	0.094	1.002	0.166	6.04	4.21	16,257
	A3	0.830	0.094	0.736	0.166	4.43	4.06	11,614
48Hpv/c	C4	0.725	0.094	0.631	0.166	3.80	3.99	9,820
	C5	0.948	0.094	0.854	0.166	5.14	4.14	13,658
	C6	0.800	0.094	0.706	0.166	4.25	4.05	11,099
	C7	1.022	0.094	0.928	0.166	5.59	4.17	14,952
	C8	0.549	0.094	0.455	0.166	2.74	3.84	6,876
3 Dpv/c	F2	0.780	0.094	0.686	0.166	4.13	4.03	10,757
	F3	0.382	0.094	0.288	0.166	1.73	3.62	4,177
	F4	0.311	0.094	0.217	0.166	1.31	3.49	3,068
	F5	0.365	0.094	0.271	0.166	1.63	3.59	3,909
	F6	0.278	0.094	0.184	0.166	1.11	3.41	2,563
4 Dpv/c	A4	0.920	0.094	0.826	0.166	4.98	4.12	13,170
	A5	0.486	0.094	0.392	0.166	2.36	3.77	5,845
	A6	0.515	0.094	0.421	0.166	2.54	3.80	6,318
	A7	0.543	0.094	0.449	0.166	2.70	3.83	6,777
	A8	0.591	0.094	0.497	0.166	2.99	3.88	7,570
5 Dpv/c	C10	0.538	0.094	0.444	0.166	2.67	3.83	6,695
	C11	0.483	0.094	0.389	0.166	2.34	3.76	5,796
	C12	0.497	0.094	0.403	0.166	2.43	3.78	6,024
	D1	0.452	0.094	0.358	0.166	2.16	3.72	5,294
	D2	0.172	0.094	0.078	0.166	0.47	3.00	1,006
7Dpv/c	F5	0.838	0.094	0.744	0.166	4.48	4.07	11,752
	F6	0.510	0.094	0.416	0.166	2.51	3.79	6,236
	F7	0.442	0.094	0.348	0.166	2.10	3.71	5,133
	F8	0.944	0.094	0.850	0.166	5.12	4.13	13,588
	F9	0.503	0.094	0.409	0.166	2.46	3.79	6,122
10Dpv/c	H6	0.312	0.094	0.218	0.166	1.31	3.49	3,083
	H7	0.360	0.094	0.266	0.166	1.60	3.58	3,830
	H8	0.317	0.094	0.223	0.166	1.34	3.50	3,160
	H9	1.229	0.094	1.135	0.166	6.84	4.27	18,622
	H10	2.206	0.094	2.112	0.166	12.72	4.56	36,644
14Dpv/c	B6	0.329	0.094	0.235	0.166	1.42	3.52	3,346
	B7	0.218	0.094	0.124	0.166	0.75	3.22	1,667
	B8	0.334	0.094	0.240	0.166	1.45	3.53	3,424
	B9	0.335	0.094	0.241	0.166	1.45	3.54	3,439
	B10	0.398	0.094	0.304	0.166	1.83	3.65	4,430

21Dpv/c	E1	0.243	0.094	0.149	0.166	0.90	3.31	2,036
	E2	0.232	0.094	0.138	0.166	0.83	3.27	1,873
	E3	0.356	0.094	0.262	0.166	1.58	3.58	3,767
	E4	1.660	0.094	1.566	0.166	9.43	4.42	26,449
	E5	1.333	0.094	1.239	0.166	7.46	4.31	20,489

BURSINE®-2 Antibody titre levels								
	Wells	*O. D.*	*NCx*	*SM -NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
BURSINE®-2								
24Hpv/c	A4	0.411	0.094	0.317	0.166	1.91	3.67	4,637
	A5	0.650	0.094	0.556	0.166	3.35	3.93	8,555
	A6	1.067	0.094	0.973	0.166	5.86	4.20	15,744
	A7	1.033	0.094	0.939	0.166	5.66	4.18	15,146
	A8	0.741	0.094	0.647	0.166	3.90	4.00	10,092
48Hpv/c	C9	0.642	0.094	0.548	0.166	3.30	3.93	8,421
	C10	0.540	0.094	0.446	0.166	2.69	3.83	6,728
	C11	0.741	0.094	0.647	0.166	3.90	4.00	10,092
	C12	0.935	0.094	0.841	0.166	5.07	4.13	13,431
	D1	1.013	0.094	0.919	0.166	5.54	4.17	14,794
3 Dpv/c	F7	0.292	0.094	0.198	0.166	1.19	3.44	2,776
	F8	0.649	0.094	0.555	0.166	3.34	3.93	8,538
	F9	0.803	0.094	0.709	0.166	4.27	4.05	11,150
	F10	0.363	0.094	0.269	0.166	1.62	3.59	3,877
	F11	0.322	0.094	0.228	0.166	1.37	3.51	3,238
4 Dpv/c	A9	0.426	0.094	0.332	0.166	2.00	3.69	4,877
	A10	0.461	0.094	0.367	0.166	2.21	3.74	5,440
	A11	0.624	0.094	0.530	0.166	3.19	3.91	8,120
	A12	0.882	0.094	0.788	0.166	4.75	4.10	12,511
	B1	0.468	0.094	0.374	0.166	2.25	3.74	5,553
5 Dpv/c	D3	0.619	0.094	0.525	0.166	3.16	3.91	8,036
	D4	0.231	0.094	0.137	0.166	0.83	3.27	1,858
	D5	0.493	0.094	0.399	0.166	2.40	3.78	5,959
	D6	0.463	0.094	0.369	0.166	2.22	3.74	5,472
	D7	0.379	0.094	0.285	0.166	1.72	3.62	4,129
7Dpv/c	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							
10Dpv/c	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							
14Dpv/c	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							
	No Sample collected							

21Dpv/c	D12	0.242	0.094	0.148	0.166	0.89	3.31	2,021
	E1	0.243	0.094	0.149	0.166	0.90	3.31	2,036
	E2	0.232	0.094	0.138	0.166	0.83	3.27	1,873
	E3	0.356	0.094	0.262	0.166	1.58	3.58	3,767
	E4	1.660	0.094	1.566	0.166	9.43	4.42	26,449

CEVAC® IBD L Antibody titre levels								
	Wells	*O. D.*	*NCx*	*S M –NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
CEVAC® IBD L								
24Hpv/c	A9	0.631	0.094	0.537	0.166	3.23	3.92	8,237
	A10	0.581	0.094	0.487	0.166	2.93	3.87	7,404
	A11	0.514	0.094	0.420	0.166	2.53	3.80	6,301
	A12	1.298	0.094	1.204	0.166	7.25	4.30	19,859
	B1	0.693	0.094	0.599	0.166	3.61	3.97	9,278
48Hpv/c	D2	0.440	0.094	0.346	0.166	2.08	3.71	5,101
	D3	0.466	0.094	0.372	0.166	2.24	3.74	5,520
	D4	0.351	0.094	0.257	0.166	1.55	3.57	3,689
	D5	0.312	0.094	0.218	0.166	1.31	3.49	3,083
	D6	0.721	0.094	0.627	0.166	3.78	3.99	9,752
3 Dpv/c	F12	0.491	0.094	0.397	0.166	2.39	3.77	5,926
	G1	0.756	0.094	0.662	0.166	3.99	4.01	10,347
	G2	0.264	0.094	0.170	0.166	1.02	3.37	2,351
	G3	0.297	0.094	0.203	0.166	1.22	3.46	2,853
	G4	0.267	0.094	0.173	0.166	1.04	3.38	2,396
4 Dpv/c	B2	0.536	0.094	0.442	0.166	2.66	3.82	6,662
	B3	0.403	0.094	0.309	0.166	1.86	3.65	4,510
	B4	0.386	0.094	0.292	0.166	1.76	3.63	4,240
	B5	0.256	0.094	0.162	0.166	0.98	3.35	2,231
	B6	0.537	0.094	0.443	0.166	2.67	3.82	6,678
5 Dpv/c	D8	0.843	0.094	0.749	0.166	4.51	4.07	11,838
	D9	0.197	0.094	0.103	0.166	0.62	3.13	1,362
	D10	0.347	0.094	0.253	0.166	1.52	3.56	3,626
	D11	0.444	0.094	0.350	0.166	2.11	3.71	5,166
	D12	0.387	0.094	0.293	0.166	1.77	3.63	4,256
7Dpv/c	F10	0.307	0.094	0.213	0.166	1.28	3.48	3,006
	F11	0.267	0.094	0.173	0.166	1.04	3.38	2,396
	F12	0.615	0.094	0.521	0.166	3.14	3.90	7,970
	G1	0.543	0.094	0.449	0.166	2.70	3.83	6,777
	G2	0.381	0.094	0.287	0.166	1.73	3.62	4,161
10Dpv/c	H10	2.206	0.094	2.112	0.166	12.72	4.56	36,644
	H11	1.772	0.094	1.678	0.166	10.11	4.46	28,517
	H12	2.124	0.094	2.030	0.166	12.23	4.55	35,096
	A1	1.674	0.094	1.580	0.166	9.52	4.43	26,707
	A2	1.579	0.094	1.485	0.166	8.95	4.40	24,961
14Dpv/c	B11	0.379	0.094	0.285	0.166	1.72	3.62	4,129
	B12	0.345	0.094	0.251	0.166	1.51	3.56	3,595
	C1	0.307	0.094	0.213	0.166	1.28	3.48	3,006
	C2	1.546	0.094	1.452	0.166	8.75	4.39	24,357
	C3	2.035	0.094	1.941	0.166	11.69	4.52	33,422

21Dpv/c	E5	1.333	0.094	1.239	0.166	7.46	4.31	20,489
	E6	1.594	0.094	1.500	0.166	9.04	4.40	25,236
	E7	0.248	0.094	0.154	0.166	0.93	3.32	2,111
	E8	1.619	0.094	1.525	0.166	9.19	4.41	25,695
	E9	1.373	0.094	1.279	0.166	7.70	4.33	21,211

IBD-VAC® Antibody titre levels								
	Wells	*O. D.*	*NCx*	*S M -NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
IBD-VAC®								
24Hpv/c	B7	0.515	0.094	0.421	0.166	2.54	3.80	6,318
	B8	0.525	0.094	0.431	0.166	2.60	3.81	6,481
	B9	2.195	0.094	2.101	0.166	12.66	4.56	36,436
	B10	1.156	0.094	1.062	0.166	6.40	4.24	17,320
48Hpv/c	D12	0.436	0.094	0.342	0.166	2.06	3.70	5,037
	E1	1.343	0.094	1.249	0.166	7.52	4.32	20,670
	E2	0.286	0.094	0.192	0.166	1.16	3.43	2,685
	E3	0.671	0.094	0.577	0.166	3.48	3.95	8,908
	E4	0.334	0.094	0.240	0.166	1.45	3.53	3,424
3 Dpv/c	G10	0.250	0.094	0.156	0.166	0.94	3.33	2,141
	G11	0.853	0.094	0.759	0.166	4.57	4.08	12,010
	G12	0.521	0.094	0.427	0.166	2.57	3.81	6,416
	H1	0.397	0.094	0.303	0.166	1.83	3.64	4,414
	H2	1.561	0.094	1.467	0.166	8.84	4.39	24,632
4 Dpv/c	B12	0.178	0.094	0.084	0.166	0.51	3.04	1,090
	C1	0.803	0.094	0.709	0.166	4.27	4.05	11,150
	C2	0.325	0.094	0.231	0.166	1.39	3.52	3,284
	C3	0.387	0.094	0.293	0.166	1.77	3.63	4,256
	C4	0.322	0.094	0.228	0.166	1.37	3.51	3,238
5 Dpv/c	E7	0.220	0.094	0.126	0.166	0.76	3.23	1,696
	E8	0.230	0.094	0.136	0.166	0.82	3.27	1,843
	E9	0.528	0.094	0.434	0.166	2.61	3.81	6,530
	E10	0.339	0.094	0.245	0.166	1.48	3.54	3,502
	E11	0.293	0.094	0.199	0.166	1.20	3.45	2,791
7Dpv/c	G8	0.223	0.094	0.129	0.166	0.78	3.24	1,740
	G9	1.888	0.094	1.794	0.166	10.81	4.49	30,672
	G10	1.755	0.094	1.661	0.166	10.01	4.45	28,202
	G11	2.298	0.094	2.204	0.166	13.28	4.58	38,387
	G12	1.818	0.094	1.724	0.166	10.39	4.47	29,370
10Dpv/c	A8	1.428	0.094	1.334	0.166	8.04	4.35	22,208
	A9	0.129	0.094	0.035	0.166	0.21	2.62	420
	A10	1.904	0.094	1.810	0.166	10.90	4.49	30,971
	A11	1.835	0.094	1.741	0.166	10.49	4.47	29,686
	A12	1.187	0.094	1.093	0.166	6.58	4.25	17,872
14Dpv/c	C9	1.693	0.094	1.599	0.166	9.63	4.43	27,057
	C10	1.692	0.094	1.598	0.166	9.63	4.43	27,038
	C11	1.651	0.094	1.557	0.166	9.38	4.42	26,283
	C12	1.624	0.094	1.530	0.166	9.22	4.41	25,787
	D1	1.619	0.094	1.525	0.166	9.19	4.41	25,695

21Dpv/c	F3	1.428	0.094	1.334	0.166	8.04	4.35	22,208
	F4	1.468	0.094	1.374	0.166	8.28	4.36	22,934
	F5	1.298	0.094	1.204	0.166	7.25	4.30	19,859
	F6	1.570	0.094	1.476	0.166	8.89	4.39	24,796
	F7	1.323	0.094	1.229	0.166	7.40	4.31	20,309

CONTROL Antibody titre levels								
	Wells	*O. D.*	*NCx*	*SM -NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
CONTROL								
24Hpv/c	B11	0.423	0.094	0.329	0.166	1.98	3.68	4,829
	B12	0.644	0.094	0.550	0.166	3.31	3.93	8,454
	C1	0.522	0.094	0.428	0.166	2.58	3.81	6,432
	C2	0.567	0.094	0.473	0.166	2.85	3.86	7,173
	C3	1.042	0.094	0.948	0.166	5.71	4.18	15,304
48Hpv/c	E5	0.300	0.094	0.206	0.166	1.24	3.46	2,899
	E6	0.237	0.094	0.143	0.166	0.86	3.29	1,947
	E7	0.814	0.094	0.720	0.166	4.34	4.05	11,339
	E8	1.109	0.094	1.015	0.166	6.11	4.22	16,487
	E9	0.247	0.094	0.153	0.166	0.92	3.32	2,096
3 Dpv/c	H4	0.360	0.094	0.266	0.166	1.60	3.58	3,830
	H5	0.137	0.094	0.043	0.166	0.26	2.72	525
	H6	0.157	0.094	0.063	0.166	0.38	2.90	797
	H7	0.122	0.094	0.028	0.166	0.17	2.52	329
	H8	0.132	0.094	0.038	0.166	0.23	2.66	459
4 Dpv/c	C5	1.095	0.094	1.001	0.166	6.03	4.21	16,239
	C6	0.825	0.094	0.731	0.166	4.40	4.06	11,528
	C7	0.445	0.094	0.351	0.166	2.11	3.71	5,182
	C8	0.404	0.094	0.310	0.166	1.87	3.66	4,525
	C9	0.334	0.094	0.240	0.166	1.45	3.53	3,424
5 Dpv/c	E12	0.264	0.094	0.170	0.166	1.02	3.37	2,351
	F1	0.181	0.094	0.087	0.166	0.52	3.05	1,133
	F2	0.155	0.094	0.061	0.166	0.37	2.89	769
	F3	0.378	0.094	0.284	0.166	1.71	3.61	4,113
	F4	0.285	0.094	0.191	0.166	1.15	3.43	2,669
7Dpv/c	H1	1.949	0.094	1.855	0.166	11.17	4.50	31,811
	H2	0.437	0.094	0.343	0.166	2.07	3.70	5,053
	H3	0.372	0.094	0.278	0.166	1.67	3.60	4,019
	H4	0.499	0.094	0.405	0.166	2.44	3.78	6,056
	H5	0.391	0.094	0.297	0.166	1.79	3.64	4,319
10Dpv/c	B1	1.063	0.094	0.969	0.166	5.84	4.20	15,674
	B2	1.340	0.094	1.246	0.166	7.51	4.31	20,616
	B3	0.390	0.094	0.296	0.166	1.78	3.63	4,303
	B4	0.305	0.094	0.211	0.166	1.27	3.47	2,975
	B5	0.354	0.094	0.260	0.166	1.57	3.57	3,736
14Dpv/c	C2	1.546	0.094	1.452	0.166	8.75	4.39	24,357
	C3	2.035	0.094	1.941	0.166	11.69	4.52	33,422
	C4	1.607	0.094	1.513	0.166	9.11	4.41	25,475
	C5	1.568	0.094	1.474	0.166	8.88	4.39	24,760
	C6	1.422	0.094	1.328	0.166	8.00	4.34	22,099

21Dpv/c	F8	1.243	0.094	1.149	0.166	6.92	4.28	18,873
	F9	1.312	0.094	1.218	0.166	7.34	4.30	20,111
	F10	0.225	0.094	0.131	0.166	0.79	3.25	1,770
	F11	1.636	0.094	1.542	0.166	9.29	4.42	26,007
	F12	1.094	0.094	1.000	0.166	6.02	4.21	16,221

CHALLENGE Antibody titre levels								
	Wells	*O. D.*	*NCx*	*SM -NCx*	*PCx-NCx*	*S/P*	*Log10 Titer*	*Titer*
CHALLENGE								
24Hpv/c	B2	0.896	0.094	0.802	0.166	4.83	4.11	12,754
	B3	0.548	0.094	0.454	0.166	2.73	3.84	6,859
	B4	0.565	0.094	0.471	0.166	2.84	3.85	7,140
	B5	0.203	0.094	0.109	0.166	0.66	3.16	1,448
	B6	0.638	0.094	0.544	0.166	3.28	3.92	8,354
48Hpv/c	D7	0.406	0.094	0.312	0.166	1.88	3.66	4,557
	D8	0.281	0.094	0.187	0.166	1.13	3.42	2,608
	D9	0.549	0.094	0.455	0.166	2.74	3.84	6,876
	D10	0.423	0.094	0.329	0.166	1.98	3.68	4,829
	D11	0.307	0.094	0.213	0.166	1.28	3.48	3,006
3 Dpv/c	G5	0.527	0.094	0.433	0.166	2.61	3.81	6,514
	G6	1.520	0.094	1.426	0.166	8.59	4.38	23,882
	G7	0.195	0.094	0.101	0.166	0.61	3.12	1,333
	G8	0.206	0.094	0.112	0.166	0.67	3.17	1,492
	G9	0.217	0.094	0.123	0.166	0.74	3.22	1,652
4 Dpv/c	B7	0.578	0.094	0.484	0.166	2.92	3.87	7,355
	B8	0.403	0.094	0.309	0.166	1.86	3.65	4,510
	B9	0.575	0.094	0.481	0.166	2.90	3.86	7,305
	B10	0.692	0.094	0.598	0.166	3.60	3.97	9,262
	B11	0.627	0.094	0.533	0.166	3.21	3.91	8,170
5 Dpv/c	E2	2.110	0.094	2.016	0.166	12.14	4.54	34,832
	E3	1.837	0.094	1.743	0.166	10.50	4.47	29,723
	E4	1.637	0.094	1.543	0.166	9.30	4.42	26,026
	E5	1.550	0.094	1.456	0.166	8.77	4.39	24,430
	E6	1.457	0.094	1.363	0.166	8.21	4.36	22,734
7Dpv/c	G3	0.235	0.094	0.141	0.166	0.85	3.28	1,917
	G4	0.224	0.094	0.130	0.166	0.78	3.24	1,755
	G5	0.443	0.094	0.349	0.166	2.10	3.71	5,149
	G6	0.163	0.094	0.069	0.166	0.42	2.94	880
	G7	1.092	0.094	0.998	0.166	6.01	4.21	16,186
10Dpv/c	A3	1.935	0.094	1.841	0.166	11.09	4.50	31,549
	A4	2.131	0.094	2.037	0.166	12.27	4.55	35,228
	A5	1.813	0.094	1.719	0.166	10.36	4.47	29,277
	A6	1.864	0.094	1.770	0.166	10.66	4.48	30,225
	A7	1.538	0.094	1.444	0.166	8.70	4.38	24,211
14Dpv/c	C4	1.607	0.094	1.513	0.166	9.11	4.41	25,475
	C5	1.568	0.094	1.474	0.166	8.88	4.39	24,760
	C6	1.422	0.094	1.328	0.166	8.00	4.34	22,099
	C7	1.443	0.094	1.349	0.166	8.13	4.35	22,480
	C8	1.700	0.094	1.606	0.166	9.67	4.43	27,186

21Dpv/c	E10	1.308	0.094	1.214	0.166	7.31	4.30	20,039
	E11	1.524	0.094	1.430	0.166	8.61	4.38	23,955
	E12	1.343	0.094	1.249	0.166	7.52	4.32	20,670
	F1	1.560	0.094	1.466	0.166	8.83	4.39	24,613
	F2	1.772	0.094	1.678	0.166	10.11	4.46	28,517

Keys:

O.D. = Optical Density

NCx = Negative Control mean

PCx = Positive Control mean

SM = Sample Mean

S/P = Sample to positive ratio

Log_{10} Titre = 1.09 (log_{10} S/P) + 3.36

Appendix III: Organ Relative Weight and Mean Weightof Different Treatment and Control Groups Recorded At Various Time Intervals

Calculation of Individual Organ Mean Weight (g)					
MB®					
Time	Sample	Organ Weight and Mean weight			
		Bursa	M BBW	spleen	M SBW
24Hpv/c	A	1.10	5.95	0.80	4.32
	B	0.80	4.35	0.60	3.24
	C	0.80	4.37	0.70	3.78
Mean Weight		0.90	4.89	0.70	3.78
48Hpv/c	A	1.40	7.57	0.90	4.86
	B	1.30	7.03	0.60	3.24
	C	1.10	5.95	0.80	4.32
Mean Weight		1.27	6.85	0.77	4.14
3 Dpv/c	A	1.70	8.50	0.80	4.00
	B	1.20	5.91	0.90	4.43
	C	1.10	5.53	0.70	3.52
Mean Weight		1.33	6.65	0.80	3.98
4 Dpv/c	A	0.70	3.43	0.50	2.45
	B	1.00	4.44	0.60	2.67
	C	1.20	6.19	0.70	3.61
Mean Weight		0.97	4.69	0.60	2.91
5 Dpv/c	A	1.60	6.87	0.70	3.00
	B	1.70	6.97	0.80	3.28
	C	1.50	6.28	0.50	2.09
Mean Weight		1.60	6.70	0.67	2.79
7Dpv/c	A	0.50	2.26	0.40	1.81
	B	0.60	2.26	0.60	2.26
	C	0.70	2.49	0.40	1.42
Mean Weight		0.60	2.34	0.47	1.83
10Dpv/c	A	0.70	2.65	0.50	1.89
	B	0.40	1.39	0.60	2.08
	C	0.60	1.75	0.40	1.17
Mean Weight		0.57	1.93	0.50	1.71
14Dpv/c	A	0.30	1.01	0.40	1.34
	B	0.60	1.68	0.50	1.40
	C	0.60	1.63	0.70	1.91
Mean Weight		0.50	1.44	0.53	1.55
21Dpv/c	A	0.80	1.58	0.60	1.19
	B	0.60	1.44	0.40	0.96
	C	0.40	1.04	0.60	1.57
Mean Weight		0.60	1.36	0.53	1.24

Calculation of Individual Organ Mean Weight (g)					
BURSINE®-2					
Time	Sample	Organ Weight and Mean weight			
		Bursa	Mean BBW	spleen	Mean SBW
	A	0.80	4.32	0.60	3.24
24Hpv/c	B	0.90	4.89	0.50	2.72
	C	1.10	6.01	0.30	1.64
Mean Weight		0.93	5.08	0.47	2.53
	A	0.90	4.43	0.50	2.46
48Hpv/c	B	0.70	3.63	0.60	3.11
	C	1.10	5.42	0.40	1.97
Mean Weight		0.90	4.49	0.50	2.51
	A	1.60	8.00	0.70	3.50
3 Dpv/c	B	1.70	8.37	0.80	3.94
	C	0.80	4.02	0.40	2.01
Mean Weight		1.37	6.80	0.63	3.15
	A	0.90	4.41	0.70	3.43
4 Dpv/c	B	1.30	5.78	0.60	2.67
	C	1.30	6.70	0.70	3.61
Mean Weight		1.17	5.63	0.67	3.24
	A	1.70	7.30	0.50	2.15
5 Dpv/c	B	1.30	5.33	0.70	2.87
	C	1.60	6.69	0.80	3.35
Mean Weight		1.53	6.44	0.67	2.79
	A	1.70	7.69	0.20	0.90
7Dpv/c	B	1.40	5.26	0.30	1.13
	C	1.80	6.41	0.50	1.78
Mean Weight		1.63	6.45	0.33	1.27
	A	0.00	0.00	0.00	0.00
10Dpv/c	B	0.00	0.00	0.00	0.00
	C	0.00	0.00	0.00	0.00
Mean Weight		0.00	0.00	0.00	0.00
	A	0.00	0.00	0.00	0.00
14Dpv/c	B	0.00	0.00	0.00	0.00
	C	0.00	0.00	0.00	0.00
Mean Weight		0.00	0.00	0.00	0.00
	A	0.60	1.13	1.40	2.64
21Dpv/c	B	0.40	0.96	0.50	1.20
	C	0.40	1.01	0.80	2.02
Mean Weight		0.47	1.03	0.90	1.95

Calculation of Individual Organ Mean Weight					
Cevac® IBD L					
Time	Sample	Organs			
		Bursa	Mean BBW	Spleen	Mean SBW
	A	1.00	5.41	0.80	4.32
24Hpv/c	B	0.90	4.89	0.60	3.26
	C	1.20	6.56	0.70	3.83
Mean Weight		1.03	5.62	0.70	3.80
	A	0.90	4.95	0.70	3.85
48Hpv/c	B	1.10	5.82	0.80	4.23
	C	0.90	4.89	0.60	3.26
Mean Weight		0.97	5.22	0.70	3.78
	A	0.70	3.50	0.70	3.50
3 Dpvi	B	1.40	6.90	0.90	4.43
	C	1.10	5.53	0.50	2.51
Mean Weight		1.07	5.31	0.70	3.48
	A	1.40	6.86	0.70	3.43
96Hpvi	B	1.40	6.22	0.40	1.78
	C	1.00	5.15	0.90	4.64
Mean Weight		1.27	6.08	0.67	3.28
	A	1.70	7.30	0.70	3.00
5 Dpv/c	B	1.30	5.33	0.80	3.28
	C	1.00	4.18	0.40	1.67
Mean Weight		1.33	5.60	0.63	2.65
	A	1.20	5.43	0.80	3.62
7Dpv/c	B	1.00	3.76	0.60	2.26
	C	1.10	3.91	0.30	1.07
Mean Weight		1.10	4.37	0.57	2.31
	A	0.30	1.14	1.00	3.79
10Dpv/c	B	1.40	4.86	1.20	4.17
	C	0.50	1.46	0.60	1.75
Mean Weight		0.73	2.49	0.93	3.23
	A	0.30	1.01	1.30	4.36
14Dpv/c	B	0.30	0.84	1.00	2.79
	C	0.70	1.91	1.40	3.81
Mean Weight		0.43	1.25	1.23	3.66
	A	0.80	2.37	1.00	2.96
21Dpv/c	B	0.40	1.06	1.00	2.65
	C	0.80	3.42	1.00	4.27
Mean Weight		0.67	2.28	1.00	3.29

Calculation of Individual Organ Mean Weight					
IBD-Vac®					
Time	Sample	Organs			
		Bursa	MBBW	spleen	MSBW
24Hpv/c	A	0.60	3.24	0.70	3.78
	B	1.10	5.98	0.60	3.26
	C	0.70	3.83	0.50	2.73
Mean Weight		0.80	4.35	0.60	3.26
48Hpv/c	A	1.20	6.22	0.70	3.63
	B	1.10	5.73	0.50	2.60
	C	1.00	5.46	0.40	2.19
Mean Weight		1.10	5.80	0.53	2.81
3 Dpv/c	A	0.80	4.00	0.50	2.50
	B	1.10	5.42	0.60	2.96
	C	0.70	3.52	0.40	2.01
Mean Weight		0.87	4.31	0.50	2.49
4 Dpv/c	A	0.90	4.41	0.40	1.96
	B	0.80	3.56	0.50	2.22
	C	1.00	5.15	0.30	1.55
Mean Weight		0.90	4.37	0.40	1.91
5 Dpv/c	A	1.90	8.15	0.80	3.43
	B	1.10	4.51	0.60	2.46
	C	1.60	6.69	0.40	1.67
Mean Weight		1.53	6.45	0.60	2.52
7Dpv/c	A	1.50	6.79	0.30	1.36
	B	1.90	7.14	0.50	1.88
	C	1.30	4.63	0.40	1.42
Mean Weight		1.57	6.19	0.40	1.55
10D Hpv/c	A	1.10	4.17	0.30	1.14
	B	2.70	9.38	0.40	1.39
	C	1.20	3.50	0.60	1.75
Mean Weight		1.67	5.68	0.43	1.42
14Dpv/c	A	2.00	6.71	0.70	2.35
	B	2.00	5.59	1.10	3.07
	C	1.80	4.90	1.00	2.72
Mean Weight		1.93	5.73	0.93	2.72
21Dpv/c	A	0.40	1.70	0.30	1.28
	B	0.40	0.88	2.00	4.42
	C	0.30	0.80	0.50	1.33
Mean Weight		0.37	1.13	0.93	2.34

Calculation of Individual Organ Mean Weight					
CONTROL					
Time	Sample	Organs			
		Bursa	M BBW	Spleen	M SBW
	A	1.20	6.49	0.50	2.70
24Hp/iv	B	0.70	3.80	0.40	2.17
	C	0.70	3.83	0.30	1.64
Mean Weight		0.87	4.71	0.40	2.17
	A	0.90	4.84	0.50	2.69
48Hpv/c	B	1.30	7.93	0.70	4.27
	C	1.10	5.42	0.30	1.48
Mean Weight		1.10	6.06	0.50	2.81
	A	1.10	5.50	0.40	2.00
3 Dpv/c	B	1.10	5.42	0.40	1.97
	C	1.40	7.04	0.40	2.01
Mean Weight		1.20	5.98	0.40	1.99
	A	1.80	1.40	0.70	1.40
4 Dpv/c	B	1.10	1.40	0.40	1.40
	C	1.00	1.00	0.40	1.00
Mean Weight		1.30	1.27	0.50	1.27
	A	1.40	6.01	0.50	2.15
5 Dpv/c	B	1.70	6.97	0.20	0.82
	C	1.40	5.86	0.60	2.51
Mean Weight		1.50	6.28	0.43	1.83
	A	1.50	6.79	0.50	2.26
7Dpv/c	B	1.20	4.51	0.40	1.50
	C	1.80	6.41	0.50	1.78
Mean Weight		1.50	5.90	0.47	1.85
	A	1.00	3.79	0.30	1.14
10Dpv/c	B	1.60	5.56	0.50	1.74
	C	1.40	4.08	0.40	1.17
Mean Weight		1.33	4.48	0.40	1.35
	A	2.50	8.39	0.70	2.35
14Dpv/c	B	2.10	5.87	1.40	3.91
	C	1.60	4.36	1.80	4.90
Mean Weight		2.07	6.20	1.30	3.72
	A	0.60	1.32	0.70	1.54
21Dpv/c	B	0.60	1.60	0.70	1.87
	C	0.50	1.34	1.20	3.23
Mean Weight		0.57	1.42	0.87	2.21

Calculation of Individual Organ Mean Weight					
CHALLENGE					
Time	Sample	Organs			
		Bursa	M BBW	Spleen	M SBW
	A	1.10	5.95	0.60	3.24
24Hpv/c	B	1.20	6.52	0.80	4.35
	C	1.00	5.46	0.90	4.92
Mean Weight		1.10	5.98	0.77	4.17
	A	0.90	4.84	0.40	2.15
48Hpv/c	B	1.30	7.93	0.50	3.05
	C	1.20	5.91	0.70	3.45
Mean Weight		1.13	6.23	0.53	2.88
	A	0.40	2.00	0.40	2.00
3 Dpv/c	B	0.60	2.96	0.50	2.46
	C	0.50	2.51	0.20	1.01
Mean Weight		0.50	2.49	0.37	1.82
	A	0.40	1.40	0.30	1.40
4 Dpv/c	B	0.80	1.40	0.30	1.40
	C	0.70	1.00	0.30	1.00
Mean Weight		0.63	1.27	0.30	1.27
	A	0.40	1.72	0.30	1.29
5 Dpv/c	B	0.30	1.23	0.20	0.82
	C	0.60	2.51	0.40	1.67
Mean Weight		0.43	1.82	0.30	1.26
	A	0.30	1.36	0.20	0.90
7Dpv/c	B	0.30	1.13	0.20	0.75
	C	0.30	1.07	0.20	0.71
Mean Weight		0.30	1.18	0.20	0.79
	A	0.10	0.38	0.40	1.52
10Dpv/c	B	0.30	1.04	0.40	1.39
	C	0.40	1.17	0.40	1.17
Mean Weight		0.27	0.86	0.40	1.36
	A	0.40	1.34	0.90	3.02
14Dpv/c	B	0.40	1.12	0.60	1.68
	C	0.60	1.63	0.50	1.36
Mean Weight		0.47	1.36	0.67	2.02
	A	0.80	1.77	0.50	1.10
21Dpv/c	B	0.70	1.45	0.80	1.66
	C	0.50	0.94	0.60	1.13
Mean Weight		0.67	1.39	0.63	1.30

Appendix IV: Bursa Body Indexof Different Treatment And Control Groups

	BURSA INDEX								
Groups/	24Hpv/c	48Hpv/c	3 Dpv/c	4 Dpv/c	5 Dpv/c	7Dpv/c*	10Dpv/c	14Dpv/c	21Dpv/c
MB®	1.04	1.13	1.11	3.69	1.07	0.40	0.43	0.23	0.96
Bursine®- 2	1.08	0.74	1.14	4.43	1.03	1.09	0.00	0.00	0.73
Cevac® IBD L	1.19	0.86	0.89	4.79	0.89	0.74	0.55	0.20	1.61
IBD-VAC®	0.92	0.96	0.72	3.44	1.03	1.05	1.27	0.92	0.79
CHALLENGE	1.27	1.03	0.42	1.00	0.29	0.20	0.19	0.22	0.98

Appendix V: Infectious Bursal Disease Vaccines used in the Research

Commercial Name	Company	Country	Strain	Batch number	Man. Date	Exp. Date
MB®	Abic	Israel	MB Intermediate	20621253A	-	5/2015
Bursine®-2	Fort-Dodge	USA	Intermediate Lukert Stock	008/13	Feb. 2013	Feb. 2015
Cevac®IBD L	Ceva	Hungary	Winterfield 2512-G-61	3910A2UKA	-	10/2014
IBD-Vaccine®	NVRI	Nigeria	Intermediate	12/2014	-	5/2015

Appendix VI: IDEXX ELISA KIT used in the Research

Item	**LOT**	**Expiry Date**
Plates	LJ807	18 May 2015
Negative Control	MJ576	11 Feb. 2016
Positive Control	AK644	18 March 2015
Diluent	KJ975	16 Oct 2015
Substrate	318131	30 Nov 2015
Stop Solution	LJ422	07 Nov 2015
Conjugate	AK646	13 June 2015
Pack	AK642	10 Jan 2015