MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS FROM BROILER FLOCKS IN LUSAKA, ZAMBIA

By

KUNDA NDASHE

Thesis submitted to the University of Zambia in fulfillment of the requirements for the award of Degree of Master of Science in Veterinary Microbiology

SCHOOL OF VETERINARY MEDICINE

UNIVERSITY OF ZAMBIA

DISEASE CONTROL DEPARTMENT

DECLARATION

I, **Kunda Ndashe** do hereby declare that the contents of the thesis being submitted herein are my original work and they have not been previously submitted to any University for the award of a degree or any other qualification.

Signature: …….

Date: ……..

CERTIFICATE OF APPROVAL

This Thesis submitted by, **Kunda Ndashe** is approved as fulfilling the requirements for the award of the degree of Master of Science in Veterinary Microbiology at the University of Zambia.

Prof. Bernard M. Hang'ombe	.....	……
Supervisor	Signature	Date
.....	.....	……
Internal Examiner 1	Signature	Date
.....	.....	……
Internal Examiner 2	Signature	Date
.....	.....	……
External Examiner	Signature	Date

……………………………………   ……………………………………   ……………………

Chairman, Board of Examiners Signature   Date

ABSTRACT

This study was aimed at determining the strains of infectious bursal disease virus (IBDV) present in Zambia causing disease in vaccinated broiler flocks. Despite routine vaccinations with appropriate IBDV vaccines, which is a control strategy of infectious bursal disease (IBD), many poultry farms still experience high mortalities to the disease (PAZ, 2013).

A molecular study on IBDV isolated from Lusaka District was performed based on partial sequences of segments A (VP2) and segment B (VP1) of the viral genome from IBD outbreaks from 20 broiler farms. In total, 16 and 11 partial nucleotide sequences of VP2 and VP1 were determined, respectively. Evolutionary analysis of both VP1 and VP2 showed that most Zambian isolates belonged to the African very virulent IBDV (VVIBDV) group which includes isolates from Nigeria, Tanzania and South Africa (Kasanga *et al.*, 2013). In contrast, it was interesting to note that one isolated strain (LUSC11-12) was genetically identical to an attenuated vaccine strain (MB) and showed 100% nucleotide sequence identity to the strain in both the VP2 and the VP1. The current study does not only demonstrate the predominance of African-like VVIBDV, but also documents for the first time the possible involvement of attenuated vaccine strains in the evolution and epidemiology of IBD in Zambia.

From the above study findings, several recommendations were made which included ;further studies, surveillance and characterization of IBDV in poultry; monitoring of live vaccine use in the field, Development of a vaccine from local or regional IBDV field strains and Strict biosecurity of poultry farms

DEDICATION

This work is especially dedicated to my son Timothy Tatenda Ndashe, my father and mother, Chola Yoram and Milika Ndashye, for the love that they shared with me in my challenges and the hardships during my studies at the University of Zambia. I thank them all immeasurably for their moral support and understanding when I could not avail myself as much as I should have done during the period of my studies. To siblings, Mumba, Esnart, Elizabeth and Mupeta, you guys are the greatest and thanks for pushing me when I thought it was hard to achieve. To you all, this is the reward of your endurance. To Yahweh, thank you for the gift of life and the abundant provisions.

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisors, Professor B.M Hang'ombe and Dr. E. Simulundu, for their guidance in the development of the proposal, during the research and the drafting of the dissertation. Their valuable contribution made it possible to finalize this study and I shall forever be grateful.

I am also indebted to Professor A. Takada for helping this research with the reagents required doing the bench work. Mr. L. Moonga, my sincere gratitude for the help and great lessons offered during the laboratory sessions. I would like to thank the Hokudai Center for Zoonosis Control in Zambia (HUCZCZ) for allowing me to use the laboratory and equipment to conduct the molecular investigation. The staff of Agrivet, Golan poultry solutions and Livestock Services cooperative Society, I appreciate help rendered in sample collection.

Drs. A. Moosa and E. Nguleka, thank you for inspiring me and shaping my interest for poultry medicine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Amino acids
Abs	Antibodies
Ag	Antigen
AGID	Agar gel immunodiffusion

AGP	Agar gel precipitin
AVIBDV	Antigenic variant infectious bursal disease virus
BF	Bursa of Fabricius
CAM	Chorio-allantoic membrane
CVIBDV	Classical virulent infectious bursal disease virus
dsRNA	double stranded RNA
ELISA	Enzyme linked immunosorbent assay
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
MDA	Maternal derived antibodies
PAZ	Poultry Association of Zambia
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
SN	Serum Neutralization
VVIBDV	very virulent infectious bursal disease virus
VP2-HVR	viral protein 2 hypervariable region
VP1	Viral protein 1

CHAPTER ONE

1.0 INTRODUCTION

During the last four decades, there has been a phenomenal growth in poultry production in Zambia as evidenced by the increase in the number of poultry input suppliers and producers and improvements through better management and disease control strategies (PAZ, 2013). Intensive poultry farms provide the optimum conditions for introduction and transmission of disease causing pathogens. Thousands of chickens crowded together in a closed, warm and dusty environment is highly conducive to the transmission of contagious diseases. These disease conditions have caused considerable economic losses to some poultry farmers and the country.

Infectious bursal disease (IBD) is a highly contagious disease of young chickens (three to six weeks) caused by IBD virus (IBDV), characterized by immunosuppression and mortality, ranging from ten percent to ninety percent depending on the pathogenicity of the virus (Muller and Becht, 1982). The disease has contributed significantly in overall losses to many poultry farms because of increased mortality due to IBD and other diseases occurring as a result of vaccination failures due to immunosuppressive effect of the disease. Before 1986 IBDV strains were of low virulence causing less than two per cent specific mortality (Van den Berg *et al*., 2004) and were satisfactorily controlled by vaccination. But from 1986 onwards, vaccination failures were described in different parts of the world (Butcher and Miles, 1994). In 1987, very virulent IBDV (VVIBDV) strains capable of causing thirty to seventy per cent mortalities in broilers and layers were isolated in Holland, Belgium and UK (Van den Berg *et al*., 1991). Since then, outbreaks of VVIBDV have

occurred in most European countries as well as in Africa, Japan, China and South East Asia. The VVIBDV were able to break through the maternal immunity as well as the active immunity induced mainly by classical or mild IBDV vaccines (Eterradossi *et al*., 1992).

IBD was first documented in Zambia in 1978 when it caused outbreaks on five farms in the Copperbelt, Central and Southern provinces. The mortality ranged between three percent and thirty percent, with the age of affected flocks being two to eight weeks (Sharma *et al*., 1977). IBD was further documented through a study conducted by Munang'andu *et al* (1996) where the researchers found that out of 67 outbreaks investigated in Lusaka Province thirty two (47.8 percent) were confirmed IBD cases using agar gel immunodiffusion test. Out of the thirty two outbreaks, five were vaccinated flocks and therefore the researchers recommended further Virological studies to elucidate the cause of the vaccine failure (Munang'andu *et al*., 1996).

In Zambia an estimated average of 1,066,153 day old broiler are produced weekly throughout the country and 1,012,673 survive for market sales resulting in a national average mortality rate of 5.01 percent at 35 days of age, although flocks affected with disease do experience higher mortality rates (PAZ, 2013). The national average mortality rate is slightly above the normal of 4.7 percent at 35 days of age for broilers. Management failures and poultry diseases including IBD cause most of the mortalities in broilers (PAZ, 2013). To control the disease, vaccination is carried out in Zambia as recommended elsewhere (OIE, 2004). Despite mandatory IBD vaccination schedules that are issued by hatcheries, farmers still record outbreaks, annually.

Cases of IBD in Zambia are currently diagnosed by postmortem examination, but this method sometimes does not offer very accurate results. Therefore, in this study IBD was accurately diagnosed using molecular method including the reverse transcriptase-Polymerase Chain Reaction (RT-PCR), from bursae of Fabricius extracted from broilers diagnosed with IBD at postmortem examination. The outcomes of this study helped in understanding the epidemiology of the disease in broilers and also helped elucidate reasons for vaccine failure in flocks in Lusaka, Zambia.

1.1.0. STUDY JUSTIFICATION

Infectious bursal disease continues to be a major problem in many poultry flocks kept intensively in Zambia (PAZ, 2013). The Poultry Association of Zambia reported in the 2011 annual general meeting report that IBD remains a challenge to many farmers in Zambia, as it is one of the major causes of mortality in broiler poultry flocks (PAZ, 2012). Currently, there is not much information on the mortality and morbidity rates of IBD. From the information received from agro-vet outlets in Lusaka, the medium and small-scale producers are the most affected. Although most farmers do vaccinate their poultry flocks, serious outbreaks of the disease are still being observed.

For this reason this study was aimed at determining the genotype of IBDV present in Zambia since IBD is being recorded in vaccinated and unvaccinated flocks. The results will influence policy makers on the exact vaccine suitable for the control of the disease in Lusaka.

To achieve the intended aim of the study, the following specific objectives were developed.

a. To detect the IBDV nucleic acid (dsRNA) from bursae tissue of chickens with suspected IBD.
b. To determine the sequence of the VP1 and VP2 fragments of IBDV of RT-PCR positive samples.
c. To conduct phylogenetic analysis of VP1 and VP2 fragments of IBDV.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1. History of infectious bursal disease

In 1957, Albert S. Cosgrove recognized peculiar clinical signs on a broiler farm near Gumboro, Delaware, United States of America. The disease was named "Gumboro disease" after the geographic location of the first recorded outbreak (Cosgrove, 1962). Gumboro disease was characterized by ruffled feathers, watery diarrhea, trembling, severe prostration, dehydration, hemorrhages in the leg and thigh muscles, increased mucus in the intestine and enlargement of the bursa of Fabricius (BF) (Winterfield, 1969). This syndrome was characterized by 10 percent flock morbidity and about 1 to 10 percent mortality. This occurred throughout the Delmarva region (Lasher and Davis, 1997). The disease spread to all thirteen poultry producing southwestern states of America in about three years of its initial recognition at the East Coast. Conventional treatments of the time such as antibiotics, vitamin supplements, molasses or different management practices did not alter the course of the disease (Lasher and Davis, 1997). The different management practices had insignificant effect on the severity of the disease (Parkhurst, 1964). It was also noticed that the disease was transmitted from farm to farm through leftover feed from the affected farms (Edgar and Cho, 1965). Early studies suggested that the etiology of IBD was a nephropathogenic strain of infectious bronchitis virus (IBV) due to similar gross changes observed in the kidney by Winterfield and Hitchner (1962). Subsequent studies (Pejkovski *et al.*, 1979 and Winterfield and Thacker, 1978), however, revealed that IBV immunized birds could still be infected with the

"infectious bursal agent" (IBA) and develop changes in their cloacal bursae of Fabricius specific for the disease. Winterfield *et al* (1962) isolated the disease agent in embryonating eggs. The isolated agent was called infectious bursal agent (IBA) and regarded as the true cause of IBD. In 1961 Edgar and Cho named the new poultry disease syndrome "Infectious bursal disease (IBD)" instead of Gumboro disease by which it was previously known (Edgar and Cho, 1965). Vaccinations with the disease agent were proposed as a control measure on the premises where IBD was already established.

The first documented case of the immunosuppressive effects of IBD was recorded when birds could not produce immune response to Newcastle disease vaccine after an IBD infection, (Allan *et al*., 1972), and in 1980 a second serotype was reported (McFerran *et al*., 1980). These factors, along with the high tendency for IBD infections to recur in successive flocks, emphasized the need for stringent measures of prevention and control. Prior to 1984, vaccination programs satisfactorily controlled the spread of clinical and subclinical forms of the disease. However, in 1984 and 1985, a significant increase in mortality, abattoir condemnations, and vaccine failures were reported in the Delmarva Peninsula broiler growing area (Saif, 1984; Hitchner, 1970). These newly emergent viruses were capable of breaking through maternal immunity against classic strains of IBDV (Rosenberger *et al*., 1987; Rosenberger and Cloud, 1985). *In vivo* reciprocal cross-challenge tests showed that unlike classic or standard strains of IBDV, the field isolates caused rapid atrophy and minimal inflammation of the cloacal bursa of Fabricius when inoculated into susceptible specific pathogen free (SPF) leghorns (Rosenberger and Cloud, 1985). Studies suggested that a major antigenic shift in serotype I viruses had occurred in the field (Synder *et al* 1992: Jackwood and Saif, 1987). The IBDV field isolates were characterized as antigenic "variants" of serotype 1 IBDV,

while the older serotype 1 viruses discovered prior to these newly emergent viruses were called classic strains of IBDV (Rosenberger and Cloud, 1985).

Outbreaks of VVIBDV were first reported in Europe in 1987-1988 (Eterradossi *et al*., 1992; Van den Berg *et al*., 1991; Chettle *et al*., 1989;). The VVIBDV infections are characterized by a peracute onset of severe clinical disease and high mortality (Tsukamoto *et al*., 1995: Nunoya *et al*., 1992; Van den Berg *et al*., 1991Chettle *et al*., 1989). Although these new serotype 1 viruses demonstrate increased virulence in their ability to break through the existing level of maternal immunity, they are antigenically similar to the classic strains of IBDV (Van den Berg *et al*., 1991; Van der Marel *et al*., 1991; Öppling *et al*., 1991). Strains of VVIBDV have rapidly disseminated to every poultry-producing country, except Canada, Mexico, Australia, and New Zealand (Hassan *et al*., 2002; Van den Berg, 2000; Di Fabio *et al*., 1999; Eterradossi *et al*., 1992).

2.2. Global occurrence of infectious bursal disease

IBD is found almost everywhere where poultry is kept intensively, and the distribution of the serotype and subtypes differs from one region to another. From the first recorded outbreaks in USA the disease has spread to various parts of the world. North America, Papua New Guinea, Australia and New Zealand are the only regions that are recorded not to have the VVIBDV (Ignjatovic and Sapats, 2002).

There is very little literature discussing the distribution of IBDV in intensive broilers productions in Africa. Kasanga *et al* (2008) endeavored to detect and characterize IBD in poultry in Tanzania

between 2000 and 2004 and showed that the results in a phylogenetic tree based on the nucleotide sequences of VP2-HVRs, all Tanzanian strains fell within the very virulent type (Kasanga *et al.*, 2008). In Central Oromia, Ethiopia, Cross-sectional study was conducted from November 2009 to May 2010 to determine the seroprevalence of IBD in backyard chickens and to identify the likely potential risk factors in selected sites. Zeryehun and Fekadu (2012) concluded that out of 276 serum samples tested, 227 were positive for IBD antigen/antibody (Ag/Ab) by indirect enzyme linked immunosorbent assay (ELISA) technique and the overall prevalence of IBD in the study area was found to be 82.2 percent (227/276). Mazengia (2008) conducted a survey on the incidence of IBD in village chicken flocks in Bahir Dar and Farta, Ethiopia. The incidence of occurrence and case fatality rates of IBD were 8.39 percent and 17.4 percent and 98.56 percent and 77.73 percent, in Bahir Dar and Farta, respectively. In that study, Mazengia *et al* (2009) confirmed IBD cases on the basis of clinical signs, pathological and serological tests. In Nigeria a study by Okwor *et al* (2012) observed high serum antibody levels against IBDV using indirect hemagglutination (IHA) test in unvaccinated village chickens reared in and around Nsukka, Southeast Nigeria. The result showed a high seroprevalence of 88.4 percent. Mbuko *et al* (2010) conducted a five-year retrospective study (2004-2008) on the prevalence of IBD and other poultry diseases diagnosed at the poultry unit of the Ahmadu Bello University Veterinary Teaching Hospital, Zaria, Kaduna Nigeria. A prevalence of 7.26 percent was recorded.

In Zambia, very little research on IBD has been undertaken, notably only by Kasanga *et al* (2008). In that study by methods of whole genome sequencing, phylogenetic analysis and amino acid comparison of genome segments A and B of the field VVIBDV it was discovered that in an isolate from Zambia reassortment of segment A and B had occurred. In this unique VVIBDV segment A

showed 98 percent nucleotide homology to the VVIBDV strain D6948, while segment B was 99.8 percent identical to the classical attenuated strain D78 (vaccine strain). The existence of heterogeneous IBDV in Zambia suggested a high risk of genome reassortment, which creates new challenges to vaccination and increased virulence of the mutated virus.

2.3. Aetiology of infectious bursal disease

IBDV is a member of the *Birnaviridae* family and genus *Avibirnavirus*. Viruses in this family have a genome consisting of 2 segments of double-stranded RNA (dsRNA), hence the name birnaviruses (Lasher and Davis, 1997).

The family *Birnaviridae* comprises three genera: *Avibirnavirus*, *Aquabirnavirus*, and *Entomobirnavirus*. IBDV is the sole member of the genus *Avibirnavirus* (Muller *et al*., 1986). Members of the genus *Aquabirnavirus* include infectious pancreatic necrosis virus of salmonid fish and related viruses of oysters and crabs (Van den Berg *et al*., 1991). Members of the genus *Entomobirnavirus* infect only insects.

Two distinct serotypes of IBDV are known, serotypes 1 and 2. Serotype 1 is the pathogenic type causing an active IBD infection in chickens, but serotype 2 produces neither disease nor immunity against pathogenic strains of serotype 1 (Lukert and Saif, 2003). Depending on the virulence and antigenicity, serotype 1 IBDV is classified into classical virulent (CVIBDV), antigenic variant (AVIBDV), and VVIBDV (Chettle *et al*., 1989).

2.4. Infectious bursal disease virus genome organization

IBDV is a double stranded (ds) RNA virus with the genome consisting two segments A and B. The large segment A (approximately 3.3 kbp) encodes the VP5 protein and, in another partially overlapping reading frame, a 110 kDa polyprotein that will yield VP2, VP3, and VP4 whereas the small segment B of the IBDV genome (approximately 2.9 kbp) codes for viral protein VP1 (Bottcher *et al.*, 1997). A high degree of sequence homology was reported between the pathogenic serotype 1 and the non-pathogenic serotype 2 viruses in the coding region of segment B; whereas lower sequence identities were observed in the coding region of segment A of serotypes 1 and 2 viruses (Saif and Lukert, 2003). In both genome segments the coding regions are flanked by short 5' and 3' untranslated regions (79 to 111 nucleotide long) (Liu and Vakharia, 2004). The secondary structure of the 3 untranslated regions appears to be critical for an efficient replication (Edison *et al*., 1980).

The virus has a non-enveloped, single icosaehedral capsid, and its size ranges from 55 to 65 nm in diameter (Azad *et al*., 1986). Five viral proteins designated VP1, VP2, VP3, VP4, and VP5 are recognized and the approximate molecular weights of the five proteins are 97 kD, 41 kD, 32 kD, 28 kD, and 21 kD, respectively. Additional proteins, such as VPX or pVP2, have been observed and have a precursor-product relationship. Becht *et al* (1988) compared isolates of serotypes 1 and 2 and reported viral proteins with molecular weights in the same range as those observed by Kibenge *et al* (1991) and Jackwood and Saif (1983). It was not possible to differentiate between strains of

serotype 1 viruses based on differences in structural proteins. VP2 and VP3 are the major structural proteins of IBDV.

VP1 is the viral RNA dependent RNA polymerase (RdRp). It is present in virus capsids both as a genome linked and as a free protein (Muller *et al*., 1979). VP2 is the main capsid protein. It forms trimers which are the basic units of the virus shell, the crystal structure of which has been recently determined with a 7Å resolution (Domanska *et al*., 2004). VP3, the other major structural protein, interacts with itself, VP2, VP1, and the viral genome, thus playing a critical role in both virion morphogenesis and encapsidation. VP4 is a viral protease that exhibits an unusual Serine-Lysine catalytic dyad (Faragher *et al*., 1972). VP4 plays a major role in the maturation of capsid protein VP2, by progressively trimming several peptides at the VP2 carboxy-terminal extremity during virus assembly. VP5 has no clearly established function, but it was suggested that it might have a regulatory function playing a role in virus release and dissemination, as well as an anti-apoptotic function at the early stages of infection (Schnitzler *et al*., 1993). Two of the peptides, X-VP2 and VP2 that arise from the maturation process of pVP2 are crucial determinants that control the geometry of the virion assembly process. One of these peptides also has a destabilizing effect on cellular membranes and has been proposed to be involved, after IBDV particles are bound to their cellular receptor, in the process of virus translocation across the cytoplasmic membrane (Bayliss *et al*., 1990).

Among all the IBDV viral proteins, VP2 protein contains important neutralizing antigenic sites and elicits protective immune response and most of the amino acid changes between antigenically

different IBDVs are clustered in the hypervariable region of VP2. Thus, this hypervariable region of VP2 is the obvious target for the molecular techniques applied for IBDV detection and strain variation studies. The region between amino acids 206 and 350 is extremely hydrophobic and contains the major neutralization site of the virus. Amino acid changes here would result in the antigenic variation of the virus (Azad *et al.*, 1987). Hydrophilicity profile of this region showed the presence of two hydrophilic peaks A and B at amino acid positions 210 to 225 and 312 to 324, respectively (Domanska *et al*., 2004). Major differences in the reactivity of neutralizing monoclonal antibodies (MAbs) result from amino acid changes in peaks A and B (Schnitzler *et al*., 1993).

2.5. Molecular determinants of pathogenicity of IBDV

Molecular characterization of IBDV has been based mainly on the study of the VP2 gene, the middle third of which contains a variable region (vVP2) (Bayliss *et al*., 1990). The VP2 stretch of hydrophilic amino acids has been recognized as the molecular basis for antigenic variation (Schnitzler *et al*., 1993). Several amino acids in vVP2 have also been proposed as putative markers for pathogenic IBDV. First, amino acid alignments of vVP2 in classical or VVIBDV isolates revealed that most of the latter shared four conserved amino acids, A222, I256, I294 and S299 (Eterradossi *et al*., 1998). These positions have since been used as the target of several molecular or antigenic tests aimed at the presumptive molecular or antigenic identification of putative VVIBDV (Eterradossi *et al*., 1998).

2.6. Epidemiology

2.6.1. Host range and potential vectors

Although turkeys, pigeons, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens, *Gallus gallus domesticus*, (van den Berg, 2000). A serotype 1 virus was isolated from two eight-week-old ostrich chicks that had lymphocyte depletion in the bursa of Fabricius, spleen, and/or thymus (McFerran *et al*., 1980). Another serotype 1 isolate was obtained from healthy ducks (McFerran *et al*., 1980). A serotype 2 IBDV was isolated from captive penguins that died without specific clinical signs (Gough *et al*., 2002). The Pekin duck (*Cairina moschata*) was also an asymptomatic carrier of serotype 1 viruses (McFerran *et al*., 1980). Anti-IBDV antibodies have been detected in common pheasants (*Phasianus colchicus)* (Louzis *et al*., 1979), guinea fowl (*Numida meleagris*) (Allan *et al*., 1984), and ostriches (*Struthio camelus*) (Cadman *et al*., 1994), which have also been demonstrated to carry serotype 2 viruses. Neutralizing 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 (Ogawa *et al*., 1998; Gardner *et al*., 1997: Wilcox *et al*., 1983).

Several researchers have studied potential vectors of IBDV. Snedeker *et al* (1967), demonstrated that the lesser mealworm (*Alphitobius diaperinus*), taken from a house eight weeks after an outbreak, harbored IBDV that was infectious to susceptible chickens when fed as a ground suspension. Pagès-Manté *et al* (2004) reported that a dog fed chicken that had died of acute IBD shed viable VVIBDV in its feces for two days after ingestion demonstrating that a dog is a potential mechanical vector of VVIBDV. Howie and Thorsen (1981) isolated non-pathogenic IBDV from

mosquitoes (*Aedes vexans*) that were trapped in an area where infected chickens were being raised. There has not been any further evidence to support a conclusion that mosquitoes act as vectors or reservoirs of the virus. Okoye and Uche (1986) detected IBDV antibodies by the agar-gel precipitin (AGP) test in six of twenty three tissue samples from rats found dead on four poultry farms that had histories of IBDV infection.

The biological significance of mechanical vectors (lesser mealworms, dogs, mosquitoes and rats) of IBDV in maintaining horizontal transmission of the virus on farm has not been studied intensively, but depopulation of these mechanical vectors is highly encouraged.

2.6.2. Susceptibility factors

The age of maximum susceptibility is between three and six weeks, corresponding to the period of maximum bursa development, during which the acute clinical signs are observed. Infections occurring prior to the age of three weeks are generally subclinical and immunosuppressive. Light strains of laying stock are more susceptible to IBD than the heavy broiler strains (Bumstead *et al*., 1993).

2.6.3. Transmission

Only horizontal transmission has been described, with healthy subjects being infected by the oral or respiratory pathway. Infected subjects excrete the virus in faeces as early as 48 hours after infection, and may transmit the disease by contact over a sixteen-day period (Vindevogel *et al*., 1976). The

disease is transmitted by direct contact with excreting subjects, or by indirect contact with any inanimate or animate (farm staff, animals) contaminated vectors. Some researchers have suggested that insects may also act as vectors (Howie and Thorsen, 1981). The extreme resistance of the virus to the outside environment enhances the potential for indirect transmission. The virus can survive for four months in contaminated bedding and premises (Benton *et al*., 1967), and up to fifty-six days in lesser mealworms (*Alphitobius* sp.) taken from a contaminated building (McAllister *et al*., 1995). In the absence of effective cleaning, disinfection and insect control, the resistance of the virus leads to perennial contamination of infected farm buildings

2.7. Pathogenesis of infectious bursal disease

After oral infection or inhalation, the virus replicates primarily in the lymphocytes and macrophages of the gut-associated lymphoid tissues (Peyer's patch and caecal tonsils) (Edgar and Cho, 1976). Then the virus reaches the bursa of Fabricius via the blood stream where the main replication will occur. The target organ of IBDV is the bursa of Fabricius at its maximum development, where B-lymphocytes mature in avian species (Lukert and Saif, 2003). The severity of the disease is directly related to the number of susceptible cells present in the bursa of Fabricius; therefore, the highest age susceptibility is between three and six weeks, when the bursa is at its maximum development.

By thirteen hours post-infection most follicles are positive for virus and by sixteen hours a second and pronounced viraemia will start, with secondary replication in other organs (Nieper and Müller, 1996). This second wave coincides with an important inflammatory response in the bursa of

Fabricius and is associated with clinical disease and death (Faragher *et al.,* 1972). Repopulation of the bursa with B lymphocytes is variable; in some cases starting by eight days and in others after three weeks post-infection, depending on the severity of the disease (Jackwood *et al*., 1984)

Recovery from disease or subclinical infection is followed by immunosuppression with more serious consequences if the strain is VVIBDV infection occurring early in life (Lukert and Saif, 2003). Chickens tend to become infected toward the age of two–three weeks, when maternal derived antibodies (MDA) declines and there is considerable evidence that the virus can have an immunosuppressive effect up to the age of six weeks at least (Kaufer and Weiss, 1980). Although the immunosuppression caused by IBDV is principally directed towards B-lymphocytes, an effect on cell-mediated immunity has also been demonstrated, thus increasing the impact of IBDV on the immunocompetence of the chicken (Lukert, 1986). In addition to its impact and role in the development of secondary infections, IBD may thus also affect the immune response of the chicken to subsequent vaccinations, essential in all types of intensive farming (Kibenge *et al.*, 1988).

2.8. Clinical observations in infectious bursal disease

The severity of the clinical signs is dependent upon the age, breed and level of MDA of the chick, as well as upon the virulence of the virus (Van den Berg, 2000). The clinical picture may differ considerably from one farm, region, country or even continent to another (Lukert and Saif, 2003). Schematically, the global IBD situation can be divided into three principal forms.

(a) The classical form, as described since the early 1960s by Faragher *et al* (1972), is caused by the classical virulent strains of IBDV. The incubation period of IBD ranges from two to four days after exposure. One of the earliest signs of the classical infection in a flock is the tendency for some birds to pick at their own vents (Cosgrove, 1962). The disease is characterized by acute onset of depression. Chickens are reluctant to move, they exhibit ruffled feathers, whitish or watery diarrhea, pericloacal feathers are stained with urates, trembling and prostration (Faragher *et al.*, 1972). Severely affected birds become dehydrated and die, mortality ranging from one percent to fifty percent (Hermann *et al*., 2003).

(b) The immunosuppressive form, principally described in the United States by Jackwood and Saif (1987), is caused by low-pathogenicity strains of IBDV, as well as by variant strains, which partially resist neutralization by antibodies against the classical strains (Snyder *et al.*, 1992).

(c) The acute and very virulent form, was described initially in Europe, and then spread to Asia, Africa and some countries in Latin America (Stuart, 1989). This form of IBD is caused by VVIBDV and is characterized by an acute progressive clinical disease that leads to high mortality rates on affected farms (Chettle *et al.*, 1989). The initial outbreaks in Europe were characterized by high morbidity (eighty percent) and significant mortality attaining twenty five percent in broilers and sixty percent in pullets over a seven-day period (Van Den Berg *et al*., 1991).

2.9. Pathology of infectious bursal disease

Although the other lymphoid organs are affected (Tanimura and Sharma, 1997; Tanimura *et al.*, 1995), the principal target of the virus is the bursa of Fabricius (Kaufer and Weiss, 1980), which is the reservoir of B-lymphocytes in birds. Indeed, the target cell is the B lymphocyte in active division, for which the infection is cytolytic (Burkhardt and Müller, 1987). Cell sorting studies have demonstrated that the B lymphocyte is susceptible in the immature stage, during which immunoglobulin M is carried on the surface of the lymphocyte (Nakai and Hirai, 1981; Hirai *et al.*, 1979). This accounts for the paradoxical immune response to IBDV, in which immunosuppression co-exists with high anti-IBDV antibody titers. The mature and competent lymphocytes will expand as a result of stimulation by the virus whereas the immature lymphocytes will be destroyed.

2.9.1. Gross lesion

Macroscopic lesions are observed principally in the bursa of Fabricius that presents all stages of inflammation following acute infection (McFerran *et al.*, 1980; Vindevogel *et al.*, 1976). Autopsies performed on chickens that died during the acute phase (three to four days following infection) reveal hyperplasia, hyperaemic and oedematous bursae of Fabricius. The most severe cases are characterized by a major infection of the mucous membrane and a serous transudate, giving the bursal surface a yellowish colour (Hiraga *et al.*, 1994). This appearance is often accompanied by petechiae haemorrhages. By the fifth day, the bursa of Fabricius reverts to normal size and by the eighth day becomes atrophied to less than a third of the normal size (Inoue *et al.*, 1994). The affected chickens are severely dehydrated, and many birds have hypertrophic and whitish kidneys containing deposits of urate crystals and cell debris. Haemorrhages in the pectoral muscles and

thighs are frequently observed, probably due to a coagulation disorder (Skeeles *et al.*, 1979). Certain variants from the USA are reported to cause rapid atrophy of the bursa of Fabricius without a previous inflammatory phase (Lukert and Saif, 2003). Moreover, in the acute form of the disease caused by VVIBDV, macroscopic lesions may also be observed in other lymphoid organs such as thymus, spleen, caecal tonsils, Harderian glands, Peyer's patches and bone marrow (Tsukamoto *et al.*, 1995).

2.9.2. Microscopic lesions (Histological lesions)

A system for evaluating microscopic lesions of the affected organs with a score ranging from one to five was developed according to severity of the disease (Henry *et al.*, 1980). The B-lymphocytes are destroyed in the follicles of the bursa of Fabricius and is infiltrated by heterophils and undergoes hyperplasia of the reticuloendothelial cells and of the interfollicular tissue (Helmboldt and Garner, 1964). As the disease evolves, the surface epithelium disappears and cystic cavities develop in the follicles. Severe panleukopenia is also observed. These microscopic lesions are exacerbated in the acute forms of the disease (Lukert and Saif, 2003).

2.10. The immunology of infectious bursal disease

2.10.1. Antigens of IBDV

IBDVs share common group antigens that may be detected by agar gel precipitation, the fluorescent antibody test, and ELISA (Chettle *et al.*, 1989; Jackwood and Saif, 1983; Hirai and Shimakura,

1974; Allan *et al.*, 1972). Both capsid proteins (VP2 and VP3) contain epitopes that are responsible for the IBDV group antigenicity (Becht *et al.*, 1988).

In addition to the common group antigens, each IBDV contains an antigen or antigens responsible for serotype specificity. Serotypes are identified by neutralization tests with serotype-specific antisera (McFerran *et al.*, 1980). It was initially thought that VP3 carried the antigenic determinant(s) of serotype specificity of IBDV (Fahey *et al.*, 1985). However, current evidence indicates that VP2 carries the serotype-specific antigens responsible for the induction of neutralizing protective antibodies (Becht *et al.*, 1988; Azad *et al.*, 1985). There are at least two virus-neutralizing epitopes on VP2, one of which is strictly serotype-specific (Becht *et al.*, 1988). The existence of additional virus-neutralizing epitopes on viral proteins of IBDV cannot be ruled out.

2.10.2. Immune response

The immunosuppressive effects of IBDV infection in chickens and turkeys have been thoroughly reviewed (Okoye and Uche, 1986). The paradox of the immune response to IBDV infection is not the simultaneous immunosuppression against many antigens (Faragher *et al.*, 1974; Allan *et al.*, 1972) but stimulation of very high antibody levels to the virus itself (Skeeles *et al.*, 1979). Indeed Kaufer and Weiss (1980) described an immune response to IBDV in surgically bursectomized chickens indicating that immunity to IBDV, at least in part, develops in the spleen and other lymphoid organs. Immunosuppression results directly from damage to the bursa of Fabricius.

(a) Active immune response

Infection with IBDV is accompanied by formation of antibodies to the group antigens and the serotype-specific antigens (Jackwood and Saif, 1983).

Humoral immunity is the primary mechanism of the protective immune response (Baxendale and Lutticken, 1981; Hitchner, 1970). Field exposure to the virus, or vaccination with either live or killed vaccines, stimulates active immunity (Wyeth and Cullen, 1978). Antibody response may be measured by several methods—Virus Neutralization (VN), agar gel precipitin (AGP), or ELISA tests. Antibody levels are normally very high after field exposure or vaccination, and VN titers greater than 1:1000 are common (Hitchner, 1970). Adult birds are resistant to oral exposure to the virus but produce antibodies after intramuscular or subcutaneous inoculation of IBDV (Hitchner, 1976).

(b) Passive immune response

Antibody transmitted from the hen via the yolk of the egg can protect chicks against early infections with IBDV, with resultant protection against the immunosuppressive effect of the virus (Hitchner, 1976). The half-life of maternal antibodies to IBDV is between three and five days (Skeeles *et al.*, 1979). Therefore, if the titer of neutralizing antibodies in the progeny is known, the time that chicks will become susceptible can be predicted. Lucio and Hitchner (1979) demonstrated that after antibody titers fell below 1:100, chicks were 100 percent susceptible to infection, and titers from 1:100 to 1:600 gave approximately 40 percent protection against challenge. Skeeles *et al* (1979) reported that titers must fall below 1:64 before chickens can be vaccinated effectively with an attenuated strain of IBDV. These figures should be considered as indicative that significant

differences have been reported in the neutralizing titers determined by different laboratories (Mekkes and de Wit, 2002). Use of killed vaccines in oil emulsions (including variant strains) to stimulate high levels of maternal immunity is extensively practiced in the field. Studies by Baxendale and Lutticken (1981) and Lucio and Hitchner (1979) indicated that oil emulsion IBD vaccines can stimulate adequate maternal immunity to protect chicks for four–five weeks, and progeny from breeders vaccinated with live vaccines are protected for only one–three weeks. As with many diseases, passively acquired immunity to IBDV can interfere with stimulation of an active immune response.

2.11. Diagnosis of infectious bursal disease

2.11.1. Clinical 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 symptoms and post-mortem examination of the pathognomonic lesions, in particular of the bursa of Fabricius (van den Berg, 2000). The conditions most liable 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 (Lukert and Saif, 2003). In all acute cases, the presence of bursal lesions allows for a diagnosis of IBD. In subclinical cases, an atrophy of the bursa of Fabricius may be confused with other diseases such as Marek's disease or infectious anaemia. A histological examination of the bursa of Fabricius will allow differentiation between these diseases (Lucio and Hitchner, 1979).

2.11.2. Histological diagnosis

Histological diagnosis is based on the detection of modifications occurring in the bursa of Fabricius. The ability to cause histological lesions in the non-bursal lymphoid organs, such as the thymus, the spleen or bone marrow has been reported as a potential characteristic of VVIBDV 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).

2.11.3. Serological diagnosis

In areas contaminated by IBDV, most broiler flocks have anti-IBDV antibodies when leaving the farm. Current serological tests cannot distinguish between the antibodies induced by pathogenic IBDV and those induced by attenuated vaccine viruses, so serological diagnosis is of little interest in endemic zones (OIE, 2004). Nonetheless, the quantification of IBDV-induced antibodies is important for the medical prophylaxis of the disease in young animals, in order to measure the titer of passive antibodies and determine the appropriate date for vaccination (de Wit, 1999) or in laying hens to verify success of vaccination (Lucio, 1987). Serology is likewise essential to confirm the disease-free status of SPF flocks. Each serological analysis must include a sufficient number (at least twenty) of individual serum samples representative of the flock under study (Meulemans *et al.*, 1987). A kinetic study requires at least two serological analyses separated by an interval of three weeks (paired sera) (Cullen and Wyeth, 1975).

The most widely used quantitative tests are the detection of precipitating antibodies by AGID (Hirai and Shimakura, 1974), ELISA, and serum neutralization (SN) (Weisman and Hitchner, 1978). AGID is the simplest, but least sensitive technique. Results are obtained after an incubation period

of forty eight hours. Variability in results may be due to the investigator, as well as the nature of the viral strain used as an antigen (Wood *et al*., 1979). Serum neutralization presents the disadvantages that specialized equipment and five days incubation is required (Jackwood and Saif, 1983). The technique is much more sensitive than AGID and correlates better with the level of protection of the chickens tested (Roney and Freund, 1988).

ELISA is the most rapid and sensitive method, and presents the fewest variations due to the viral strain used as an antigen (Roney and Freund, 1988). Considerable inter- and intra-laboratory variability can occur with certain commercial kits (Kreider *et al*., 1991). Although the correlation between results obtained using SN and ELISA is high, ELISA remains less sensitive, and does not detect low neutralizing titers that are sufficient to block vaccine administration (residual maternal antibodies). Ag-ELISA, which uses a recombinant VP2-protein, as the sole antigen may be better, correlated with protection (Jackwood *et al*., 1984).

2.11.4. Virological diagnosis

IBDV 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 (Lukert and Saif, 2003).

A. Isolation

A filtered homogenate of the bursa of Fabricius is inoculated in nine to eleven-day old embryonated eggs originating from hens free of anti-IBDV antibodies (Hitchner, 1970). The most sensitive route of inoculation is the chorio-allantoic membrane (CAM); the yolk sac route is also practicable, and the intra-allantoic route is the least sensitive. The specificity of the lesions observed must be

demonstrated by neutralizing the effect of the virus with a monospecific anti-IBDV serum (Lukert and Saif, 2003). Isolation in embryonated eggs does not require adaptation of the virus by serial passages, and is suitable for VVIBDV. In the absence of lesions, the embryos from the first passage should be homogenized in sterile conditions and clarified, and two additional serial passages should be performed (Rosenberger and Cloud, 1985).

B. Detection of viral antigens

Antigens produced during virus infections can be detected either in cells collected from the site of infection or in secretions and blood. Viral antigens specific to IBDV can be detected from the bursa of Fabricius, and cases of VVIBDV from other lymphoid tissue such as spleen, caecal tonsils, and Peyer's patch.

a. Thin sections of the bursa of Fabricius

The viral antigens specific to IBDV may be detected by direct and indirect immunofluorescence (Allan *et al*., 1984) or by immunoperoxidase staining (Cho, 1970) in the bursal follicles of infected chickens between the fourth and sixth day after inoculation. No viral antigen is detectable from the tenth day. However, the virus can be isolated from bursae of Fabricius sampled from the second to the tenth day, with a maximum infectious titer after four days (Winterfield and Thacker, 1978). The use of monoclonal antibodies for detection of the virus enhances the specificity of the test (Cho, 1970).

b. Suspensions of the bursa of Fabricius

The basis for the agar-gel immunodiffusion (AGID) test is the concurrent migration of antigen and antibodies toward each other through an agar gel matrix. When the antigen and specific antibodies come in contact, they combine to form a precipitate which is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentration of antigen and antibodies is optimum. An extreme variation in the concentration of antigen or antibodies will alter the location of the line or cause it to be dissolved. Electrolyte concentration, buffer, pH and temperature also affect precipitate formation (Hirai and Calnek, 1979).

Agglutination tests, using latex beads coated with an anti-IBDV monoclonal antibody (Nieper and Müller, 1996) or sheep red blood cells coupled with anti-IBDV immunoglobulins are also possible (Nunoya *et al*., 1992).

Antigenic capture as revealed by ELISA (AC-ELISA) consists of capturing the viral antigens present in the suspensions, using anti-IBDV antibodies and a polystyrene support. The viral antigens captured are detected through a sandwich ELISA with an anti-IBDV antibody conjugated with peroxidase (Tsukamoto *et al.*, 1995), or with an anti-IBDV antibody followed by adapted anti-species conjugate (Kim *et al*., 2004). The use of a polyclonal serum for capture enhances the sensitivity of the test. The use of monoclonal antibodies in the capture or detection stages allows for more precise antigenic characterization of the captured viruses. Different batteries of monoclonal antibodies enable a tentative identification of the variants from the USA (Synder *et al*., 1992) or VVBDV (Eterradossi *et al*., 1998).

C. Detection of the viral genome

The sensitivity and versatility of nucleic acid hybridization techniques have been expanded rapidly such that probing for the viral genome has overtaken probing for antigen as the diagnostic method of choice in many laboratories (Eterradossi *et al*., 1992). More dramatic still has been the extraordinary adoption of PCR as an amplification step for enhancing the detection of viral nucleic acid in clinical specimens (Hiraga *et al.*, 1994). It is now theoretically possible for a single laboratory with access to a complete panel of oligonucleotide primers to use PCR to provide a comprehensive diagnostic service within 24 hours from specimen submission (Ismail and Saif, 1991).

a. Deoxyribonucleic acid probes

Deoxyribonucleic acid (DNA) probes labeled with 32 P (Jackwood, 1988), biotin (Jackwood and Sommer, 1997) or digoxigenin have been used on prints of infected tissues to detect the multiple virus strains of serotypes 1 and 2. There is no genomic probe that enables differentiation between variant viruses or VVIBDV that has yet been described, undoubtedly owing to the very high degree of genetic resemblance between serotype 1 and 2 strains of the virus (Lukert and Saif, 2003).

b. Reverse transcription and genetic amplification by polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) allows the detection of viral RNA in homogenates of infected organs or embryos, as well as in cell cultures, irrespective of the viability of the virus present (Sapats and Ignjatovic, 2000). The choice of amplified genomic zones depends on the objective. When the only objective is to detect multiple strains of the virus, primers are selected in the highly preserved zones (Wyeth and Cullen, 1978).

When the characterization of the amplified fragment is to allow for identification of the virus strains, the central, so-called variable portion of VP2 is generally chosen (Liu and Vakharia, 2004). The amplified fragment may then be characterized by direct sequencing (Brown and Skinner, 1996), and the analysis of the coded aminopeptide sequence. The simultaneous presence of four amino acids (alanine 222, isoleucine 256, isoleucine 294 and serine 299) is considered as indicative of VVIBDV (Yamaguchi *et al*., 1981). The electrophoretic profile of the amplified fragment may also be studied after digestion with different restriction endonucleases (RT-PCR/RE) (Liu and Vakharia, 2004). The value of the results obtained will depend on the choice of endonucleases. In a given virus, the absence of restriction sites for enzymes *BstNI* and *StyI*, located respectively at codons 222 and 253 of the gene coding for VP2, has been correlated with an atypical antigenicity, such as that found in the variant viruses from the USA (Jackwood, 1988).

2.12. Prevention and control of infectious bursal disease

Like most viral infections IBD does not have direct treatment to control the course and progression of the disease. Since the IBDV invades the B-lymphocytes in bursa of Fabricius for viral replication this results in immunosuppression thereby rendering the chickens vulnerable to secondary opportunistic infections (Parkhurst, 1964; Cosgrove, 1962). The complication secondary infections therefore render the management of IBD challenging.

2.12.1. Eradication

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

necessitates hygiene measures and medical prophylaxis. No vaccine can solve the problem if major sanitary precautions are not taken. These precautions include '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) (Mekkes and de Wit, 2002). 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. 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 (Saif and Lukert, 2003). 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 washed using hot water (60°C) with a detergent, at a pressure of 80 bar to 150 bar (Eterradossi *et al.*, 1998). 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 (Cosgrove, 1962). Disinfection is to be undertaken only after all the buildings have been cleaned. All disinfectants are more active at a temperature above 20°C, however, chlorinated and iodinated disinfectants cannot be heated above 43°C. The quantity of disinfectant solution to be used is approximately four liters per fifteen square meters (Mickael and Jackwood, 2005).

2.12.2. Immunization

Attenuated live virus vaccines and oil-emulsion inactivated virus vaccines are used against IBDV (Tham and Moon, 1996). The general principles governing the choice and the use of these vaccines have been developed and presented (Tsai and Saif, 1992) and remain valid. The ideal vaccine must

offer the correct balance between efficacy and innocuity (Gough *et al.*, 2002). The vaccine must not cause disease or bursal lesions, must not be immunosuppressive or excreted, and must confer long-lasting immunity even in birds with a high level of maternal immunity. Unfortunately, such a vaccine does not exist (McFerran *et al*., 1980).

2.12.2.1. Live virus vaccines

Live virus vaccines are widely used in broilers, layers and breeder flocks. These are made from strains of virus that have been attenuated by serial passages in embryonated eggs or cell cultures. Depending on the degree of attenuation, the vaccine strains cause histological lesions of varying severity to the bursae of SPF chickens, and are classified as mild, intermediate or hot (OIE, 2004). The hot strains induce histological lesions in SPF chickens which are comparable to those caused by pathogenic strains, the only difference being that the hot strains do not cause mortality.

Intermediate vaccines are used for vaccinating broilers and pullets (Mazengia, 2008). These are also administered to chicks in breeder flocks that are at risk of challenge by highly pathogenic strains at an early age. Although intermediate vaccines are also sensitive to neutralization by passive antibodies, these vaccines may be administered at day-old by nebulisation in order to protect a chick that may not have a sufficient level of specific antibodies (Saif and Lukert, 2003). Another reason for such early vaccination is to bring about replication of the vaccine virus in the chicks, and the dissemination of the virus within the farm; this would, at least partially, provide indirect vaccination to the other chicks at a time when they become sensitive to the infection. In high-risk farms, two vaccinations are generally performed (Butcher and Miles, 1994). The age at vaccination depends on the maternal antibody titers present in the chicks at hatch. Vaccines are usually

administered through drinking water, although nebulisation is also possible.

The mild strains are used chiefly for the vaccination of breeder flocks. These are very sensitive to interference by homologous maternal antibodies, and are administered when these antibodies have disappeared, i.e. between the fourth and eighth week of age, depending on whether the grandparent flocks have or have not been vaccinated with an oil-emulsion inactivated vaccine before lay (Eterradossi *et al*., 1998).

Live IBDV vaccines are compatible with other avian vaccines. However, the strains that cause serious lesions to the bursa of Fabricius may also provoke immunosuppression and exacerbate the pathogenicity of other immunosuppressive viruses such as Marek's disease virus (MDV) and chicken anaemia virus (CAV) and jeopardize the immunization of poultry against other diseases (DeWit, 1999). Registration procedures for these vaccines must include tests to verify the absence of interference with other vaccinations as well as the absence of reversion to virulence in the course of serial passages in three- to six-week-old SPF chickens.

A vaccine for *in ovo* vaccination of embryos has recently been developed. The vaccine is a mixture of virus and specific antibody, and is injected into eighteen-day-old embryos. Broiler chicks hatched from these eggs are immunised against IBDV throughout the growing period. This method avoids interference by parental antibodies (Hassan *et al*., 2002).

Various vaccines using recombinant viruses expressing the VP2 protein of IBDV have been described and have proven efficacy in laboratory tests (Tsukamoto *et al*., 1995). The advantages of

these vaccines are the absence of residual pathogenicity, sensitivity to maternal antibodies and risk of selection of mutants, as well as the possibility of use in *ovo* and of differentiation between infected and vaccinated animals (DeWit, 1999). No commercial version of these vaccines is currently available.

2.12.2.2. Inactivated vaccines

Inactivated vaccines are essentially used to produce high titers, uniform and persistent antibodies in hens prior to lay that have been vaccinated with a live virus or have been naturally infected through exposure to the virus on the farm (Cullen and Wyeth, 1975). These vaccines are administered by the subcutaneous or intramuscular route at the age of sixteen to twenty weeks.

Progeny of hens that have been vaccinated in this way have protective antibodies until the age of approximately thirty days (Hassan and Saif, 1996). The chicks are thus protected during the period of susceptibility to the IBDV strains that only provoke immunosuppression. However, the chicks are not protected from other highly pathogenic strains that may inflict high mortality rates at later stages (Wyeth and Cullen, 1975). The decision to use an inactivated vaccine will thus depend on the epidemiological context, namely: presence or absence of highly pathogenic strains requiring vaccination of broilers with live virus vaccines (Briggs *et al*., 1986). Where no risk of infection with VVIBDV exists, boosting of laying hens with an inactivated vaccine just before lay is fully justified. However, the duration and uniformity of the immunity thus conferred upon chicks will, to a great extent, depend on the concentration and the antigenic specificity of the virus present in the vaccine (Van den Berg *et al*., 1991). These vaccines are obtained either from bursal homogenates of infected chicks, or from viral cultures on embryonated eggs or fibroblasts, which are then

inactivated by formaldehyde and presented as oil emulsions. Sub-unit vaccines produced in yeast (Fahey *et al*., 1985) or insect cell cultures have also been described, but are not currently in use.

2.12.2.3. Immune complex vaccines

An immune complex vaccine is made by mixing a live intermediate IBDV with IBD antibody contained in whole hyperimmune serum (Chang *et al*., 2001; Haddad *et al*., 1997; Butcher and Miles, 1994). This coating of the antigen protects it from attack by the MDA. At a point when the MDA levels drop to a critical level, the antigen emerges and starts to trigger an immune response. Each chick responds as an individual as the immune system within the chick is unique in terms of individual MDA levels (Haddad *et al*., 1997). This also overcomes the problem of the MDA levels interfering with vaccine take. The vaccine virus attaches to the bursal tissue and effectively blocks attachment of field virus as per the process of the classic IBD vaccines (Lukert and Saif, 2003). The current vaccine available is a Winterfield strain (intermediate plus), which has a rapid growth rate of two days to colonize the bursal and effectively block field viral attachment to the bursa.

The vaccine was originally designed for use as an in-ova application at day eighteen of embryonation but can now be given as a day of hatch vaccine (Chang *et al*., 2001). The earliest that the vaccine antigen could be released is at 8 days in MDA free birds (SPF birds) and in birds with variable MDA levels, may well be later. Under field conditions, where challenge loads are moderate to high, an additional live IBD (intermediate) must be given at about day ten (Butcher and Miles, 1994). Under variant IBDV challenges this vaccine does not offer protection because the Winterfield strain used in production of the vaccine is a classic vaccine type and not a variant strain (Haddad *et al*., 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study site

Lusaka District is the main region of intensive poultry activity in Zambia, having five hatcheries that produce day old chicks (broilers and pullets). The hatcheries produce the following breeds; Ross 308, Cobb 500, Indian River, Hubbard, Lohmann Brown and Isa Brown. The combined production capacity of all the hatcheries in 2012 was 1,155,000-day-old broiler chicks (DOC) weekly. Of the total chick production, Sixty five percent were sold in Lusaka District and therefore being the region with highest production of broilers in Zambia (PAZ, 2013). This study was focused on the broiler chickens showing signs of IBD clinically and confirmed by gross necropsy lesions. The study area consisted of farmers categorized as commercial large scale, medium scale and small-scale, as show in Table 1.

Table 1: Farmer categorization by size of poultry flocks

Category of farmers	**Size of poultry flock**
Small scale	1000 and below
Medium scale	1001 to 10,000
Large scale	10,001 and above

3.2. Study design

A purposive (biased) sampling design was employed, where the selection parameter were based on the lesions on the chickens. This means that broilers not showing any clinical signs of IBD were excluded from the study. On the other hand, the broilers showing IBD-like lesions were included in the study. The following gross necropsy lesions of IBD were considered for diagnosis; petechiae hemorrhages on the pectoral and thigh muscles, enlargement of bursa of Fabricius, presence of milky exudates on the serosa of the bursa of Fabricius and presence of hemorrhages in the lumen of the bursa of Fabricius.

3.3. Sampling Procedure

Four veterinary outlets were incorporated in diagnosis and sample collection of IBD infected chickens. Farmers coming from various locations of Lusaka District, as shown in Table 2, submitted dead or live moribund chickens to the veterinary outlets for disease diagnosis and advise on treatment and control. Veterinarians at the outlets conducted post mortems on the chickens and those diagnosed with IBD had their whole bursa of Fabricius excised and collected for RT-PCR. The pathological lesions considered pathognomic for IBD included petechiae hemorrhages on the pectoral and thigh muscles, bursa enlargement, presence of milky exudates on the serosa of the bursa and presence of hemorrhages in the lumen of the bursa of Fabricius.

After initial processing, the bursae of Fabricius were collected aseptically and packaged in a properly labeled plastic container and stored under ice. From each outbreak, information on the

location, management system, type of vaccine used, and age of flock were collected and entered in the data sheet as shown in Table 2. From the veterinary outlets, the samples were transported on ice to the University of Zambia, School of Veterinary Medicine, Department of Disease Control laboratory where they were stored at -20°C till analysis.

Table 2: Location, age, bird type, flock size and collection date of bursa of Fabricius for molecular investigation

Case ID No.	Date of collection	Bird type	Age of chickens (days)	IBD vaccine	Flock size	Location of farm
LUS 01-12	22/08/2012	Broiler	28	Intermediate	200	Lusaka North
LUS02-12	24/08/2012	Broiler	30	Intermediate	5500	Lusaka East
LUS03-12	25/08/2012	Broiler	22	Mild	300	Lusaka North
LUS04-12	28/08/2012	Broiler	35	Intermediate	1000	Lusaka South
LUS05-12	1/09/2012	Broiler	28	Intermediate	1000	Lusaka East
LUS06-12	3/09/2012	Broiler	26	Intermediate	1600	Lusaka South
LUS07-12	6/09/2012	Broiler	35	Intermediate	4000	Lusaka West
LUS08-12	7/09/2012	Broiler	24	Mild	1750	Lusaka East
LUS09-12	10/09/2012	Broiler	42	Mild	200	Lusaka East
LUS10-12	13/09/2012	Broiler	35	Intermediate	4600	Lusaka West
LUS11-12	14/09/2012	Broiler	20	Mild	650	Lusaka South
LUS12-12	17/09/2012	Broiler	42	Intermediate	200	Lusaka East
LUS13-12	18/09/2012	Broiler	21	Intermediate	390	Lusaka East
LUS14-12	21/09/2012	Broiler	35	Intermediate	250	Lusaka East
LUS15-12	22/09/2012	Broiler	14	Mild	1000	Lusaka West
LUS16-12	27/09/2012	Broiler	24	Intermediate	300	Lusaka East
LUS17-12	29/09/2012	Broiler	30	Mild	100	Lusaka East
LUS18-12	2/10/2012	Broiler	28	Intermediate	5700	Lusaka West
LUS19-12	2/10/2012	Broiler	25	Intermediate	700	Lusaka East
LUS20-12	5/10/2012	Broiler	22	Mild	500	Lusaka East

3.4. Specimen processing

3.4.1. RNA extraction

The bursae of Fabricius from twenty outbreaks, (four-five bursae of Fabricius from each IBD outbreak were pooled as one sample) were cut into small pieces and homogenized in a 1.5

appendorf tube, 50 mg of homogenized pooled bursae were mixed with 1 ml of Trizol reagent followed by incubation for five minutes at room temperature. Then 200 µl of chloroform was added to the mixture and was then shaken vigorously for fifteen seconds and incubated for three minutes at room temperature. The twenty samples were centrifuged for fifteen minutes at 12,000 *xg* at 4°C. After centrifugation process, the aqueous phase was transferred to fresh tubes and then 0.5 ml of isopropanol was added to each tube. The aqueous phase and isopropanol mixture were then incubated at room temperature for ten minutes, followed by centrifugation for ten minutes at 12,000 *xg* at 4°C. The supernatant was discarded and the RNA pellet were mixed with 1 ml 75% ethanol then vortexed and thereafter centrifuged at 7,500 *xg* for five minutes at 4°C. The ethanol was then discarded and the RNA pellets air-dried for five minutes and re-dissolved by adding 50 µl of nuclease free water in each of the 20 tubes. The RNA was then stored at -30°C until analysis.

3.4.2. Reserve transcription

The first strand cDNA was synthesized using ReverTra Ace kit (Toyobo, Osaka, Japan) and gene specific primers following the manufacturer's instructions with few modifications. The gene specific primers (Toyobo, Japan) targeting the VP2 (segment A) and VP1 (segment B) domains of IBDV genome were V1/V2 and BF1/BF2 in accordance with the original articles cited, (Kasanga *et al*, 2008) as shown in Table 3.

Table 3**:** Primers used to amplify VP1 and VP2 genes of IBDV

Primer name	**Sequence**
V1 forward primer	5'-CCAGAG TCT ACA CCATAA-3'
V2 reverse primer	5'-CCTGTTGCCACTCTTTCGTA-3'
BF1	5'-CCTCTTCTTGATGATTCTACCA-3'
BR1	5'-GAC CATATGTTACGGGTCTT-3'

In detail, three µl of each of the twenty-extracted RNA were mixed with 1.5 µl of dimethyl sulfoxide (DMSO), incubated for five minutes at 97^{o}C and then chilled on ice. To the RNA-DMSO mixture, the following were added: 2.5 µl of 5 × RT reaction buffer, 2.5 µl of 2.5 mM dNTPs, 0.25 µl of 10 µM of each of forward and reverse primers (Table 3), 0.25 µl of ReverTra Ace RT enzyme (100 U/µl) and 0.25 µl of RNase-free water to make a final volume of 11 µl. Reaction tubes were incubated for 45 minutes at 42^{o}C, then for five minutes at 94^{o}C, and cooled at 4^{o}C. The synthesized cDNAs were then used as templates for PCR.

3.4.3. Polymerase chain reaction (PCR)

The cDNA templates were amplified using *Thermus aquaticus* Ex-Taq DNA polymerase (Takara, Shiga, Japan) and V1/V2 and BF1/BF2 primers in a Thermo Cycler.

To 2.5 µl of 10 × Ex-Taq DNA PCR buffer 1.0 µl of each cDNA reaction mix was added, 2.0 µl of 2.5mM dNTPs, 0.5 µl of each of forward and reverse primers (Table 3), 0.125 µl of Ex Taq DNA polymerase enzyme and 18.375 µl of nuclease free water to make a total volume of 25 µl. Amplification consisted of 35 cycles for denaturing (30 seconds at 94^{o}C), primer annealing (30 seconds at 55^{o}C), and primer extension (two minutes at 72^{o}C), with a final single extension step of seven minutes at 72^{o}C.

3.4.4. Visualization of PCR products

The amplified PCR products were run on 1.2 percent agarose gel and visualized with ethidium bromide staining.

3.4.5. DNA recovery from gel for sequencing

The PCR products were gel-purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, USA) according to the manufacturer's protocol. The purified DNA was then used in the Sequencing reaction.

3.4.6. PCR amplicon sequencing

Recovered DNA was sequenced directly using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequences were assembled and edited using GENETYX ATGC software, version 4.0.10

(GENETYX Co., Tokyo, Japan). The sequences were finally deposited in the DDBJ/GenBank/EMBL database.

3.4.7. Amino acid sequence comparison and phylogenetic analysis

The assembled nucleotide sequences were uploaded and analyzed using BioEdit (Ibis Bioscience, Carlsbad, USA) and molecular evolutionary genetics analysis (MEGA) 6.0 (MEGA, Arizona, USA) software packages to produce phylogenetic trees and amino acid sequences.

The nucleotide sequences from the study were blasted on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to search for IBDV nucleotide sequences from other studies that were identical to those of the present study. All nucleotide sequences that were above 96 percent identity were selected for phylogenetic analysis and amino acid sequence comparison of VP1 and VP2. For amino acid sequence comparison some known CVIBDV, AVIBDV, VVIBDV were included in the analysis.

CHAPTER FOUR

4.0 RESULTS

4.1. Detection of infectious bursal disease virus genome

From twenty IBD outbreaks confirmed at necropsy, 100 bursae were collected, 5 bursae pooled from each IBD outbreak, sixteen and eleven bursae were positive for VP2-HVR and part of VP1 regions of IBDV genome, respectively as shown in the electrophoresis pictures in Figure 1 and 2.

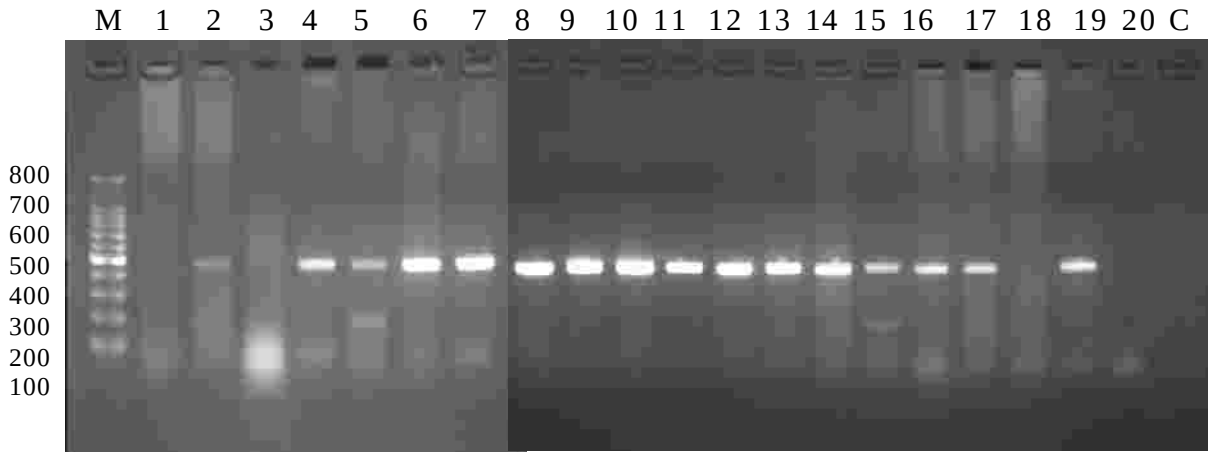

Figure 1: RT-PCR amplification of the IBDV VP2-HVR of broiler bursal homogenate samples. The positive samples are 472 base pair (bp) in size. Lane M, 100-bp molecular ladder; Lanes 1–20, identification numbers of tested samples namely Lus01-12, Lus02-12, Lus03-12, Lus04-12, Lus05-12, Lus06-12, Lus07-12, Lus08-12, Lus09-12, Lus10-12, Lus11-12, Lus12-12, Lus13-12, Lus14-12, Lus15-12, Lus16-12, Lus17-12, Lus18-12, Lus19-12, Lus20-12; Lane C, negative control (distilled water).

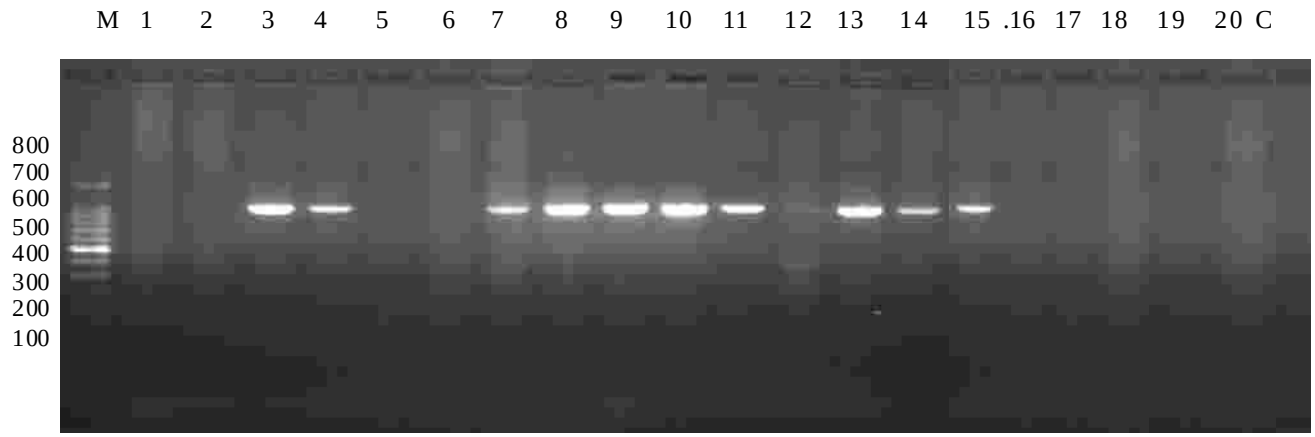

Figure 2: RT-PCR amplification of part of the IBDV VP1 of broiler bursal homogenate samples. The positive samples are 643 base pair (bp) in size. Lane M, 100-bp molecular ladder; Lanes 1–20, identification numbers of tested samples namely Lus01-12, Lus02-12, Lus03-12, Lus04-12, Lus05-12, Lus06-12, Lus07-12, Lus08-12, Lus09-12, Lus10-12, Lus11-12, Lus12-12, Lus13-12, Lus14-12, Lus15-12, Lus16-12, Lus17-12, Lus18-12, Lus19-12, Lus20-12; Lane C (not shown), negative control (distilled water).

All the bursae of Fabricius collected at postmortem came from birds that had the following gross lesion; petechiae hemorrhages on the pectoral and thigh muscles, enlargement of bursa of Fabricius, presence of milky exudates on the serosa of the bursa of Fabricius and presence of hemorrhages in the lumen of the bursa of Fabricius. As shown in Table 4 from the twenty samples that were analyzed, ten were positive for both VP1 and VP2-HVR, while one was positive for VP1 only and six were positive for VP2-HVR only. Three samples were negative for both VP1 and VP2-HVR.

Table 4: VP1 and VP2 results of the bursae of Fabricius extracted from broilers diagnosed with IBD at necropsy

ISOLATE	VP1	VP2
LUS 01-12	-VE	-VE
LUS02-12	-VE	+VE
LUS03-12	+VE	-VE
LUS04-12	+VE	+VE
LUS05-12	-VE	+VE
LUS06-12	-VE	+VE
LUS07-12	+VE	+VE
LUS08-12	+VE	+VE
LUS09-12	+VE	+VE
LUS10-12	+VE	+VE
LUS11-12	+VE	+VE
LUS12-12	+VE	+VE
LUS13-12	+VE	+VE
LUS14-12	+VE	+VE
LUS15-12	+VE	+VE
LUS16-12	-VE	+VE
LUS17-12	-VE	+VE
LUS18-12	-VE	-VE
LUS19-12	-VE	+VE
LUS20-12	-VE	-VE

4.2. Deduced amino acid sequence comparison

Amino acids sequences were deduced from nucleotide sequences using BioEdit 7.0 and MEGA 6. Comparison of the amino acid sequence of VP2- HVR of isolates of this study with those of selected serotype 1 IBDV strains revealed that almost all of the Lusaka IBDVs' (from this study) had conserved putative virulence marker amino acids at positions 222(A), 256(I), 279(D), 284(A), 294(I), and 299(S), (Table 4) which are found in most reported VVIBDV (Kasanga *et al*., 2008).

Unique amino acid residues were observed at positions 254(G), 272(T), and 279(N) in isolate Lus11-12, and at position 280(S) in isolate Lus15-12 (Figure 3). The unique amino acid residue at positions 279(N) in Lus11-12 was also found in CVIBDVs' (1/10Et, 002/73), AVIBDV (variant E) and attenuated strains (228E, MB, Lukert and CEVAC-Gumbo-L) (Figure 3). The unique amino acid at position 280(S) in isolate Lus15-12 was not found in any of the compared sequences (Figure 3).

Amino acid residues 253(Q) and 284(A) have been confirmed to contribute to the high pathogenicity of IBDV and they were conserved in all the isolates that caused IBD in broilers in this study (Table 4). Less pathogenic IBDV are known to have histidine and threonine at positions 253 and 284, respectively.

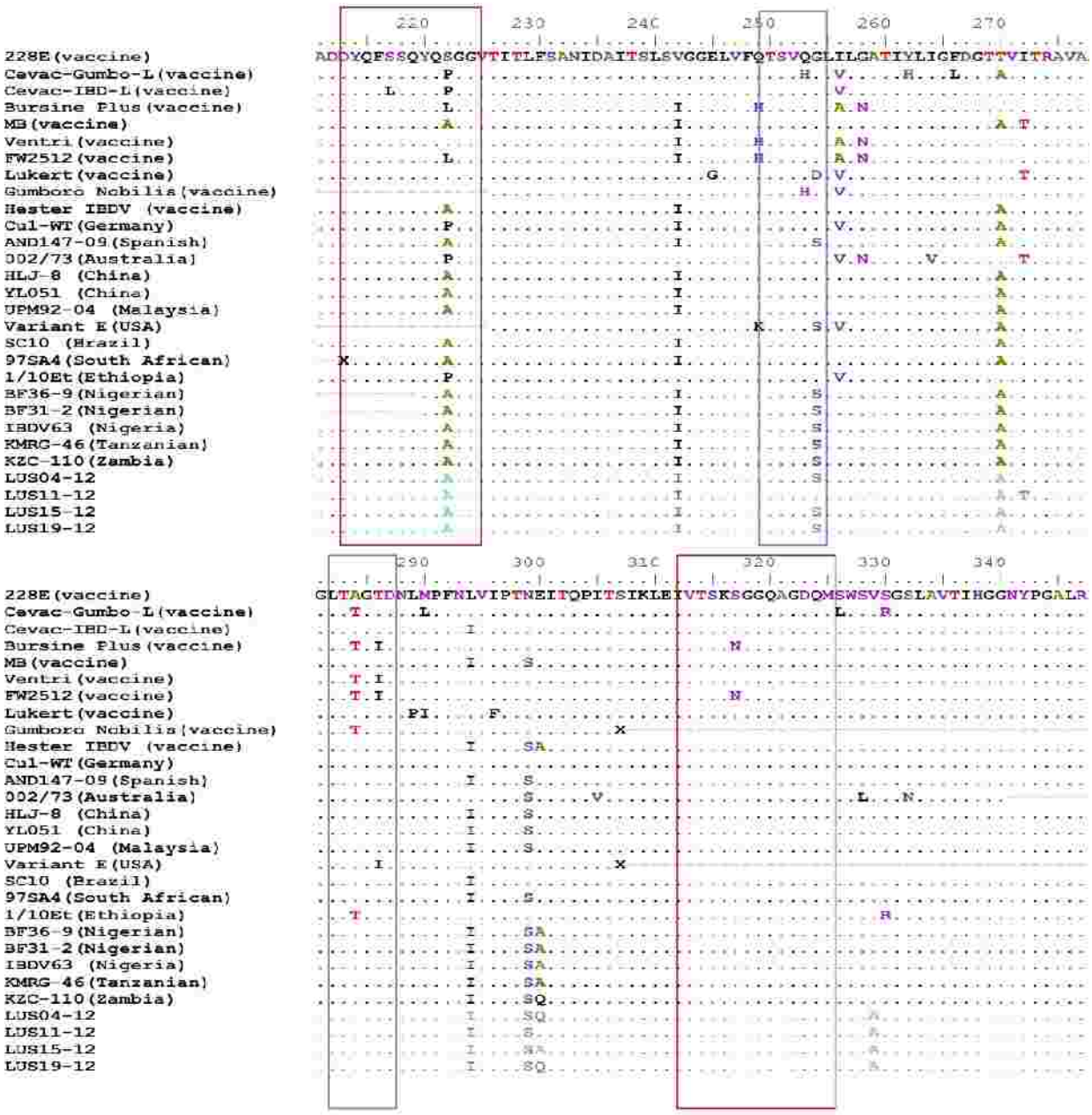

Figure 3: Alignment and comparison of deduced amino acid sequences from position 210 to 350 (numbering according to Bayliss *et al.*, 1990) of the hypervariable region of ORF2 of very virulent strains (Lus04-12, Lus11-12 Lus15-12, Lus19-12, 97SA4, BF36-9, BF31-2, IBDV63, KMRG-46, KZC-110, UPM92-04, YL051, HLJ-8 and AND147-09), non-very virulent serotype 1 strains (002-73, 1/10Et, Cu1-wt and Var-E) and attenuated strains (228E CEVAC-IBD-L, CEVA-GUMBO-L, Bursine plus, FW2512, Lukert and Hester IBD). Amino acid differences with respect to the 228E strain are shown and identical amino acid residue is indicated with a dot. The large boxes

indicate the Major hydrophilic peaks A and B, 212 to 225 and 312 to 326, respectively. The smaller boxes highlight the minor hydrophilic peaks 1 and 2, 249 to 255 and 282 to 287, respectively.

Table 5: Vaccine and Lusaka isolates comparison of amino acids responsible for antigenicity and pathogenicity in VVIBDV

	Amino acids								
	222	**253**	**254**	**256**	**279**	**284**	**294**	**299**	**300**
Virus Isolates									
Nobilis 228E	Ser	Gln	Gly	Ile	Asn	Ala	Leu	Asn	Glu
Cevac-Gumbo-L	Pro	His	Gly	Val	Asn	Thr	Leu	Asn	Glu
Cevac-IBD-L		Gln	Gly	Val	Asp	Ala	Ile	Asn	Glu
Bursine Plus		Gln	Gly	Ala	Asp	Thr	Leu	Asn	Glu
MB	Ala	Gln	Gly	Ile	Asn	Ala	Ile	Ser	Glu
Ventri	Ser	Gln	Gly	Ala	Asp	Thr	Leu	Asn	Glu
FW 25-12		Gln	Gly	Ala	Asp	Thr	Leu	Asn	Glu
Lukert		Gln	Asp	Val	Asn	Ala	Leu	Asn	Glu
Hester IBDV	Ala	Gln	Gly	Ile	Asp	Ala	Ile	Ser	Ala
Egypt/09		Gln	Gly	Ile	Asp	Ala	Ile	Ser	Glu
Lus02-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus04-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Glu
Lus05-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus06-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus07-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus08-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus09-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus10-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus11-12	Ala	Gln	Gly	Ile	Asn	Ala	Ile	Ser	Glu
Lus12-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus13-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus14-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus15-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Ala
Lus16-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus17-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
Lus19-12	Ala	Gln	Ser	Ile	Asp	Ala	Ile	Ser	Gln
	222	**253**	**254**	**256**	**279**	**284**	**294**	**299**	**300**
VVIBDV	Ala			Ile	Asp	Ala	Ile	Ser	
CVIBDV	Pro			Val	Asn	Thr	Leu	Asn	

4.3.0 Phylogenetic analysis

For phylogenetic analysis, eleven and sixteen sequences of the VP1 and VP2, respectively, detected from 20 clinical samples and 40 additional sequences obtained from the NCBI Genbank were included in this study. Among the isolates included were those from the following countries or regions; South Africa, Tanzania, Ethiopia, Nigeria, Malaysia, Egypt, China, Iran, Spain, Brazil and France. The analysis was done using BioEdit and Mega 6.0 with 1000 bootstrap resampling and the Neighbor Joining method. The Phylogenetic trees were formed based on the data from nucleotide sequences along with the amino acid sequences.

The phylogenetic tree based on VP2-HVR included sixteen field isolates from this study and 34 reference strains from Africa, Asia, Australia, Europe and America. Briefly, the sequences used in the analysis clustered into two main clades, VVIBDV and non-VVIBDV serotype 1 strains (Figure 4). The first cluster VVIBDV was further subdivided into four groups. The B1 sub-cluster contained three South African (DQ916235, DQ916233 and AF159217) isolates and 1 Brazilian isolate (JN982268). The B2 sub-cluster contained three isolates, two from China (EU042144 and DO656506) and one from Malaysia (AF262030). One isolate from this study, Lus11-12, was genetically close to two intermediate plus attenuated strains (KJ547670 and DQ656512) and they formed the sub-cluster B3. The African VVIBDV as previously described by Kasanga *et al* (2008) were sub-clustered in B4 and the group contained isolates from this study, Nigeria, Tanzania, Ethiopia and Zambia (from a previous study) (Figure 4). The clustering of the VVIBDV in the phylogenetic tree shows some form of geographical specificity for the isolates in this group.

The non-VVIBDV contained seven CVIBDV and two AVIBDV and they appeared to be sub-

clustered according to geographical region (Figure 4). The seven CVIBDV isolates were two Australian (AF148073 and AF069577), two European (AJ878886 and AY321953), two Ethiopian (JQ684021 and JQ684020) and one Tanzania (FJ607166) and the two AVIBDV were both from USA (AF293792 and AF293788).

In the phylogenetic tree based on VP1 (Figure 5), the IBDV strains were mainly divided into two clusters based on bootstrap values, VVIBDV and the non-VVIBDV isolates. The first cluster, VVIBDV, was further subdivided into four groups; C1, C2, C3 and C4. The sub-cluster C1 contained eight isolates from this study, three Zambian isolates (AB368958, AB368959 and AB368960) from previous study (Kasanga *et al*., 2008) and one isolate from Tanzania (AB368971). In the second sub-cluster, C2, two isolates from the study (Lus8-12 and Lus19-12) were genetically close to a Dutch strain (DQ679811). Like in segment A, Lus11-12 was again clustered with attenuated strains (DQ927041 and AF083094) forming subgroup C3. The fourth subgroup consisted strains from Ethiopia (JQ241033 and JQ241035), Nigeria (AY099457) and Tanzania (AB368955 and AB368957).

The non-VVIBDV cluster of VP1 contained five CVIBDV and two AVIBDV and two serotype strains (Figure 5). The five CVIBDV isolates were two Australian (AJ878682 and AJ878639), two European (AF362748 and AJ878643), one Zambian (AB368969). The AVIBDV isolates were variant A (AJ878475) and variant E (AJ878676) while the two serotype 2 strains were OH (U30819) and 23/82 (AJ878684).

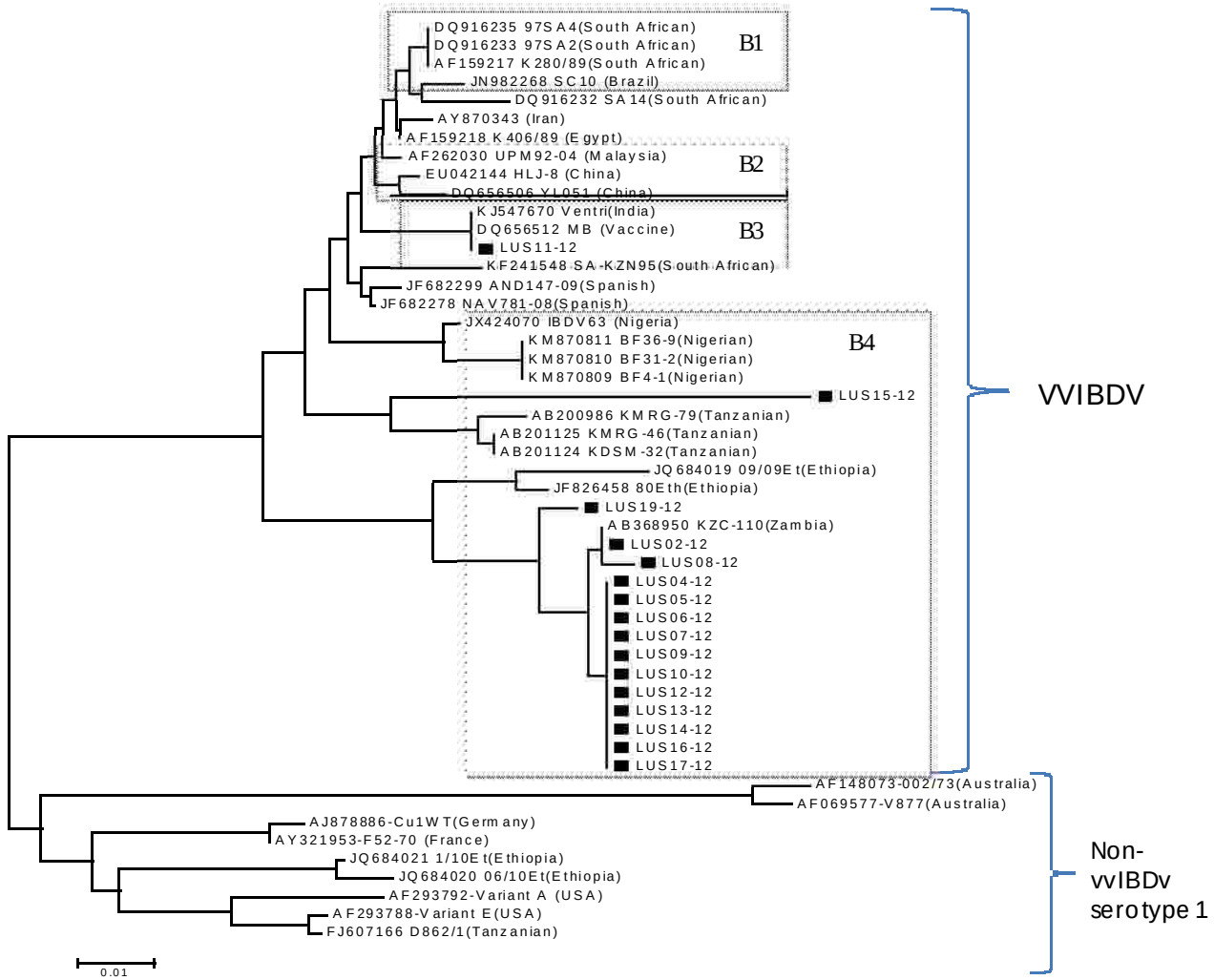

Figure 4: Phylogenetic tree constructed by Neighbor-Joining methods based on 243-bp sequences in VP2-HVR of IBDV serotype 1 and attenuated strains using nucleotide alignment created in BioEdit and MEGA 6.0. Sequences were obtained from this study (black square) including IBDV VP2 from Africa, Asia, Europe, Australia and America. The sequences included in the phylogenetic analysis show accession numbers, common name and country of origin. The analysis composed of CVIBDV, AVIBDV and VVIBDV. In the phylogenetic tree, two clusters of the IBDV clade were observed (VVIBDV and non-VVIBDV serotype 1) based on the bootstrap value. The VVIBDV cluster had four sub clusters; B1, B2, B3, and B4. The Non-VVIBDV group composed AVIBDV and CVIBDV strains.

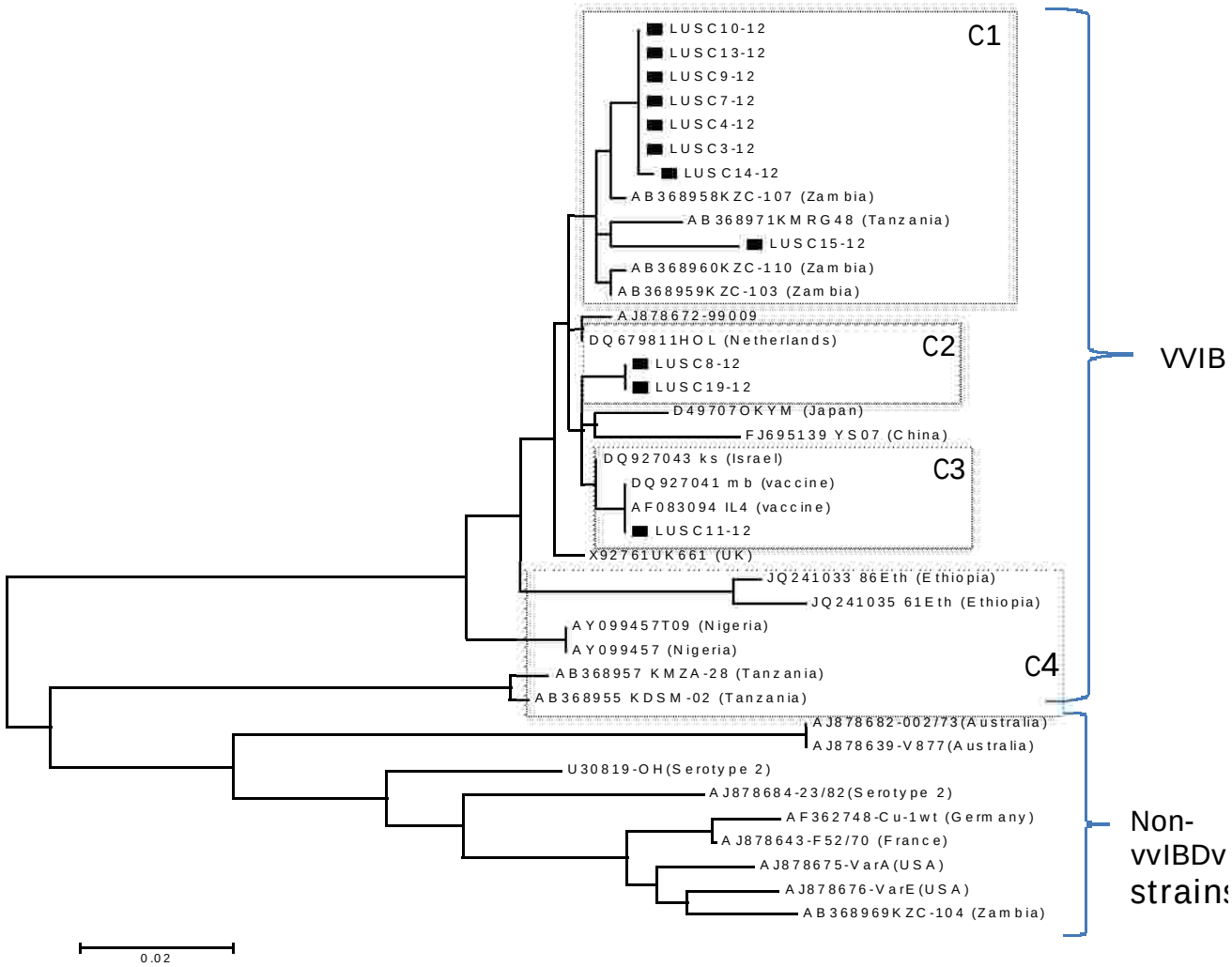

Figure 5: Phylogenetic tree constructed by Neighbor-Joining methods based on 534-bp sequences in VP1 of IBDV serotype 1 and 2 and attenuated strains using nucleotide alignment created in BioEdit and MEGA 6.0. Sequences were obtained from 11 broiler outbreaks in Lusaka (black square) including IBDV VP1 from Africa, Asia, Europe, Australia and America. The included sequences show accession numbers, common name and country of origin. The analysis composed of CVIBDV, AVIBDV, VVIBDV and serotype 2 strains. In the phylogenetic tree, two clusters of the IBDV clade were observed (VVIBDV and non-VVIBDV serotype 1) based on the bootstrap value. The VVIBDV cluster had four sub clusters; C1, C2, C3, and C4. The Non-VVIBDV group composed AVIBDV, CVIBDV and serotype 2 strains.

CHAPTER FIVE

5.0 DISCUSSION

IBD is still a major threat to the poultry industry worldwide. One of the major problems observed in the field is that frequent outbreaks of IBD occur in spite of the repeated usage of available IBD vaccines in Zambia. Many factors may contribute to vaccine failures in the field, such as immunological pressure or genome reassortment as a consequence of mixed infections with different genotypes of IBDV, allowing new field strains to emerge (He *et al*., 2014). It is therefore important to diagnose these new strains and track their evolutionary changes at the molecular level by sequencing the critical viral genes continually. The hypervariable region within VP2 has been widely used for molecular diagnosis and genotyping of IBDV (Kasanga *et al*., 2008). However, recently it has been shown that VP1 of VVIBDV is essential for the in vivo pathogenicity of IBDV (Jackwood, 2011; Le Nouen *et al*., 2006; Le Nouen *et al*., 2005). It has been shown that the characterization of IBDV strains based on both genome segments would be appropriate, but the complete sequencing of both segments is impractical in routine practice and may not be necessary. Recently, VP2-HVR in segment A and the 5' terminus of VP1 in segment B have been demonstrated to be sufficient to assess their genetic relatedness in IBDV isolates (Jackwood, 2011; Le Nouen *et al*., 2006; Le Nouen *et al*., 2005).

5.1 Confirmation of infectious bursal disease virus

Since the first report of IBD in Zambia, very few studies have focused on the molecular characterization and mutations of field isolates (Kasanga *et al*., 2008; Sharma *et al*., 1977).

However, the molecular characteristics of field IBDV isolates from Zambia have not yet been extensively studied, with only a few reports available on IBDV genotype and genome reassortment in this country (Kasanga *et al.*, 2008, 2013)

The result of this study clearly indicated the presence of IBDV in broiler chickens in Lusaka, Zambia. Using RT-PCR of IBDV of VP1 and VP2, it was confirmed that from the twenty outbreaks diagnosed with IBD at postmortem, eleven were positive for VP1 and sixteen were positive for VP2. The confirmation of VVIBDV in broilers is in agreement with previous studies of IBDV in poultry in Zambia by Kasanga *et al (*2013). We were unable to amplify a part of genome segment B (VP1) of Lus02-12, Lus05-12, Lus06, Lus16-12, Lus17-12 and Lus19-12 although the corresponding segment A (VP2-HVR) was successfully amplified. Our inability to amplify a part of genome segment B of Lus02-12, Lus05-12, Lus06, Lus16-12, Lus17-12 and Lus19-12 may be because segment B has undergone genetic recombination. Therefore, the genetic nature and origin of genome segment B of Lus02-12, Lus05-12, Lus06, Lus16-12, Lus17-12 and Lus19-12 remain to be investigated (Kasanga *et al*., 2013)

All the VVIBDV isolates were extracted from bursae of Fabricius of broilers between ages of two to five week-old and is in agreement with results by other researchers (Howie and Thorsen, 1981; Vindevogel *et al*., 1976). The age of maximum susceptibility is between three and six weeks, corresponding to the period of maximum bursa development, during which the acute clinical signs are observed (Bumstead *et al*., 1993).

Currently, poultry veterinarians in Zambia diagnose IBD based on the following postmortem gross lesions that are considered pathognomic to the disease; dehydration, with petechial haemorrhages of the thigh and pectoral muscles, increased mucus in the intestine, renal changes and a swollen, hyperemic bursa of Fabricius. Necropsy is not definitive in the diagnosis of diseases such IBD and therefore from this study out of the twenty outbreaks confirmed at postmortem only sixteen were found to be positive for VP2-HVR and eleven positive for VP1 by RT-PCR method. The RT-PCR results were a confirmation that 80 percent of positive cases at necropsy in this study were actually IBD infections and the other twenty percent could have been other poultry diseases such as Infectious bronchitis (IB) (Lukert and Saif, 2003). In early studies by Cosgrove (1962) identification of the etiological agent of IBD was clouded by the presence of infectious bronchitis virus (IBV) and because of the similarity with the kidney lesions in the two diseases, it was thought that IBD was just another presentation of IB (Cosgrove, 1962). Therefore a likely hood of mistaking the two poultry viral diseases is very much possible. These nucleic acid extraction based techniques are useful tool for detecting IBDVs as the virus can be detected and typed without isolation and propagation in cell cultures or embryonated eggs (Jackwood, 2011). Furthermore nucleic acids can be extracted even when the virus is present in very minute quantity and has lost its infectivity (OIE, 2008; Eterradossi *et al*., 1998). This study therefore demonstrated the diagnosis of IBD infections by necropsy is not very definitive because the disease being immunosuppressive does subject the affected chickens to other opportunistic infections. More accurate diagnostic techniques such as PCR are therefore recommended for more accurate diagnosis of IBD.

5.2 Phylogenetic analysis of infectious bursal disease virus

Phylogenetic analysis of IBDV was done using VP1 and VP2 genes of the virus. The phylogenetic tree based on VP2-HVR included 16 field isolates from this study and 34 reference strains from Africa, Asia, Australia, Europe and America. In the tree, 15 of Zambian strains from this study were clustered among the very virulent (vv) type while one strain, Lus11-12, was clustered with attenuated strains (vaccines) (Figure 4). Phylogenetic analysis indicated that Lus02-12, Lus04-12, Lus05-12, Lus06-12, Lus07-12, Lus08-12, Lus09-12, Lus10-12, Lus12-12, Lus13-12, Lus14-12, Lus15-12, Lus16-12, Lus17-12 and Lus19-12 were closely related to Ethiopian strains (JQ200986 and JF826458) and a Zambian strain, KZC-110 (AB368450) clustered in VVIBDV (Figure 4). KZC-110 (AB368450) was isolated from broilers in Lusaka by Kasanga *et al* (2013), therefore this is an indication that it has a common ancestor with the Lusaka isolates from this present study. Lus11-12 was closely clustered with two attenuated strains (KJ547670 and DQ656512) and it is an interesting finding that highlights the involvement of IBD vaccines in causing disease.

From the total 20 outbreaks, only eleven were positive for IBDV VP1 using RT-PCR. Phylogenetic analysis showed that ten isolates were clustered with VVIBDV while one, Lus11-12, was clustered with two attenuated strains (DQ927041 and AF083094). Lus11-12 demonstrated similar relationship to attenuated strains in phylogenetic tree for VP1 and VP2. From the eleven isolates that were positive for VP1, seven isolates (Lus3-12, Lus4-12, Lus7-12, Lus9-12, Lus10-12, Lus13-12, and Lus14-12) clustered with KZC-107 (AB368958) isolated from broiler in Lusaka (Kasanga *et al.*, 2013). The close relationship between the Lusaka isolates of this study and KZC-17 by Kasanga *et al* (2013) is as indications of common ancestry therefore posing a question of the quality biosecurity existing on farms in Lusaka. Lus15-12 was closely clustered with KMRG-48

(AB368971), which was isolated from a chicken in Tanzania.

A close relationship between Zambian, Tanzanian and Ethiopian strains was demonstrated in phylogenetic analysis of VP1 and VP2 suggesting possibilities of a common ancestor. It has been postulated that the migratory birds could be responsible for spreading the virus between Zambia, Tanzania and Nigeria (Adamu *et al*., 2013). Several researchers have isolated IBDV from migratory birds and are reported to be responsible for spreading a South African IBDV strain to Canada. Natural IBDV infections in different free-living and migratory bird species carrion crows (*Corvus corone*) and rock pigeons (*Columba livia*) (Kasanga *et al*., 2008; Wang *et al*., 2003; Wilcox *et al*., 1983), including the Sooty Tern (Wilcox *et al*., 1983), have been documented and were found around barns and domestic chicken flocks. The extreme resistance of the virus to the outside environment enhances the potential for indirect transmission. The virus can survive for four months in contaminated bedding and premises (Benton *et al*., 1967), and up to fifty-six days in lesser mealworms (*Alphitobius* sp.) taken from a contaminated building (McAllister *et al*., 1995).

Lus11-12 was identical to the vaccine strains in both VP1 and VP2, therefore it can be hypothesized that the isolate was a mutation of the attenuated strains. Detection of such a vaccine-like virus from broilers diagnosed with IBD implied that replication and accidental spread of live vaccine virus resulting in reversion of the virus to the virulent form. Although not observed in this study, reassortment has been reported in Zambia (Kasanga *et al*., 2008) and in China (He *et al*., 2014). Many factors may contribute to the reassortment process: vaccine selective pressure (using different kinds of vaccine such as inactivated vaccines and attenuated live vaccines), environment (temperature, light, humidity, and breeding density), and immune functions of birds (such as

response to vaccine and environment) (Wei *et al*., 2008). Therefore, we can postulate that Lus11-12 was in the initial stages of reassortment at the time of investigation.

5.3. Assessment of key amino acids of viral protein 2 (VP2)

Residues present in the VP2 region at positions 222, 242, 253, 256, 279, 284, 294, 299 and 329 are key amino acids believed to be responsible for viral antigenicity and pathogenicity (Liu *et al*., 2004; Eterradossi *et al*., 1998). In VVIBDVs conserved amino acids are A222, I256, D279, A284, I294 and S299 While in CVIBDV they are P222, V256, N279, T284, L294 and N299 (Brown and Skinner, 1996). The VP2-HVR of fifteen isolates from this study encoded all amino acids that are conserved among the VVIBDV strains. One isolate, Lus11-12, had asparagine at position 279 while the rest of the conserved amino acids in that isolate were identical to those of VVIBDV strains. The main mechanisms of evolution of dsRNA viruses (and negative-strand segmented RNA viruses) are often sequential point mutations and reassortment (Wei *et al*., 2008), as well as recombination, which is seen only infrequently. Therefore, the change at 279 in Lus11-12 of the signature amino acid Aspartic acid to asparagine could be attributed to sequential point mutation.

It is known that isolates with a glutamine at position 253 and Alanine at 284 have increased pathogenicity while those with a histidine at position 253 and threonine at 284 are much less pathogenic (Mundt, 1999). All the strains isolated in this study had amino acids glutamine at position 253 and alanine at 284 (Table 4) indicating that they are highly pathogenic in nature corresponding with pathological lesions observed at necropsy in the IBD outbreaks. Comparison of the Zambian isolates in this study with those from other African countries showed that isolates from Tanzania, South Africa and Nigerian all had glutamine at position 253 indicating they were highly

pathogenic as well. Although the nucleotide sequences of the African VVIBDVs showed some differences in the VP2 hypervariable domain, the deduced amino acid sequences of this region, which was reported as critical for antigenicity, were not different. The amino acid sequences of the isolates from this study were also identical to those of VVIBDVs previously detected in Zambia (Kasanga *et al*., 2008).

Following comparison of the VP2-HVR sequences from Lusaka VVIBDV isolates of this study and attenuated strains, it was observed that two vaccine strains (MB and Hester IBDV) had putative amino acids consistent with the Lusaka isolates. Lus11-12 had amino acids A222, G253, G254, I256, N279, A284, I294 and S299 and was similar to those on the vaccine MB and Hester IBDV. This result of the present study suggests that widespread use of live vaccines from Europe or other parts of the world may have contributed to the spread of IBDV strains from one region to another with the escape mutant vaccine strains adapting to the new region alongside indigenous field strains. In fact, Tong *et al* (1993) showed that IBD was more likely to occur in vaccinated chickens than in non-vaccinated chickens. There have been earlier reports of field isolates having sequences similar to IBDV vaccine strains (Jackwood *et al*., 2011 Dolz *et al*., 2005; Ignjatovic and Sapats, 2002).

CHAPTER SIX

6.0 CONCLUSIONS

a. The confirmation of VVIBDV from broiler chickens in this study reaffirmed the reports of similar studies in Lusaka, Zambia indicating presence of the VVIBDV in poultry.

b. Molecular diagnostic methods provided better and more accurate IBD results than necropsy.

c. The Zambian VVIBDV strains have a common ancestor with the strains from Tanzania and Nigeria.

d. The study demonstrated that attenuated strains could acquire pathogenicity and eventually cause IBD infections and mortality in broiler flocks.

e. Detection of such a vaccine-like virus from broilers diagnosed with IBD implied that replication and accidental spread of live vaccine virus resulting in reversion of the virus to the virulent form.

CHAPTER SEVEN

7.0 RECOMMENDATIONS

7.1 Poultry Breeders and Hatcheries

a. The use the local VVIBDV strains to produce live and inactivated vaccines for priming and boosting of breeder flocks to provide adequate passive immunity is highly recommended. Passive immunity to local VVIBDV strains will be transferred to broiler progeny (day old chicks) providing them some level of early protection against field challenge.

b. Hatcheries should regularly determine the day of vaccination for day old chicks from different breeder flocks. The vaccination date information should then be disseminated to the farmers through regular bulletins in sales outlets or in training seminars. The timing of broiler vaccination depends on the level of MDAs present in the chicks. It is not possible to know accurately without serological examination of antibodies how much MDAs a day old chick receives from the breeder hen. High levels of MDAs at the time of vaccination will neutralize the IBD vaccine. Several formulae for estimating the optimal age of vaccinating are used in the field.

c. Hatcheries should vaccinate day-old chick they produce with an antigen-antibody immune-complex vaccine. The emergence of VVIBDV necessitated the development of an IBD vaccine capable of working in the presence of relatively high level of MDAs. Recently, an

immune complex vaccine (Transmune™) has been developed by CEVA, (Palya *et al*., 2004), which combines an intermediate plus IBDV complexed with serum (antibodies) against IBDV. The vaccine is administered either by in-ovo or day-old subcutaneous applications. The vaccine is designed to release antigens while the MDAs wane-off from the broilers immune system, protecting them in the presence of field VVIBDVs

7.2 Broiler farms

a. Farmers should vaccinate broiler flocks at right time using the appropriate IBD vaccine strain which are maintained under cold chain. Live IBDV vaccines are given to broilers in an effort to gain active immunity against IBDV (Giambrone *et al*., 1977). The information on the right timing for vaccination can be provided by the hatchery as mentioned above. Repeated vaccination of commercial broiler flocks reared in houses associated with frequent poor performance and in which subclinical IBD has been identified is effective in significantly increasing flock performance (McAllister *et al*., 1995).

b. Biosecurity plays an important role in the control of poultry diseases. In a study by Ndashe *et al*., (unpublished) it was observed that high incidence of IBD was recorded in Lusaka District during rainy seasons and it was attributed to poor biosecurity. In rainy seasons, farm environments are usually very wet and contaminated with chicken faecal dropping that may harbor IBDV for months. Thorough cleaning of poultry houses with appropriate soaps and formaldehyde disinfectants has proven to be successful in the control of IBD outbreaks. Wild birds have been reported transmit IBDV to domestic poultry flocks it is therefore important to ensure that poultry houses are bird-proof. Control of movement of farm staff and equipment

within the farm is very important in the control of transmission of IBDV. Proper standard operating procedures need to be put in place to ensure that farm staff and equipment are adequately disinfected and their usage limited to a single poultry house.

7.3 National policy and awareness

a. Further studies, surveillance and characterization of circulating IBDV in poultry flocks. Losses due to IBD significantly affect the economy of the poultry farmers and ultimately the nation due to mortalities and poor performance of chicken. Recent research has shown that IBDV does undergo mutation by reassortment or point mutation creating strains that antigenically differ from available vaccines on the market. The poultry industry through PAZ and local universities should invest in investigating circulating IBDV genotypes and such activities should be done regularly in a period of five years. In this regard a national database on the circulating genotypes of IBDV needs to be created.

b. Awareness of the economic devastating nature of IBD needs to be created by PAZ through the hatcheries by holding seminars to educate the farming community on the prevention and control of IBD. Poultry producers, mainly small scale, have very little knowledge of the disease and so have no information on its control.

REFERENCES

Abdel-Alim, G. A. and Saif Y. M. (2001). Immunogenicity and antigenicity of very virulent strains of infectious bursal disease viruses. Avian Dis 45:92–101.

Adamu, J, Owoade A. A, Abdu P. A, Kazeem H. M. and Fatihu M. Y. (2013) Characterization of field and vaccine infectious bursal disease viruses from Nigeria revealing possible virulence and regional markers in the VP2 minor hydrophilic peaks. Avian. Path. 42:5:420-433,

Allan, G.M, McNulty M.S, Connor T.J, McCracken R.M. and McFerran J.B. (1984). Rapid diagnosis of infectious bursal disease infection by immunofluorescence on clinical material. Avian Path. 13:419-427.

Allan, W. H, Faragher, J. T. and G. A. Cullen. (1972). Immunosuppression by the infectious bursal agent in chickens immunized against Newcastle disease. Vet Rec 90:511–512.

Alexander, D. J. and Chettle N. J. (1998). Heat inactivation of serotype 1 infectious bursal disease virus. Avian Path 27: 97–99.

Ashraf, S., Abdel-Alim G. and Saif Y. M. (2006). Detection of anti- bodies against serotypes 1 and 2 infectious bursal disease virus by commercial ELISA kits. Avian Dis 50:104–109.

Azad, A. A, Fahey K. J, Barrett S. A, Erny K. M. and Hudson P. J. (1986). Expression in

Escherichia coli of cDNA fragments encoding the gene for the host-protective antigen of infectious bursal disease virus. Virology. 149:190-8.

Azad, A. A, Barrett, S. A. and Fahey K. J. (1985). The characterization and molecular cloning of the double-stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology 143:35–44.

Baxendale, W. and Lutticken D. (1981). The results of field trials with an inactivated Gumboro vaccine. Dev Biol Stand 51:211–219.

Bayliss, C. D, Spies U, Shaw K, Peters, R. W Papageorgiou A, Muller H. and Boussnell M. (1990). A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. J Gen Virol 71: 1303–1312.

Becht, H. Müller H. and Müller H.K. (1988). Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. J Gen Virol 69:631–640.

Benton, W. J. Cover M.S. and Rosenberger J.K (1967). Studies on the transmission of the infectious bursal agent (IBA) of chickens. Avian Dis 11:430–438.

Boot, H. J, ter Hurne A.A, Hoekman A.J, Pol J.M, Gielkens A.L. and Peeters B.P. (2002). Exchange of the C-terminal part of VP3 from very virulent infectious bursal disease virus results in an attenuated virus with a unique antigenic structure. J Virol 76:10346–55.

Bottcher, B, Kiselev N. A, Stel'Mashchuk V. Y, Perevozchikova N. A, Borisov A. V. and Crowther R. A. (1997). Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. J Virol. 71:325-30.

Briggs, D. J, Whitfill C. E, Skeeles J. K, Story J. D. and Reed K. D. (1986). Application of the positive/negative ratio method of analysis to quantitate antibody responses to infectious bursal disease virus using a commercially available ELISA. Avian Dis 30:216–218.

Brown, M. D. and Skinner M. A. (1996). Coding sequences of both genome segments of a European “very virulent” infectious bursal disease virus. Virus Res 40:1–15.

Bumstead, N, Reece R.L. and Cook J.K.A. (1993). Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. Poult. Sci. 72: 403- 410.

Butcher, G. and Miles R. (1994). Vaccine failure in poultry: factors to consider. UF/IFAS Extension. VM82:1-5.

Burkhardt, E. and Muller H. (1987). Susceptibility of chicken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). Arch Virol. 94:297-303.

Bygrave, A. C. and Faragher J. T. (1970). Mortality associated and Gumboro disease. Vet Rec 86:758–759.

Cadman, H.F, Kelly P.J, Zhou R, Davelaar F. and Mason P.R. (1994). A Sero survey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in ostriches {Struthiocamelus) from Zimbabwe. Avian Dis.38:621-625.

Campbell, G. (2001). Investigation into evidence of exposure to infectious bursal disease virus and infectious anaemia virus in wild birds in Ireland. Proceedings II International Symposium on Infectious Bursal Disease and Chicken Infectious Anaemia. Rauisch- holzhausen, 230–235.

Chang, H. C, Lin T. L. and Wu C.C. (2001). DNA-mediated vaccination against infectious bursal disease in chickens. Vaccine 20:328–35.

Chettle, N, Stuart J. C. and Wyeth P. J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. Vet Rec 125:271–272.

Chevalier, C, Lepault J, Erk I, Da Costa B. and Delmas B. (2002). The maturation process of pVP2 requires assembly of infectious bursal disease virus capsids. J Virol 76:2384–2392.

Cheville, N. F. (1967). Studies on the pathogenesis of Gumboro disease in the bursa of Fabricius, spleen and thymus of the chicken. Am J Pathol 51:527–551.

Cho, B. R. (1970). Experimental dual infections of chickens with infectious bursal and Marek's disease agents. I. Preliminary observation on the effect of infectious bursal agent on Marek's

disease. Avian Dis 14:665–675.

Cho, Y. and Edgar S. A. (1969). Characterization of the infectious bursal agent. Poult Sci 48:2102–2109.

Chui, C. H. and Thorsen J. J. (1984). Experimental infection of turkeys with infectious bursal disease virus and the effect on the immunocompetence of infected turkeys. Avian Dis 28:197–207.

Cosgrove, A. S. (1962). An apparently new disease of chickens avian nephrosis. Avian Dis 6:385–389.

Craft, D. W, Brown J. and Lukert P.D. (1990). Effects of standard and variant strains of infectious bursal disease virus on infections of chickens. Am J Vet Res 51:1192–1197.

Cullen, G. A. and Wyeth P. J. (1975). Quantitation of antibodies to infectious bursal disease. Vet Rec 97:315.

De Wit, J. J, Heijmans J. F, Mekkes D. R. and van Loon A. A. W. M. (2001). Validation of five commercially available ELISAs for the detection of antibodies against infectious bursal disease virus (serotype 1). Avian Pathol 30:543–549.

DeWit, J.J. (1999). Gumboro disease: optimising vaccination.Int.Poult.Prod.7:1921-1926

Delmas, B, Kibenge F. S. B, Leong J.C, Mundt E, Vakharia V. N. and Wu J. L (2004). Birnaviridae. In Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball (eds). Academic Press: London, 561–569.

DiFabio, J, Rossini L, Eterradossi N, Toquin D. and Gardin Y. (1999). European-like pathogenic infectious bursal disease virus in Brazil. Vet Record 145:203–204.

Dobos, P. (1979). Peptide map comparison of the proteins of infectious bursal disease virus. J Virol 32:1046–1050.

Dohms, J. E. and J. S. Jaeger. (1988). The effect of infectious bursal disease virus infection on local and systemic antibody responses following infection of 3-week-old broiler chickens. Avian Dis 32:632–640.

Dolz R, Majó N, Ordóñez G. and Porta R. (2005). Viral Genotyping of Infectious Bursal Disease Viruses Isolated from the 2002 Acute Outbreak in Spain and Comparison with Previous Isolates. Avian Dis. 49.3. 332-339

Domanska, K, Mato T, Rivallan G, Smietanka K, Minta Z, de Boisseson C, Toquin D, Lomniczi B, Palya V. and Eterradossi N. (2004). Antigenic and genetic diversity of early European isolates of Infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of Infectious bursal disease virus revisited? Arch Virol.

149:465-80.

Edgar, S. A. and Cho Y. (1976). The epizootiology of infectious bursal disease and prevention of it by immunization. Dev Biol Stand. 33:349-356.

Edgar, S. A. and Cho Y. (1965). Avian nephrosis (Gumboro disease) and its control by immunization. Poult Sci.44:1366 (abstract).

Edison, C. S, Gelb J, Villegas P, Page R. K, Lukert P. D. and Kleven S. H. (1980). Comparison of inactivated and live infectious bursal disease virus vaccines in White Leghorn breeder flock. Poult Sci. 59:2708-16.

El-Bagoury, G F, El-Nahas E M, and El-Habbaa A S. (2015). Isolation and molecular characterization of IBDV from Qualubyia governorate, Egypt. Benha.Vet.Med.J.28:283-294.

Eterradossi, N, Arnauld C, Toquin D. and Rivallan G. (1998). Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. Arch Virol. 143:1627-36.

Eterradossi, N, Picault J.P, Drouin P, Guittet M, L'Hospitalier R. and Bennejean B. (1992). Pathogenicity and preliminary antigenic characterization of six infectious bursal disease virus strains isolated in France from acute outbreaks. Zentralbl Veterinaermed Reihe. 39:683–691.

Eterradossi, N. Gauthier C, Reda L, Comte S, Rivallan G, Toquin D, de Boisseson, C, Lamande J, Jestin V, Morin Y, Cazaban C. and Borne P. M. (2004). Extensive antigenic changes in an- atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. Avian Pathol 33:423–431.

Fadly, A. M, Winterfield R. W. and Olander H. J. (1976). Role of the bursa of Fabricius in the pathogenicity of inclusion body hepatitis and infectious bursal disease virus. Avian Dis 20:467–477.

Fahey, K. J O'Donnell I. J. and Azad A. A. (1985). Characterization by western blotting of the immunogens of infectious bursal disease virus. J Gen Virol 66:1479–1488.

Faragher, J. T, Allan W. H. and Wyeth C. J (1974). Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. Vet Rec 95:385–388.

Faragher, J. T, Allan W. H. and Cullen G. A. (1972). Immunosuppressive effect of the infectious bursal agent in the chicken. Nat New Biol. 237:118-9.

Fernandez-Arias, A, Martinez S. and Rodriguez J.F. (1997). The major antigenic protein of infectious bursal disease virus, VP2, is an apoptotic inducer. J Virol 71:8014–8018.

Gardner, H, Kerry K, Riddle M, Brouwer S. and Gleeson L. (1997). Poultry virus infection in Antarctic penguins. Nature 387:245.

Giambrone, J. J, Donahoe J.P, Dawe D.L. and Eidson C. S. (1977). Specific suppression of the bursa-dependent immune system of chicks with infectious bursal disease virus. Am J Vet Res38:581–583.

Gough, R.E, Drury S. E. N, Welchman D. D. B, Chitty J. R. and Summerhays G. E. S. (2002). Isolation of birnavirus and reovirus-like agents from penguins in the United Kingdom. Vet Rec 151:422–424.

Gorbaleny, A.E, Pringle F.M, Zeddam J.L, Luke B.T, Cameron C.E, Kalmakoff J, Hanzlik T.N, Gordon K.H.J. and Ward J.L. (2002). The palm sub domain-based active site is internally permuted in viral RNA-dependent RNA polymerases of an ancient lineage. J Mol Biol 324: 47–62.

Haddad E.E, Whitfill C.E, Avakian A.P, Ricks C.A, Andrews P.D, Thoma J.A. and Wakenell P.S. (1997). Efficacy of a novel infectious bursal disease virus immune complex vaccine in broiler chickens. Avian Dis.41:882–889.

Hassan, M. K, Afify M. and Aly M.M. (2002). Susceptibility of vaccinated and unvaccinated Egyptian chickens to very virulent infectious bursal disease virus. Avian Path. 31:149–156.

Hassan, M. K. and Saif Y. M. (1996). Influence of the host system on the pathogenicity, immunogenicity, and antigenicity of infectious bursal disease viruses. Avian Dis 40:553–561.

He, X, Xiong Z, Yang L, Guan D, Yang X. and Wei P. (2014). Molecular epidemiology studies on partial sequences of both genome segments reveal that reassortant infectious bursal disease viruses were dominantly prevalent in southern China during 2000-2012. Arch Virol.159:3279–3292.

Heine, H. G, Haritou M, Failla P, Fahey K.J. and Azad A.A. (1991). Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus, which can circumvent vaccination with standard, serotype 1 strains. J Gen Virol 72:1835–1843.

Helmboldt, C. F. and Garner E. (1964). Experimentally induced Gumboro disease (IBA). Avian Dis 8:561–575.

Hermann, M, Islam M.D. and Raue R. (2003). Research on infectious bursal disease: The past, the present and the future. Vet. Microbio. 97.153–165.

Henry, C.W, Brewer R.N, Edgar S.A. and Gray B.W. (1980). Studies on infectious bursal disease of chickens:2-scoring microscopic lesions in the bursa of Fabricius, thymus, spleen and kidney in gnotobiotic and battery reared white Leghorns experimentally infected with infectious bursal disease virus. Poult.Sci. 59:1006-1017.

Hiraga M, Nunoya T, Otaki Y, Tajima M, Saito T. and Nakamura T. (1994). Pathogenesis of highly virulent infectious bursal disease virus infection in intact and bursectomized

chickens.J.vet.med.Sci. 56:1057-1063.

Hirai, K. and Calnek B. W. (1979). In vitro replication of infectious bursal disease virus in established lymphoid cell lines and chicken B lymphocytes. Infect Immun 25:964–970.

Hirai, K. and Shimakura S. (1974). Structure of infectious bursal disease virus. J Virol 14:957–964.

Hirai, K, Kunihiro K. and Shimakura S. (1979). Characterization of immunosuppression in chickens by infectious bursal disease virus. Avian Dis 23:950–965.

Hitchner, S. B. (1976). Immunization of adult hens against infectious bursal disease virus. Avian Dis 20:611–613.

Hitchner, S. B. (1970). Infectivity of infectious bursal disease virus for embryonating eggs. Poult Sci 49:511–516.

Howie, R. I. and Thorsen J. (1981). Identification of a strain of in- fectious bursal disease virus isolated from mosquitoes. Can J Comp Med 45:315–320.

Ignjatovic, J. and Sapats S. (2002). Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. Aust Vet J. 80:689-94

Inoue, M, Fukuda M. and Miyano K. (1994). Thymic lesions in chicken infected with infectious bursal disease virus. Avian Dis.,38: 839-846.

Ismail, N. and Saif Y. M. (1990). Differentiation between antibodies to serotypes 1 and 2 infectious bursal disease viruses in chicken sera. Avian Dis 34:1002–1004.

Ismail, N. and Saif Y. M. (1991). Immunogenicity of infectious bursal disease viruses in chickens. Avian Dis 35:460–469.

Ivanyi, J. (1975). Immunodeficiency in the chicken II. Production of monomeric IgM following testosterone treatment of infection with Gumboro disease. Immunology 28:1015–1021.

Ivanyi, J. and Morris R. (1976). Immunodeficiency in the chicken. IV. An immunological study of infectious bursal disease. Clin Exp Immunol 23:154–165.

Jackwood, D.J. (2011). Viral competition and maternal immunity influence the clinical disease caused by very virulent infectious bursal disease virus. Avian Dis. 55, 398–406.

Jackwood, D. J. and Sommer S. E. (1997). Restriction length polymorphism in the VP2 gene of infectious bursal disease viruses. Avian Dis 41:627–637.

Jackwood, D. J. (1988). Detection of infectious bursal disease virus using nucleic acid probes. J Am Vet Med Assoc 192:1779.

Jackwood, D. H. and Saif Y. M. (1987). Antigenic diversity of infectious bursal disease viruses. Avian Dis 31:766–770.

Jackwood, D. J, Saif Y. M. and Hughes J. H. (1984). Nucleic acid and structural proteins of infectious bursal disease virus isolates belonging to serotypes I and II. Avian Dis 28:990–1006.

Jackwood, D. J. and Saif Y. M. (1983). Prevalence of antibodies to infectious bursal disease virus serotypes I and II in 75 Ohio chicken flocks. Avian Dis 27:850–854.

Johnson, D. C, Lukert P. D. and Page R.K. (1980). Field studies with convalescent serum and infectious bursal disease vaccine to control turkey coryza. Avian Dis 24:386–392.

Kasanga, C. J, Yamaguchi T, Munang'andu H. M, Ohya K. and Fukushi H. (2013). Genomic sequence of an infectious bursal disease virus isolate from Zambia: classical attenuated segment B reassortment in nature with existing very virulent segment A. Arch Virol158:685–689

Kasanga, C.J, Yamaguchi T, Wambura P.N, Munang'andu H.M, Ohya K. and Fukushi H. (2008). Detection of infectious bursal disease virus (IBDV) genome in free-living pigeon and guinea fowl in Africa suggests involvement of wild birds in the epidemiology of IBDV, Virus Genes 36:521–529

Kaufer, I. and Weiss E. (1980). Significance of bursa of Fabricius as target organ in infectious

bursal disease of chickens. Infect Immun 27:364–367.

Kibenge, F. S, McKenna P. K. and Dybing J. K. (1991). Genome cloning and analysis of the large RNA segment (segment A) of a naturally avirulent serotype 2 infectious bursal disease virus. Virology. 184:437-40.

Kibenge, F. S, Dhillon A. S. and Russell R. G. (1988). Growth of serotypes I and II and variant strains of infectious bursal disease virus in Vero cells. Avian Dis. 32:298-303.

Kim, S. J, Sung H. W, Han J. H, Jackwood D. and Kwon H. M. (2004). Protection against very virulent infectious bursal disease virus in chickens immunized with DNA vaccines. Vet Microbiol 101:39–51.

Kosters, J, Becht H. and Rudolph R. (1972). Properties of the infectious bursal agent of chicken (IBA). Med Microbiol Immunol 157:291–298.

Kreider, D.L, Skeeles J.K, Parsley M, Newberry L.A. and Story J.D. (1991). Variability in a commercially available enzyme-linked immunosorbent assay system. II. Laboratory variability. Avian Dis. 35:288-293.

Landgraf, H, Vielitz E. and Kirsch R. (1967). Occurrence of an infectious disease affecting the bursa of Fabricius (Gumboro disease). Dtsch Tieraerztl Wochenschr 74:6–10.

Lasher, H.N. and Davis V.S. (1997). History of infectious bursal disease in the U.S.A. The first two decades. Avian Dis 41: 11-19.

LeNouen, C, Rivallan G, Toquin D,Darlu P,Morin Y, Beven V, de Boisseson C, Cazaban C, Comte S, Gardin Y. and Eterradossi N. (2006). Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment-B-reassorted isolate. J. Gen.Virol. 87, 209–216.

Le Nouen, C, Rivallan G, Toquin D. and Eterradossi N. (2005). Significance of the genetic relationships deduced from partial nucleotide sequencing of infectious bursal disease virus genome segments A or B. Arch. Virol. 150:313–325.

Lee, L. H. and Lukert P. D. (1986). Adaptation and antigenic variation of infectious bursal disease virus. J Chin Soc Vet Sci 12:297–304.

Lim, B, Cao Y, Yu T. and Mo C. (1999). Adaptation of very virulent infectious bursal disease virus to chicken embryonic fibro- blasts by site-directed mutagenesis of residues 279 and 284 of viral coat protein VP2. J Virol 73:2854–2862.

Liu, M. and Vakharia V. N. (2004). VP1 protein of infectious bursal disease virus modulates the virulence in vivo. Virology 330:62–73.

Liu, M. and Vakharia V. N. (2006). Non-structural protein of infectious bursal disease virus inhibits apoptosis at the early stage of virus infection. J Virol 80:3369–3377.

Lombardo, E.A, Maraver A, Espinosa I, Fernadez-Arias A. and Rodriguez J.F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. J. Virol. 73:6973–6983.

Lombardo, E.A, Maraver A, Espinosa I, Fernadez-Arias A. and Rodriguez J.F. (2000). VP5, the non-structural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virology 277:345–357.

Louzis, C, Gillet J.P, Irgens K, Jeannin A. and Picault J.P. (1979). Gumboro disease onset in the pheasant of livestock .Bull.mens.Soc.vét.prat.Fr 63:3-7

Lucio B. (1987). Quantitative agar gel precipitation test:an alternative for monitoring infectious bursal disease vaccination programs.InProc.36thWestern PoultryDisease Conference,3-5March,Davis,California.University of California, Davis,116-119.

Lucio, B. and Hitchner S.B. (1979). Infectious bursal disease emulsified vaccine:effect upon neutralizing antibody levels in the dam and subsequent protection of the progeny. Avian Dis. 23:466-478.

Lukert, P. D. and Saif Y. M. (2003). Infectious bursal disease. In: Diseases of Poultry, 11th ed. Y. M. Saif, H.J.Barns, A.M. Fadly, J.R. Glisson, L.R. McDougald and D. E. Swayne, eds. Iowa State

Press, Ames, IA:161-179.

Lukert, P. D. (1986). Serotyping recent isolates of infectious bursal disease virus. Proc 21st Natl Meet Poult Health Condemn: Ocean City, MD, 71–75.

Lukert, P. D, Leonard J. and Davis R. B. (1975). Infectious bursal disease virus: Antigen production and immunity. Am J Vet Res 36:539–540.

MacDonald, R. D. (1980). Immunofluorescent detection of double- stranded RNA in cells infected with reovirus, infectious pancreatic necrosis virus, and infectious bursal disease virus. Can J Microbiol 26:256–261.

Martinez-Torrecuadrada, J. L, Saubi N, Pagès-Manté A, Caston J. R, Espuna E. and Casal J.I. (2003). Structure dependent efficacy of infectious bursal disease virus (IBDV) recombinant vaccines. Vaccine 21:3342–3350.

Mazengia, H, Tilahun S B. and Negash T. (2009). Incidence of infectious bursal disease in village chickens in two districts of Amhara Region, Northwest Ethiopia. J. Livest. Res. Rural Dev. 21:12.

Mazengia, H. (2008). Newcastle disease and Infectious Bursal Disease in chickens among households of Bahir Dar and Farta districts, Northwest Ethiopia, DVM Thesis,
FVM, AAU, Ethiopia.

Mbuko, I.J, Musa W.I, Ibrahim S, Sa'idu L, Abdu P.A, Oladele S.B. and. Kazeem H.M. (2010). A Retrospective Analysis of Infectious Bursal Disease Diagnosed at Poultry Unit of Ahmadu Bello University, Nigeria. Int. J. Poultry. Sci. 9: 784-790,

McAllister, J. C, Steelman C. D, Newberry L. A. and Skeeles J. K. (1995). Isolation of infectious bursal disease virus from the lesser mealworm, Alphitobius diaperinus (Panzer). Poult Sci 74:45–49.

McFerran, J. B, McNulty M. S, McKillop E. R, Conner T. J, McCracken R. M, Collins D. S. and. Allan G. M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkey and duck: Demonstration of a second serotype. Avian Pathol 9:395–404.

Mekkes, D.R. and de Wit J.J. (2002). Report of the second international ring trail for Infectious Bursal Disease Virus (IBDV) antibody detection in serum. Annual report and proceedings 2002 of COST Action 839: Immunosuppressive viral diseases in poultry, 210–226.

Meroz, M. (1966). An epidemiological survey of Gumboro disease. Ref Vet 23:235–237.

Meulemans, G, Decaesstecker M, Halen P. and Froyman R. (1987). Comparison of ELISA and serum neutralization test for the detection of antibodies against the virus of Gumboro disease. ELISA practical applications.Rec.Méd.vét.,163:561-565.

Mickael, C. S. and Jackwood D. J. (2005). Real time RT-PCR analysis of two epitope regions encoded by the vP2 gene of infectious bursal disease viruses. J Virol Met 128:37–46.

Moody, A, Sellers S. and Bumstead N. (2000). Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. J Virol Met 85:55–64.

Müller, H. (1986). Replication of infectious bursal disease virus in lymphoid cell. Arch Virol 87:191–203.

Müller, H, Lange H. and Becht H. (1986). Formation, characterization and interfering capacity of a small plaque mutant and of in- complete virus particles of infectious bursal disease virus. Virus Res 4:297–309.

Müller, H. and Becht H. (1982). Biosynthesis of virus-specific proteins in cells infected with infectious bursal disease virus and their significance as structural elements for infectious virus and incomplete particles. J Virol 44:384–392.

Muller, H, Scholtissek C. and Becht H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. J Virol. 31:584-9.

Munang'andu, H.M, Syachaba M.Z. and Sharma R.N. (1996). A study of Infectious Bursal Disease outbreaks in Lusaka Province of Zambia. Zam J of Vet Sci 1, 8-10.

Mundt, E. (1999). Tissue culture infectivity of different strains of infectious bursal disease virus is determined by distinct amino acids in VP2. J Gen Virol 80:2067–2076.

Mundt, E. and Müller H. (1995). Complete nucleotide sequences of 5' and 3' non-coding regions of both segments of different strains of infectious bursal disease virus. Virology 209:10–18.

Mundt, E. and Vakharia V. N. (1996). Synthetic transcripts of double-stranded birnavirus genome are infectious. Proc Natl Acad Sci USA 93:11131–11136.

Nakai, T. and Hirai K. (1981). *In vitro* infection of fractionated chicken lymphocytes by infectious bursal disease virus. Avian Dis 25:831–838.

Nieper, H. and Müller H. (1996). Susceptibility of chicken lymphoid cells to infectious bursal disease virus does not correlate with the presence of specific binding sites. J Gen Virol 77:1229–1237.

Nunoya, T, Otaki Y, Tajima M, Hiraga M. and Saito T. (1992). Occurrence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in specific-pathogen-free chickens. Avian Dis. 36:597-609.

Ogawa, M, Yamaguchi T,Setiyono A, Ho T, Matsuda H, Furasawa S, Fukushi H. and Hirai K. (1998). Some characteristics of a cellular receptor for virulent infectious bursal disease virus using flow cytometry. Arch Virol 143:2327–2341.

Okoye, J. O. A. and Okpe G. C. (1989). The pathogenicity of an isolate of infectious bursal disease virus in guinea fowls. Acta Vet Brno 58:91–96.

Okoye, J. O. A. and Uche U. E. (1986). Serological evidence of infectious bursal disease virus infection in wild rats. Acta Vet Brno 55:207–209.

Okwor, E C, Eze D C. and Okonkwo K. (2012). Serum antibody levels against infectious bursal disease virus in Nigerian village chickens using indirect hemagglutination test. Pak Vet J, 32: 286-287.

Öppling, V, Müller H. and Becht H. (1991). The structural polypeptide VP3 of infectious bursal disease virus carries group- and serotype- specific epitopes. J Gen Virol 72:2275–2278.

Owoade, A. A, Mulders M. N, Kohnen J, Ammerlaan W. and Muller C. P. (2004). High sequence diversity in infectious bursal disease virus serotype 1 in poultry and turkey suggests West-African origin of very virulent strains. Arch Virol 149:653–672.

Pagès-Manté, A, Torrents D, Maldonado J. and Saubi N. (2004). Dogs as potential carriers of infectious bursal disease virus. Avian Path 33:205–209.

Palya, V, Korgach K, Suveges T, Kelemen M, Meszaros J. and Benyeda J. (2004). Control of infectious bursal disease by an immune complex vaccine. 22nd World poultry congress, june 8-13,

Istanbul, Turkey.

Parkhurst, R. T. (1964). On-the-farm studies of Gumboro disease in broilers. Avian Dis 8:584–596.

Pattison, M, Alexander D. J. and Harkness J. W. (1975). Purification and preliminary characterization of a pathogenic strain of infectious bursal disease virus. Avian Pathol 4:175–187.

Pejkovski, C, Davelaar F.G. and Kouwenhoven B. (1979). Immunosuppressive effect of infectious bursal disease virus on vaccination against infectious bronchitis. Avian Pathol. 8:95-106.

Perelman, B. and Heller E. D. (1983). The effect of infectious bursal disease virus on the immune system of turkeys. Avian Dis 27:66–76.

Petek, M, D'Aprile P. N. and Cancellotti F. (1973). Biological and physicochemical properties of the infectious bursal disease virus (IBDV). Avian Path 2:135–152.

Peters, G. (1967). Histology of Gumboro disease. Berl Munch Tierarztl Wochenschr 80:394–396.

Peters, M.A, Lin T. L. and Wu C. C. (2005). Real-time RT-PCR differentiation and quantitation of infectious bursal disease virus strains using dual-labeled fluorescent probes. J Virol Met 127:87–95.

Phenix, K.V Wark K, Luke C. J, Skinner M. A, Smyth J. A, Mawhinney K. A. and Todd D. (2001). Recombinant Semliki Forest virus vector exhibits potential for avian virus vaccine development. Vaccine 19:3116–23.

Poultry Association of Zambia, Annual General Meeting Report, 2011

Poultry Association of Zambia, Annual General Meeting Report, 2012

Poultry Association of Zambia, Annual General Meeting Report, 2013

Poultry Association of Zambia websites (http://www.paoz.org/index.php/directions) and (http://www.paoz.org/index.php/13-news-and-events/20-the-poultry-industry-in-zambia) 12/7/2012

Pous, J, Chevalier C, Ouldali M, Navaza J, Delmas B. and Lepault J. (2005). Structure of birnavirus-like particles determined by com- bined electron cryomicroscopy and X-ray crystallography. J Gen Virol 86:2339–2346.

Rautenschlein, S. and Haase C. (2005). Differences in the immunopathogenesis of infectious bursal disease virus (IBDV) fol- lowing in ovo and post-hatch vaccination of chickens. Vet Immunol Immunopathol 106:139–150.

Rautenschlein, S, Yeh H. Y, Njenga M. K. and Sharma J. M. (2002). Role of intrabursal T cells in infectious bursal disease virus infec- tion: T cells promote viral clearance but delay follicular

recovery. Arch Virol 147:285–304.

Reddy, S.K, Silim A. and Frenette D. (1992). Biological roles of the major capsid proteins and relationships between the two existing serotypes of infectious bursal disease virus. Arch Virol 127:209–222.

Roney, C.S. and Freund R.C. (1988). A comparison of infectious bursal disease antibody titers using different antigens in the serum neutralization and enzyme-linked immunosorbent assay tests. InProc.37thWesternPoultry Disease Conference,29 February-2 March, Davis, California. University of California, Davis.17-20.

Rosenberger, J. K. and Cloud S. S. (1985). Isolation and characterization of variant infectious bursal disease viruses. J Am Vet Med Assoc 189:357.

Rosenberger, J. K. and Gelb J. (1978). Response to several avian respiratory viruses as affected by infectious bursal disease virus. Avian Dis 22:95–105.

Rosenberger, J. K, Klopp S, Eckroade R. J. and Krauss W. C. (1975). The role of the infectious bursal agent and several avian adenoviruses in the hemorrhagic-aplastic-anemia syndrome and gangrenous dermatitis. Avian Dis 19:717–729.

Rosenberger, J. K, Cloud S. S. and Metz A. (1987). Use of infectious bursal disease virus variant vaccines in broilers and broiler breeders. Proc 36th West Poult Dis Conf, 105–109.

Saif, Y. M. (1984). Infectious bursal disease virus types. Proc 19th Natl Meet Poult Health Condemn: Ocean City, MD, 105–107.

Sapats, S. I. and Ignjatovic J. (2000). Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. Arch Virol 145:773–785.

Sapats, S. I, Trinidad L, Gould G, Heine H G, Van den Berg T P, Eterradossi N, Jackwood D, Parede L, Toquin D. and Ignjatovic J. (2006). Chicken recombinant antibodies specific for very virulent infectious bursal disease virus. Arch Virol 151:1551–1566.

Schnitzler, D, Bernstein F, Müller H. and Becht H. (1993). The genetic basis for the antigenicity of the VP2 protein of the infectious bursal disease virus. J Gen Virol 74:1563–1571.

Sharma, J. and Rosenberger J. K. (1987). Infectious bursal disease and reovirus infection of chickens: Immune responses and vaccine control. Avian Immun. Basis Pract. 2:149–150

Sharma, J. M. (1984). Effect of infectious bursal disease virus on protection against Marek's disease by turkey herpes virus vaccine. Avian Dis 28:629–640.

Sharma, J. M. and Lee L. F. (1983). Effect of infectious bursal disease virus on natural killer cell activity and mitogenic response of chicken lymphoid cells: Role of adherent cells in cellular

immune suppression. Infect Immun 42:747–754.

Sharma, J. M, Dohms J. E. and Metz A. L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and the effect of those viruses on humoral and cellular immune competence of specific pathogen free on chickens. Avian Dis 33:112–124.

Sharma, J.M, Benko l. and D'cruz S. (1977). Infectious bursal disease in Zambia. Vet. Rec. 101: 158,

Shirai, J, Seki R, Kamimura R. and Mitsubayashi S. (1994). Effects of invert soap with 0.05% sodium hydroxide on infectious bursal disease virus. Avian Dis 38:240–243.

Skeeles, J. K. and Lukert P. D. (1980). Studies with an attenuated cell-culture-adapted infectious bursal disease virus: Replication sites and persistence of the virus in specific-pathogen-free chickens. Avian Dis 24:43–47.

Skeeles, J. K, Lukert P. D, De Buysscher E. V, Fletcher O. J. and Brown J. (1979). Infectious bursal disease virus infections. II. The relationship of age, complement levels, virus-neutralizing antibody, clotting and lesions. Avian Dis 23:107–117.

Snedeker, C, Wills F. K. and Moulthrop I. M. (1967). Some studies on the infectious bursal agent. Avian Dis 11:519–528.

Snyder, D. B, Marquardt W. W, Mallinson E. T, Russek-Cohen E, Savage P. K. and Allen D. C. (1986). Rapid serological profiling by enzyme-linked immunosorbent assay. IV. Association of infectious bursal disease serology with broiler flock performance. Avian Dis 30:139–148.

Snyder, D. B, Vakharia V.N. and Savage P. K. (1992). Naturally occurring neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch. Virol. 127:89–101.

Steger, D, Müller H. and Riesner D. (1980). Helix-core transitions in double-stranded viral RNA: Fine resolution melting and ionic strength dependence. Biochem Biophys Acta 606:274–285.

Snyder, D. B, Vakharia V.N. and Savage P. K. (1992). Naturally occurring neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch. Virol. 127:89–101.

Stuart, J.C. (1989). Acute infectious bursal disease in poultry.Vet.Rec.,125:281.

Tacken, M.G.J, Peeters B.P.H, Thomas A.A.M, Rottier P.J.M. and Boot H.J. (2002). Infectious bursal disease virus capsid protein VP3 in- teracts both with VP1: The RNA dependent RNA polymerase, and with double-stranded RNA. J Virol 76:11301–11311.

Takase, K, Baba G. M, Ariyoshi R. and Fujikawa H. (1996). Susceptibility of chicken embryos to highly virulent infectious bur- sal disease virus. J Vet Med Sci 58:1129–1131.

Tanimura, N. and Sharma J. M. (1997). Appearance of T cells in the bursa of Fabricius and cecal tonsils during the acute phase of infectious bursal disease virus infection in chickens. Avian Dis. 41:638-45.

Tanimura, N, Tsukamoto K, Nakamura K, Narita M. and Maeda M. (1995). Association between pathogenicity of infectious bursal disease virus and viral antigen distribution detected by immunochemistry. Avian Dis 39:9–20.

Tham, K. M. and Moon C. D. (1996). Apoptosis in cell culture induced by infectious bursal disease virus following in vitro infection. Avian Dis 40:109–113.

Thayer, S. G, Villegas P. and Fletcher O.J. (1987). Comparison of two commercial enzyme-linked immunosorbent assays and conventional methods for avian serology. Avian Dis 31:120–124.

Todd, D. and McNulty M. S. (1979). Biochemical studies with infectious bursal disease virus: Comparison of some of its properties with infectious pancreatic necrosis virus. Arch Virol 60:265–277.

Tong, J.C, Umoh J.U, Abdu P.A. and Sa'idu L. (1993). Retrospective studies of Gumboro disease seen in Ahmadu Bello University Veterinary Teaching Hospital Zaria, Nigeria (1985_1990). Bull. Animal.Health.Prod.Africa. 41.173-179.

Tsai, H. J. and Saif Y. M. (1992). Effect of cell-culture passage on the pathogenicity and immunogenicity of infectious bursal diseasevirus. Avian Dis 36:415–422.

Tsukamoto, K, Matsumura T, Mase M. and Imai K. (1995). A highly sensitive, broad-spectrum infectivity assay for infectious bursal disease virus. Avian Dis. 39:575-86.

Ture, O. and Saif Y. M. (1992). Structural proteins of classic and variant strains of infectious bursal disease viruses. Avian Dis 36:829–836.

Vakharia, V. N, Ahamed B. and Snyder D. B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. Vir Res 31:265–273.

Vakharia, V. N, Snyder D. B, Lutticken D, Mengel-Whereat S. A, Savage P. K, Edwards G. H. and Goodwin M. A. (1994). Active and passive protection against variant and classic infectious bursal disease virus strains induced by baculovirus expressed structural proteins. Vaccine 12:452–456.

Van den Berg, T. P, Morales D, Eterradossi N, Rivallan G, Toquin D, Raue R, Zierenberg K, Zhang M.F, Zhu Y.P , Wang C.Q, Zheng H.J , Wang X, Chen G.C, Lim B.L. and Müller H. (2004). Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. Avian Path 33:470–476.

Van den Berg, T. P. (2000). Acute infectious bursal disease in poultry: a review. Avian Path. 29:175-194.

Van den Berg, T, Gonze M. and Meulemans G. (1991). Acute infectious bursal disease in poultry: Isolation and characterization of a highly virulent strain. Avian Path 20:133–143.

Van der Marel, R., Snyder D. B. and Luetticken D. (1991). Antigenic characterization of IBDV field isolates by their reactivity with a panel of monoclonal antibodies. Dtsch Tieraerztl Wochenschr 97:81–83.

Vindevogel, H, Gouffaux M, Meulemans G,Duchatel J.P. and Halen P.(1976). Gumboro disease: Distribution and persistence of the virus in the chick inoculated. Studies on transmission of disease .Avian Path 5:31-38.

Wang, X, Jiang P, Deen S, Wu J, Liu X. and Xu J. (2003). Efficacy of DNA vaccines against infectious bursal disease virus in chickens enhanced by co-administration with CpG oligodeoxynucleotide. Avian Dis 47:1305–1312.

Wei, Y, Yu X, Zheng J, Chu W, Xu H, Yu X. and Yu L. (2008). Reassortant infectious bursal disease virus isolated in China. Virus Res. 131: 279–282.

Weisman, J. and Hitchner S.B. (1978). Infectious bursal disease virus infection attempts in turkeys and coturnix quail. Avian Dis 22:604–609.

Wilcox, J. E, Flower R. L. and Baxendale W. (1983). Serological survey of wild birds in Australia for the prevalence of antibodies to egg drop syndrome 1976 and infectious bursal disease viruses. Avian Path 12:135–139.

Winterfield, R. W. and Thacker H. L. (1978). Immune response and pathogenicity of different strains of infectious bursal disease virus applied as vaccines. Avian Dis. 22:721-31.

Winterfield, R. W. (1969). Immunity response to the infectious bursal agent. Avian Dis 13:548–557.

Winterfield, R. W. and Hitchner S. B. (1962). Etiology of an infectious nephritis-nephrosis syndrome of chickens. Am J Vet Res 23:1273–1279.

Winterfield, R. W, Hitchner S. B, Appleton G.S. and Cosgrove A. S. (1962). Avain nephrosis, nephritis and Gumboro disease, in L&M news Views. 3:130.

Wood, G.W, Muskett J.C, Hebert C.N. and Thornton D.H. (1979). Standardization of the quantitative agar gel precipitin test for antibodies to infectious bursal disease. J.biol.Standard. 7:89-96.

World Organization for Animal Health (Office International des Epizooties). 2004. Chapter 2.7.1 Infectious bursal disease. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Fifth Edition. Edited by OIE, Paris, France, 817–832.

Wyeth, P. J. (1975). Effect of infectious bursal disease on the response of chickens to S. typhimurium and E. coli infections. Vet Rec 96:238–243.

Wyeth, P. J. and Chettle N. J. (1990). Use of infectious bursal disease vaccines in chicks with maternally derived antibodies. Vet Rec 126:577–578.

Wyeth, P. J. and Cullen G. A. (1978). Transmission of immunity from inactivated infectious bursal disease oil-emulsion vaccinated parent chickens to their chicks. Vet Rec 102:362–363.

Yamaguchi, S, Imada I. and Kawamura H. (1981). Growth and infectivity titration of virulent infectious bursal disease virus in established cell lines from lymphoid leucosis. Avian Dis 25: 927–935.

Yao, K. and Vakharia V. N. (2001). Induction of apoptosis in vitro by the 17kDa non-structural protein of infectious bursal disease virus: possible role in viral pathogenesis. Virology 285:50–58.

Zambia National Farmers Union. (2011). Annual General Meeting Report.

Zeryehun, T. and Fekadu G. (2012). Seroprevalence of infectious bursal disease in chickens managed under backyard production system in Central Oromia, Ethiopia. African J of Microbio Res. 6:6736-6741