PATHOLOGICAL EVALUATION OF GUMBORO VACCINE PREPARED FROM FREEZE-DRIED CULTURE OF THE "LIVE GUMBORO VIRUS GM97 STRAIN" (HIPRA GUMBORO® – GM97) IN COMMERCIAL CHICKENS

A THESIS

BY

ROZINA MURMU

SEMESTER: MARCH- AUGUST, 2010
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SESSION: 2009-2010

MASTER OF SCIENCE (M. S.) IN

PATHOLOGY

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

AUGUST, 2010

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DEDICATED
TO MY
BELOVED PARENTS

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The author
August 2010

ABSTRACT

A modified form of Intermediate plus of Infectious Bursal Disease Vaccine (HIPRAGUMBORO®-GM97) prepared from the GM97 strain of Infectious Bursal Disease virus was tested for its pathogenicity in commercial chickens. A total of 500 unvaccinated Cobb-500 commercial chicks, raised in relative isolation from day old. 21 chicks were collected from experimental farm at Day_{11} (D_{11}), D_{13}, D_{15}, D_{17}, D_{20}, D_{23} and D_{26} respectively three birds were collected from each respective day. Vaccine was administered at ocular route at Day_{11} and boosting was done at Day_{17} with drinking water. A typically affected flock was included in this study for the comparison of the study. Clinical signs were observed. All the sampled birds were subjected to detailed necropsy. The visible gross morbid lesions, Bursa/Body weight ratios was recorded. The bursae were collected, preserved at 10% formalin, processed, sectioned and stained for histopathology including determination of bursal lesion scores. Data's were analyzed statistically.

There was no visible gross morbid lesions observed during necropsy and bursa/body weight ratios were 1.99, 2.98, 3.19, 2.44, 2.45, 2.24, 2.38 and 2.45 at D_{11}, D_{13}, D_{15}, D_{17}, D_{20}, D_{23}, D_{26} and the affected flock respectively. Histopathological lesions were characterized as normal to severe lymphatic cell depletion with varying degrees of follicular atrophy and the score's of bursal lesions were 1.33, 1.33, 2.00, 0.67, 1.33, 3.33, 1.33 and 1.33 at D_{11}, D_{13}, D_{15}, D_{17}, D_{20}, D_{23} , D_{26} and the affected flock respectively. There was no significant changes in the histological structures in the bursal follicles. No out breaks was noted the vaccinated flock, but significant changes were found in the affected flock.

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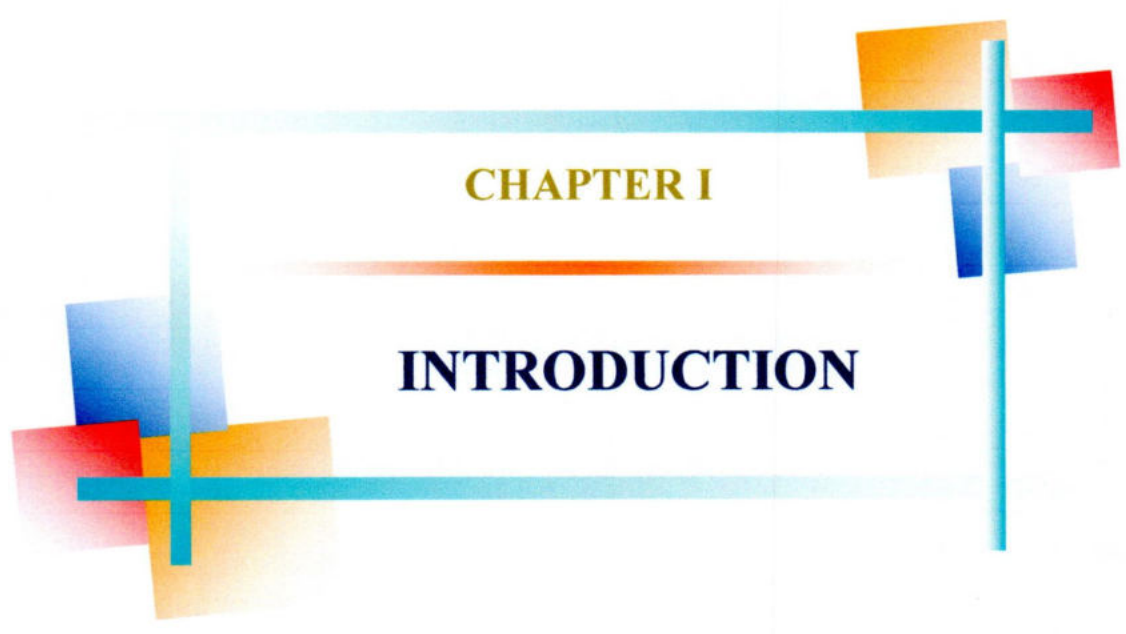
ABBREVIATION AND SYMBOLS

α	:	Alpha
γ	:	Gamma
μg	:	Microgram
μl	:	Microlitre
@	:	At the rate of
AGPT	:	Agar gel precipitation test
Ala	:	Alanine
B/BW	:	Bursa/body weight
HEPES	:	Hydroxy Ethyl Piperazine Ethene Sulfonic acid
BF	:	Bursa of Fabricius
CAM	:	Chorio-allantoic membrane
cDNA	:	Complementary deoxy ribonucleic acid
CEF	:	Chicken embryo fibroblast
D	:	Day
d.p.i.	:	Days post inoculation or days post infection
ds	:	Double-stranded
ELISA	:	Enzyme -linked immunosorbent assay
g	:	Gram
Glu	:	Glutamine
His	:	Histidine
H.S.T.U.		Hazee Mohammad Danesh Science and Technology University
IBD	:	Infectious Bursal Disease
IBDV	:	Infectious Bursal Disease Virus
IFN	:	Interferon
lbs	:	Pounds

Ltd.	:	Limited
MDA	:	Maternally derived antibody
mg	:	Milligram
ml	:	Milliliter
nm	:	Nanometer
°C	:	Degree centigrade
ORF	:	Open reading frame
p.i.	:	Post inoculation or post infection
PBS	:	Phosphate buffered solution
RNA	:	Ribonucleic acid
SIBW	:	Spleen/body weight
SPF	:	Specific pathogen free
Sq.	:	Square
TCID50	:	50% tissue culture infective dose
Thr	:	Threonine
TNF	:	Tumor necrosis factor
VNT	:	Virus neutralization test
VP	:	Virus protein
vv	:	Very virulent
vvIBDV	:	very virulent Infectious Bursal Disease Virus
W/V	:	Weight/volume
et al	:	And his associates
etc.	:	Etcetera
Fig.	:	Figure
H & E	:	Hematoxylin and Eosin
Min	:	Minute



CHAPTER I

INTRODUCTION

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Infectious bursal disease (IBD) or Gumboro disease is an acute, highly contagious viral disease of young chickens characterized mainly by severe lesions in the bursa of Fabricius (BF) followed by immunosuppression (Fadley *et al.*,1976; Rosenberger and Gelb, 1978; Saif, 1994; Lukert and Saif, 1997). It is a major poultry pathogen in the poultry industry (Hein *et al.*, 2002). Infectious bursal disease virus (IBDV), the oetiological agent of Gumboro disease, belonging to the genus Birnavirus (Murphy *et al.* 1995), sub-genus Avibirnavirus (Pringle, 1998), family Birnaviridae (Dobos *et al.*, 1979; Brown, 1986). IBD is economically important for the poultry industry in function of the immune depression that it causes (Moraes *et al.*, 2004). One of the significant components of the control of the disease is its vaccination which if improved may help in lowering the incidence of the disease in poultry (Zaheer *et al.*, 2003). IBD has been widely studied mainly for two reasons:

Firstly, the highly contagious virus can cause severe economic losses in poultry industries resulting from both the high mortality in the acute course of the disease and the consequences of B cell-dependent immunodeficiency (Muller *et al.*, 1992; Lasher and Shane, 1994; Lukert and Saif, 1997; Nagarajan and Kibenge, 1997; van den Berg, 2000).

Secondly, the pathological mechanism of IBDV is yet difficult to explain and interesting since only one organ system, the bursa of Fabricius, is almost exclusively involved (Hirai and Calnek, 1979; Kaufer and Weiss, 1980).

The effects of IBDV in chickens have been extensively reviewed (Lukert and Saif, 1997, van den Berg, 2000). The severity of these effects varies with the virulence of the field virus, age of the birds, and the maternally derived antibodies (MDA) (Lucio and Hitchner, 1979).

There are two distinct serotypes of IBDV: serotype1 and serotype II. Both serotypes can infect chickens and turkeys, but clinical disease is recognized only in chickens (Jackwood and Saif, 1987; Lana *et al.* 1992; Hassan and Saif, 1996; Yamaguchi *et al.* 1996a). Only serotype 1 viruses are virulent for chickens, replicating in and eventually destroying maturing B lymphocytes in the bursa of Fabricius (Cheville, 1967), inducing immunosuppression (Faragher *et al.*, 1972). Serotype I has four pathotypes: classical virulent, attenuated strains,very virulent and antigenic variant (van den Berg, 2000; Lukert and Saif, 2003). Very severe clinical outbreaks with high mortality rates caused by very virulent IBDV (vvIBDV) have been reported in Europe (van den Berg *et al.*, 1991; van den Berg, 2000), Africa (Zierenberg *et al.*, 2000), South America (Di Fabio *et al.*, 1999), Asia (Nunoya *et al.*, 1992; Chen *et al.*, 1998; To *et al.*, 1999) including Bangladesh (Rahman, 1994; Chowdhury *et al.*, 1996; Islam *et al.*, 1997). Bangladeshi strains of IBDV have been found to be antigenically and genetically similar to other very virulent strains (Islam *et al.*, 2001a; Hoque *et al.*, 2001). IBDV is now the major killer disease in the poultry farms of Bangladesh.

IBDV is exclusively a lymphotrophic virus targeting and destroying the growing B lymphocytes bearing cell-surface IgM (Hirai and Calnek, 1979; Nakai and Hirai, 1981), developing the severe morphological alteration of BF (Winterfield and Hitchner, 1962; Lukert and Saif, 1997), and producing a profound immunosuppression (Ivan *et al.*, 2001). The immunosuppression prevents the birds from optimally responding to vaccine (Winterfield and Thacker, 1978; Sharma *et al.*, 1984), and ultimately leads to increase in the incidence of numerous concurrent bacterial (Wyeth, 1975), viral (Giambrone *et al.*, 1977; Rosenberger and Gelb, 1978), protozoal (Anderson *et al.*, 1977)

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and fungal (Chowdhury *et al.,* 1996) infections as well as microbial toxicosis (Somvanshi and Mohanty, 1993).

IBDV is highly infectious and very resistant to inactivation. There is no alternative of vaccination in the prevention of IBD or Gumboro disease (Lukert and Saif, 1997), although the clinical outbreaks in vaccinated flocks are also reported (Chettle *et al.* 1989; van den Berg *et al.,* 1991; Eterradossi *et al.,* 1992; Muhammad *et al.,* 1996; Hafez *et al.,* 2002). In order to control IBD with live vaccine, it is critical to vaccinate commercial chickens that have maternal antibodies at optimum time. Live vaccines have the ability to overcome the maternal antibodies at certain level, vaccination during low maternal antibody titre shows better immune response than high maternal antibody titre (Giasuddin *et al.,* 2003). Neutralization of vaccine virus by the neutral antibodies is considered to be one factors causing vaccination failure. To overcome this problem stronger vaccine with higher residual pathogenicity has been developed to withstand maternal antibodies (Kouwenhoven and van den Bos, 1994). The antigenic variation among viruses also may causes vaccination failure, mainly when antigenic structures among field and vaccine strains no longer coincide (Jackwood and Saif, 1987: Cao *et al.,* 1998; van den Berg, 2000). No vaccine based on vvIBDV is yet commercially available.

The immunogenicity of virus may differ between strain to strain (Rosales *et al.,* 1989a, b,c; Abdel-Alim and Saif, 2001). The intermediate vaccine strain produced moderate to severe bursal lesions reported by many researchers (Franciosini and Coletti, 2001). The better protection with more virulent strain of IBDV is due to more antigenic stimulation based on higher and longer replication in lymphoid tissues (Rautenschlein *et al.,* 2001).

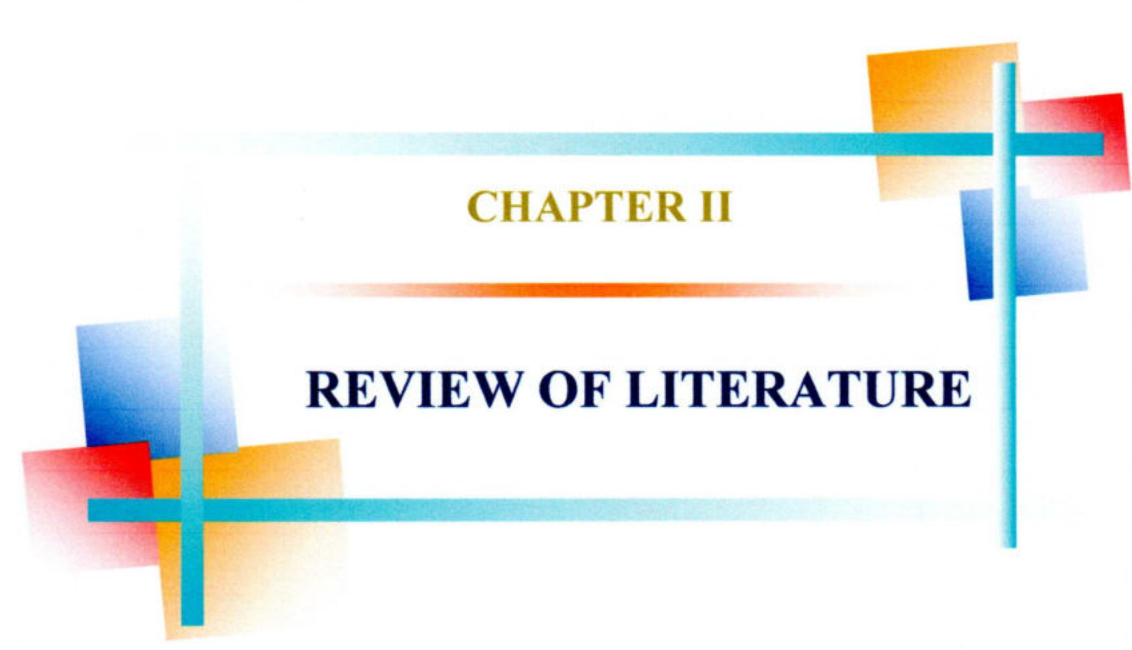
The present study is proposed to investigate the pathogenicity of the GM-97 strain of HIPRA GUMBORO®-GM97 in commercial chickens. The time of vaccination, type of the vaccine, maternally derived antibodies in the progeny chicks and pathogenicity of IBDV field challenge are the important factors determining the efficacy of the vaccination (Hair- Bejo *et al.*, 2004).

Objectives

- To study the gross morbid lesions including Bursa/Body weight ratios of the vaccinated flock.
- To study the sequential histopathology of bursa of Fabricius of birds vaccinated with GM-97 strain of IBDV including bursal lesion scores.
- Plotted bursal lesion scores towards understanding the level of immunesuppression.

Goal

- Evaluation of the vaccine prepared by live "GM-97 strain" of infectious bursal disease virus (IBDV), HIPRA GUMBORO®-GM97 in commercial chickens.


CHAPTER II
REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Available literature for the determination of the pathogenicity of a intermediate plus strain of HIPRA GUMBORO®-GM97 vaccine in this part of the thesis after a brief overview on the history, epidemiology, etiology, pathogenesis and pathology, clinical manifestations, immunosuppressive effects, and immunization strategies against IBD.

2.1. HISTORY OF IBD AND IBDV

The syndrome which emerged in 1957 (Cover, 1960) was formally documented by Cosgrove (1962) in broiler flocks located near the town of Gumboro, southern Delware, USA, while gave the common eponym of the malady as 'Gumboro disease'. Originally the condition was referred to as 'avian nephrosis' or 'nephritis-nephrosis syndrome of chickens' because of prominent kidney lesions (Cosgrove, 1962). Subsequently, the disease was called infectious bursal disease (IBD) because of the consistent involvement of the bursa of Fabricius. The term infectious bursal was proposed by Hitchner (1970). The etiological viral agent was isolated by Winterfield in 1962 (Lukert and Saif, 1997) who differentiated the disease from a previously established disease known as nephrotoxic viral infection of chickens. Following the initial outbreaks, the disease had been brought under control by extensive vaccination until the antigenic variant strains emerged in early 1980s in the USA (Snyder *et al.*, 1990).

Prior to 1987 the European strains of IBDV were of low pathogenicity, causing less than 1% mortality (Cavanagh, 1992). In 1987, the picture changed, a very virulent (vv) pathotype of IBDV emerged, which caused an acute disease with very high mortality (van den Berg *et al.*, 1991).

The acute disease first described in Europe at the end of the 1980s (Chettle *et al.*, 1989; van den Berg *et al.*, 1991; Eterradossi *et al.*, 1992), then described in Japan as acute form in the early 1990s (Nunoya *et al.*, 1992; Lin *et al.*, 1993), and they rapidly spread all over the major parts of the world (Eterradossi, 1995).

The first outbreaks of IBD occurred in Bangladesh at the end of 1992 (Islam *et al.*, 1994a and 1994b; Rahman *et al.*, 1996; Chowdhury *et al.*, 1996) with high mortality in the poultry farms (Bhattacharjee *et al.*, 1996; Chowdhury *et al.*, 1996; Islam *et al.*, 1997;Talha *et al.*, 2001). The virus has been isolated from the field outbreaks (Chowdhury *et al.*, 1996; Islam *et al.*, 2001a) and their pathogenicity has been tested (Islam *et al.*, 1997). IBDV isolates from Bangladesh were also characterized at antigenic and molecular level and had been found to be antigenitically and genetically related to other very virulent strains isolated earlier in Europe, Asia and Africa (Islam, *et al.*, 2001a). The complete nucleotide sequence of both genome segments of a vvIBDV from Bangladesh (BD-3/99) has established and full-length cDNA clones corresponding to the both segments have been established (Islam *et al.*, 2001 b).

Subsequent studies indicated that birds immune to infectious bronchitis virus (Gray virus) could still be infected with the Infectious bursal disease (IBD) virus and would develop changes in the cloacal bursa like IBD (Lukert *et al.*, 2003).

2.2. EPIDEMIOLOGY

2.2.1. Geographical distribution and prevalence of IBD

IBDV are of worldwide distributed, occurring in all major poultry producing areas (Eterradossi, 1995; Lukert and Saif, 1997). Australia, Newzealand, Canada and the US are so far unaffected (Snyder, 1990; Proffitt *et al., 1999;* Sapats and Ignjatovic, 2000). Variant IBD viruses were first reported in the Delmarva Peninsula region of the eastern United States in 1984. Variant strains are the predominant viruses in the United States (Lukert and Saif 2003).

Australia has remained free of vvIBDV mainly due to geographical isolation and strict quarantine barriers, but a disease outbreak during which IBD virus was isolated occurred in 1999 (Ignjatovic *et al.*, 2004).

The infection is endemic in nature and birds are constantly exposed to the virus. Breeder flocks are vaccinated against the virus to provide maternal immunity to the off-springs, so, all chicken flocks are seropositive for the virus (Lukert *et al.*, 2003).

2.2.2. Host ranges

Domestic fowls are the natural host of IBDV (Helmboldt and Garner, 1964). Natural infection of turkeys and ducks have also been recorded (Page *et al.*, 1978; McNulty *et al.*, 1979; McFerran *et al.*, 1980; Johnson *et al.*, 1980). IBDV infections of turkeys are subclinical in 3-6 weeks old poults, producing microscopic lesion in the bursa (Giambrone *et al.*, 1978). The couturnix quail is not infected with a chicken strain of IBDV (Weisman and Hitchner, 1978). Experimental inoculation of pheasants, partridges, guinea fowls and quails showed no signs of disease (van den Berg *et al.*, 2001). IBD virus has been isolated from a goose in China (Wang *et al.*, 2007).

Antibodies against IBDV have been detected in various wild birds like penguins (Gardner *et al.*, 1997), commercially raised ostrich (Ley *et al.*, 2000), wild ducks, crows, goose (Wilcox *et al.*, 1983; Hollmen *et al.*, 2000), which may mean that wild birds may act as targets or reservoirs (Wilcox *et al.*, 1983; Gardner, *et al.*, 1997; Ogawa *et al.*, 1997a; Hollmen, *et al.*, 2000).

In one study, dogs were evaluated as a potential carrier of the virus since viable virus persisted in the feces two days after initial ingestion and maintained its original characteristics (Torrents *et al.*, 2004).

2.2.3. Breeds susceptibility

The population at risk includes broiler flocks and young pullets destined for breeder and commercial egg laying flocks. Lighter breeds (laying breeds) show severe reaction to IBDV infection than heavier broiler breeds (Dr. Yonatan, 2009) and the highest susceptibility (about 80% mortality) was recorded in a Brown Leghorn line (Bumstead *et al.,* 1993). On the other hand, Meroz (1966) found no difference in the mortality between heavy and light breeds in a survey of 700 outbreaks of the disease.

There is no report of IBD in the native breeds. Moreover, indigenous chickens also can be infected experimentally (Okoye *et al.,* 1999).

2.2.4. Susceptible age

Chickens of 3-6 weeks of age are more commonly affected (Cosgrove, 1962; Winterfield and Hitchner, 1964; Hanson, 1967; Ley *et al.,* 1983; Shamaila Ashraf, 2005; Dr. Yonatan, 2009). Sub-clinical infection has been reported to occur in chicks before three weeks of age (Allan *et al.,* 1972; Ley *et al.,* 1979; Lukert and Saif, 1997) and even in newly hatched chicks (Fadley and Nazerian, 1983). Clinical disease also occurred in chickens up to 18 weeks of age (Ley *et al.,* 1979 and 1983).

2.2.5. Sources and transmission of infection

The incubation period (time between infection and the appearance of clinical disease) of IBDV in chickens is about 2 to 4 days (Dr. Yonatan, 2009). Infected chickens shed IBDV two days after infection and can transmit the disease for at least 14-16 days (Vindevogel *et al.,* 1976; Baxendale, 2002; Dr. Yonatan, 2009) but not exceeding 16 days (Winterfield *et al.,* 1972). Indirect transmission of virus most probably occurs on fomites (clothing and litter) or through airborne, virus laden feathers and poultry house dust (Benton *et al.,* 1967a). Virus can remain viable for up to 60 days in poultry house litter (Vindevogel *et al.,* 1976). Fishmeal in the feed contaminated with IBDV may act as a transmitter of the disease (Yongshan *et al.,* 1994), while lesser mealworm

as well as mosquito may act as a reservoir of IBDV (Snedeker *et al.*, 1967; Howie and Thorson, 1981). IBD virus has recently been isolated from a sparrow in China, suggesting that wild birds could act as carriers (Wang *et al.*, 2007).

According to another report, houses that contained infected birds were infective for innate birds after 54 and 122 days (Benton *et al.*, 1967a). No egg transmission of IBDV has yet been reported.

2.2.6. Seasons

IBD occurred round the year in Assam of India (Sami and Baruah, 1997), although IBD is more common during the winter months in Botswana (Binta *et al.*, 1995).

2.2.7. Morbidity and mortality rates

Striking features of this disease are the sudden and high morbidity rate, spiking death curve, and rapid flock recovery (Lukert and Hitchner, 1984).

Morbidity could be 100% (Islam *et al.*, 2008) and mortality could reach up to 80% in field outbreaks (Chowdhury *et al.*, 1996; Islam *et al.*, 1997; Hoque *et al.*, 2001; Islam *et al.*, 2008). Experimentally, infection to SPF chickens with vvIBDV causes 90-100% mortality (Chettle *et al.*, 1989; van den Berg *et al.*, 1991; Wenky *et al.*, 1994). The genetically engineered tissue culture adapted vvIBDV did not show any mortality in SPF chickens (van Loon *et al.*, 2001).

Mortality due to IBD on various farms ranged from 1 to 40% in broilers and from 2 to 40% in layers (Kurade *et al.*, 2000) and from 1.5 to 30% in native and broiler flocks respectively (Saif *et al.*, 2000).

2.2.8. Factors influencing the pathogenicity

Several viruses and host related factors can influence the pathogenicity of IBDV.

Table 1: Factors influencing the pathogenicity of IBDV

Factors influencing the pathogenicity		Reference(s)
Virus factors	Genetic variation	Sharma *et al.*,1989;Nunoya *et al.*, 1992; Jing *et al.*, 1995; Yamaguchi *et al.*, 1996b; van Loon *et al.*, 2001; Hoque *et al.*, 2001
	Virus antigen distribution in the nonbursal lymphoid organs	Tanimura *et al.*, 1995
Host factors	Species	Brown and Grieve, 1992
	Age	Winterfield and Hitchner, 1964
	Breeds	Lukert and Hitchner, 1984; Bumstead *et al.*, 1993
	Serial passaging in cell culture	Yamaguchi *et al.*, 1996a; Hassan *et al.*, 1996
	Levels of MDA	Iordanides *et al.*, 1991

2.3. OETIOLOGY

2.3.1. Classification of IBDV

Family: Birnaviridae

Genus: Birnavirus

Sub-genus: Avibirnavirus

Species: Infectious bursal disease virus

The etiological agent of the disease is Infectious bursal disease virus (IBDV) belonging to the family Birnaviridae of the genus Avibirnavirus. The genus name Birnavirus was proposed to describe viruses with two segments of double stranded RNA. Other viruses included in this group are Infectious Pancreatic Necrotic Virus (IPNV) of fish, Tellina virus, oyster virus, blotched snakehead virus (BSVN) (Da Costa *et al.*, 2003) and crab virus of bivalve mollusks belonging to Aquabirnavirus while Drosophila X virus belongs to genus Entomobirnavirus. All of these contain two segments of double stranded RNA surrounded by a single protein capsid of icosahedral symmetry (Dobos *et al.*, 1979).

2.3.2. Morphology of the virus

IBDV is a small, non-enveloped virus with icosahedral symmetry (Hirai and Shimakura, 1974). IBDV particles have a diameter of 55-60 nm (Hirai and Shimakura, 1974; Nick *et al.,* 1976) and posses a bisegmented, double-stranded RNA genome (Dobos *et al.,* 1979; Muller *et al.,* 1979a; Muller and Becht, 1982; Kibenge *et al.,* 1988). The molecular weight of the virus ranged from 2.2 to 2.5 X 106 Daltons (Nick *et al.,* 1976; Müller *et al.,* 1979) with the buyoant density of 1.34 g/ml (Hirai and Shimakura, 1974; Nick *et al.,* 1976; Dobos *et al.,* 1979; Jackwood *et al.,* 1982).

The virus consists of four structural proteins, VP1 to VP4 (Nick *et al.,* 1976; Dobos *et al.,* 1979) and the molecular weight of VP1, VP2, VP3 and VP4 polypeptides is 11000, 50000, 35000 and 25000 Daltons, respectively (Nick *et al.,* 1976). The capsid proteins (VP2 and VP3) arranged in the capsid, a single capsid shell composed of 32 capsomeres arranged in a 5:3:2 symmetry (Hirai and Shimakura, 1974).

The three dimensional structure of IBDV virion has been determined by electron cryomicroscopy. The outer and inner surfaces of the capsid are made of trimeric subunits (Bottcher *et al.,* 1997). Capsid is 9 nm thick and non-spherical in shape since the subunits close to the 5 fold symmetry axes are at a larger radius than those close to 2-3 fold axes. The VP2 forms the external trimeric subunits and protrude out of the shell forming a honeycomb surface. The VP3 forms the inner Y- shaped trimers that are packed closely to form a continuous shell and are connected to VP1. VP4 formed the rim around each 5 fold axis on the inner surface of the capsid (Bottcher *et al.,* 1997). This model suggests 780 copies of VP2, 600 copies of VP3, 60 copies of VP4 and is in accordance with the observed composition of 51% VP2, 40% of VP3, 6% VP 4 and 3% VP1 (Dobos *et al.,* 1979).

2.3.3. Serotypes and pathotypes of IBDV

There are two distinct serotypes of IBDV: serotype I and serotype II (Lukert *et al.*, 1979; McFerran *et al.*, 1980; Jackwood *et al.*, 1982), Serotype I is isolated from both chickens and turkeys while serotype II is isolated mainly from turkeys (Jackwood *et al.*, 1980) and also from chickens (Ismail *et al.*, 1988). A serotype I IBD virus has been isolated from the faeces of clinically healthy adult ducks, but the significance of the isolation is uncertain (Wang *et al.*, 2007). Serotype I viruses differ significantly in their pathogenicity and antigenicity (Winterfield and Thacker, 1978; McFerran *et al.*, 1980; Rosenberger and Cloud, 1986; Jackwood and Saif, 1987), whereas, serotype II is apathogenic to chickens (Brown and Grieve, 1992; Ashraf, 2005). Antibody has been detected but no clinical disease has been reported in chickens or turkeys as a result of infection with IBD virus serotype II (Lukert and Saif, 2003). Serotype I viruses can be further categorized into 4 groups on the basis of their pathogenicity: Classical strains, variants strains, attenuated strains and very virulent strains (Lim *et al.*, 1999; van den Berg *et al.*, 2000; Lukert and Saif, 2003) depending on their pathogenicity and/or antigenicity (Jackwood and Saif, 1987; Lasher and Shane, 1994).

Classical IBDV has traditionally affected poultry worldwide since the first reported incident from Gumboro. Classical strains of IBD virus vary in pathogenicity (Ignjatovic *et al.*, 2004). Classical strains cause bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20 -30% in specific pathogen free (SPF) chicken (Lim *et al.*, 1999).

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

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

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

Recently, emerged very virulent pathotypes of IBDV are closely related to classical serotype I strain of IBDV (Box, 1991; van der Marel *et al.*, 1991; van den Berg *et al.*, 1991; Tsukamoto *et al.*, 1995b; Abdel-Alim and Saif, 2001), but molecularly distinct from classical strains (Brown *et al.*, 1994). Molecular and antigenic characterization of Bangladeshi isolates of IBDV demonstrates their similarities with recent European, Asian and African vvIBDV strains (Islam *et al.*, 2001a).

Serotype I also includes many attenuated vaccine strains with different degrees of residual pathogenicity. They are designated as mild, intermediate and intermediate plus strains. A serotype II strain causes neither mortality nor bursal lesions in SPF birds. Serotype I vaccine causes no mortality but possess residual pathogenicity with bursal lesions varying from mild to moderate or even severe. Virulent serotype I field strains induce both mortality and bursal lesions.

2.3.4. Physico-chemical properties

The virus is non-enveloped and highly resistant to physical conditions and chemical agents. Due to the stability and hardiness of the virus, it persists in poultry premises even after thorough cleaning and disinfection. IBDV is resistant to a temperature of 56°C for 5 hours (Benton *et al.*, 1967b), at 60°C for 90 minutes, at room temperature

25°C for 21 days (Cho and Edgar, 1969), viable for up to 60 days in poultry house litter (Vindevogel *et al.*, 1976) and outside the host for at least four months (Baxendale, 2002). Petek (1973) observed that IBDV was more resistant then Reovirus to heat, ultraviolet irradiation and photodynamic inactivation. The hardiness of the virus makes it difficult to eradicate it from poultry houses after outbreaks of IBD (Alexander *et al.*, 1998). The IBDV can tolerate acidity as low as pH2, but inhibited in pH12 (Benton *et al.*, 1967b). The virus is inhibited by formalin and wescodyne but not by chloroform, phenol, ether, thimerosal and hyamine 2389 treatments (Benton *et al.*, 1967b). There is a marked reduction in the virus infectivity when exposed to 0.5% formalin for 6 hours (Lukert and Hitchner, 1984). Cho and Edgar (1969) reported that the virus was inactivated by exposure for 1 hour to 1% formalin, 1% cresol and 1% phenol. Chloramine (0.5%) killed the virus after 10 minutes (Landgraf *et al.*, 1967). The virus could survive outside the host for at least four months (Allan *et al.*, 1982).

2.4. CLINICAL MANIFESTATIONS

The clinical signs of the affected birds were more or less similar to the signs generally developed due to the infection with vvIBDV (Islam *et al.*, 2008). The virus causes immunosuppression in young chickens whereas clinical signs and death may be evident in older chickens at a time when the BF is more developed (Lukert and Saif, 1991). The exact cause of clinical symptoms and death is still unclear, but the signs do not seem to be related only to the severity of the lesions and the bursal damage (van den Berg, 2000).

The incubation period of IBD is 2-3 days (Cho and Edgar, 1972; Hirai *et al.*, 1974). During the acute phase of IBDV infection, the symptoms are similar to that observed in a septic shock like syndrome (Stocquardt *et al.*, 2001) or very similar to what observed in acute coccidiosis. It has been shown that ChIFN (Yun *et al.*, 2000; Rothwell *et al., 2000)* and TNF (Zhang *et al.*, 1995) might play an important role in the onset of the clinical signs. The disease is characterized clinically by marked depression, prostration, ruffled feathers, whitish or watery diarrhoea, inappetance or anorexia,

dehydration, emaciation, progressive weakness, reluctance to move, vent picking, soiled-vent feathers significantly elevated body temperature at 48 hours of infection but dropped below normal later, lateral recumbence before death and coma. Similar observations were also obtained from many literatures (Cosgrove, 1962; Snedeker *et al., 1967;* Cho and Edgar, 1972; Islam *et al.*, 1997; Thangavelu *et al.*, 1998; van den Berg, 2000; Islam *et al.*, 2008). Morbidity could be 100% and mortality could reach upto 80% in field outbreaks (Chowdhury *et al.*, 1996; Islam *et al.*, 1997; Hoque *et al.*, 2001; Islam *et al.*, 2008). Experimentally, infection to SPF chickens with vvIBDV causes 90-100% mortality (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). The wild-type vvIBDV strain and a virus generated by reverse genetics technology showed 100% morbidity but a tissue culture adapted vvIBDV strain did not show any clinical manifestation in SPF birds (van Loon *et al.*, 2001).

2.5. PATHOGENESIS AND/OR IMMUNOPATHOGENESIS OF IBD

Pathogenesis is defined as the method used by the virus to cause injury to the host with mortality, disease or immunosuppression as a consequence (van den Berg, *et al.*, 2000). The injuries can be evaluated at the level of host, the organ and the cell. IBDV usually infects young chickens between 3-6 weeks of age (Asraf, 2005; Dr. Yonatan, 2009) and causes a clinical disease, while sub-clinically infecting older birds. The outcome of IBDV infection is dependent on the strain and amount of the infecting virus, the age and breed of the birds, route of inoculation and presence or absence of neutralizing antibodies (Muller, *et al.*, 2003).

Sequential studies of tissues from orally infected chickens using immuno-florescence detected the viral antigen in macrophages and lymphoid cells in the caecum at 4 hr PI and in the lymphoid cells of duodenum and jejunum at 5 hr PI (Muller, *et al.*, 1979). The virus reaches the liver at 5 hrs PI and enters the bloodstream from where it is distributed to other organs; the bursal infection is followed by second viremia (Lukert, *et al.*, 2003). The virus persists in the bursa of experimentally inoculated SPF chickens till 3 weeks of age but the presence of maternal antibodies in the commercial chicken decreases the duration of its existence in bursa (Abdel-Alim, *et al.*, 2001).

2.5.1. Apoptosis

Apoptosis or the programmed cell death has shown to be one of the major mechanisms by which IBDV causes lesions (Eterradossi, 2001). Some IBDV strains induce apoptosis of bursal lymphocytes (Vasconcelos and Lam, 1995), but this was not confirmed with another IBDV strains (Hill and Sharma, 1999). Only 20% of the lymphoid cells in the BF contain replicating IBDV. The severe damage to the bursa can be ascribed to apoptosis. In addition to necrosis, marked atrophy of the BF occurs without eliciting an inflammatory response that is a characteristic sign of the apoptotic process. Replication of the virus in bursa of fabricius results in secondary viremia thus spreading the virus to other tissues (Ashraf, 2005).

It has been suggested that early after infection, the cells containing the viral antigen are protected from apoptosis to ensure viral replication. Anti- viral mechanisms kick in and destroy the neighboring cells to prevent the spread of the virus. During the late infection, the infected cells undergo apoptosis thus seeding the virus to other cells. The IBDV infection of a susceptible chicken has been shown to induce apotosis in the bursa as well as thymus (Vasconcelos, *et al.*, 1995; Lam, 1997; Ojeda, *et al.*, 1997; Tanimura, *et al.*, 1997, Tanimura, *et al.*, 1998).

Apoptosis has also been demonstrated in peripheral blood lymphocytes (Vasconcelos and Lam, 1995) and chickens embryo fibroblasts (Tham and Moon, 1996) when infected *in vitro* with IBDV. Both IBDV positive and IBDV negative cells of bursa of fabricius (Tanimura and Sharma, 1998; Nieper *et al.*, 1999), and antigen negative cells of thymus (Tanimura and Sharma, 1998) are died by apoptosis in IBDV infected chickens. IBDV probably induces apoptosis indirectly in nonbursal organs (Eterradossi, 2001). IBDV induced protein VP5 plays the crucial role in the pathogenesis of IBD (Yao *et al.*, 1998) and the degree of intensity of apoptotic death is mediated by this protein (Yao *et al.*, 1998; Raue *et al.*, 2000). During the replication of IBDV in growing B lymphocytes the viral proteins induce apoptosis, resulting in a rapid depletion of B lymphocytes (Vasconcelos and Lam, 1995; Jungmann *et al.*, 2001).

2.5.2. Role of T cells in the pathogenesis

IBDV infection leads to the dramatic accumulation of T cells (Tanimura and Sharma, 1997; Kim *et al.,* 1999; Sharma *et al.,* 2001; Kim *et al.,* 2008) around the site of virus replication, concurrently to B cells depletion in the bursa (Kim *et al.,* 2000), but IBDV does not multiply within the T lymphocytes (Cursiefen, 1980). CD^{4+} and CD^{8+} cells are present in the bursa in similar proportion in the early infection, but later, mainly the CD^{8+} cells remain (Sharma *et al.,* 2000). Early after IBDV infection the role of bursal T cells are as follows:

- Expression of high levels of MHC class II and IL-2 receptors
- Elevated expression of cytokine genes like IFN-γ and IL-6 like factor
- Proliferation when stimulated *in vitro* with IBDV antigens but have a reduced response to T cell mitogens such as ConA (Sharma *et al.,* 2000)
- Inhibition of the mitogenic response of normal splenocytes by a soluble fact produced by themselves (Sharma *et al., 2001*) or CD^{4+} or CD^{8+} cells (Kim and Sharma, 2000)

In late stage of IBDV infection, bursal T cells play an important role in the recovery (Kim *et al.,* 2000). The possible role of IBDV on antigen presenting cells or impairment of T cells function need to be further investigated. Indeed, the effect of IBDV infection on cell-mediated immunity is still not fully understood (Eterradossi, 2001). IBDV modulates T cells function (Sharma *et al.,* 2001; Stocquart *et al.,* 2001).

Experimentally induced T cell immunodeficiency modulates the IBDV pathogenesis as follows (Kim *et al.,* 2000; Kautenschlein *et al.,* 2001; Sharma *et al.,* 2001):

- The viral antigen load in the BF becomes significantly higher.
- The severity of local inflammatory response in the bursa is increased.
- The incidences of apoptotic bursal cells are increased.
- The follicular recovery becomes significantly faster.

2.5.3. Role of chemokines in the pathogenesis

There are various chemical mediators such as IFN-γ (Kim *et al., 2000)*, TNFα (Klasing and Peng, 1990; Kim *et al.*, 1998), nitric oxide (NO) (Green *et al.*, 1982; Kim *et al.*, 1998), interleukins (Kim *et al., 1998)* that are produced by the biological interaction between IBDV and host cells. The acute IBDV infection induce the development of a septic shock like syndrome as in acute coccidiosis where IFN-γ (Yun *et al.*, 2000; Rothwell *et al.*, 2000) and TNFα (Zhang *et al.*, 1995) might play an important role in the onset of the clinical signs and be involved in the susceptibility to infection. Nitric oxide (NO), TNFα may promote the cellular destruction (Kim *et al.*, 1998) and ChIFNα is able to activate macrophages (Digby and Lowenthal, 1995; Karaca *et al.*, 1996). Excessive or insufficient production of cytokine may contribute significantly to the pathophysiology of the disease (Koghut, 2000).

2.5.4. Role of immune complexes in the pathogenesis

Previously the disease was recognized as avian nephrosis as because of its prominent kidney lesions (Cosgrove, 1962). Lodging of immune complexes in the glomeruli of IBDV infected chicks reveals its important role in the pathogenesis of IBDV infection in chickens (Ley and Yamamoto, 1979).

2.5.5. Role of bursal secretory dendritic cells (BSDC) in the pathogenesis

Principally, the BSDC plays the role in the transportation of IBDV to the different organs (Olah *et al.*, 2001).

2.5.6. General cyclic sequence of IBD

IBDV first infect the lymphocytes and macrophages of the gut-associated tissues (duodenum, jejunum, caeca) (Muller *et al.*, 1979b; Weis and Kaufer-Weis, 1994). These organs are considered as the organs of primary replication or organs of primary affinity. The virus containing cells or virus particles reach the BF, the target organ of IBDV (Kaufer and Weis, 1976), producing transient viremia (Winterfield *et al.*, 1972; Weis and Kaufer-Weis, 1994) and by way a considerable part of them are

phagocytized by kupffer cells of liver, but the virus materials are not trapped in the liver (Weis and Kaufer-Weis, 1994). Presumably the virus is first taken up by the follicle-associated epithelium (bursal tufts) and then reaches the medulla of the follicles (Kaufer and Weis, 1976). The failure of the electron microscope to demonstrate adsorption and uptake of the virions is due to the fact that the follicle-associated epithelium normally contains numerous vacuoles, filled with electron-densed granular material, making it almost impossible to identify phagocytized virus particles (Kaufer and Weis, 1976).

After entering into the follicles, the virus infect and replicate within the B lymphocytes (Nakai and Hirai, 1981; Muller,1986) and then a second and pronounced viremia occur with secondary replication in other organs leading to the development of the clinical signs and sometimes death (Weis and Kaufer-Weis, 1994; van den Berg, 2000).

2.5.7. Effect of IBDV on innate immunity

IBDV have been shown to modulate the macrophage function by altering the in vitro phagocytic activity (Lam, 1998). Macrophages from the infected chicken have upregulated cytokine gene expression and produce increased levels of NO (Kim, *et al.*, 1998).

2.6. PATHOLOGY

2.6.1. Organs affected

The principal target organ for pathogenic IBDV is the bursa of Fabricius (BF) (Cheville, 1967; Hirai and Calnek, 1979; Kaufer and Weis, 1980; Lukert and Saif, 1991; Tsukamoto *et al.*, 1995b; Tanimura *et al.*, 1995; Elankumaran *et al.*, 2001). The BF reaches the maximum development between 3-6 weeks of age and at this time chickens are most susceptible to the disease. The bursectomized chicken survives the IBDV infections lethal for normal chicken (Kaufer, 1980). But other lymphoid organs such as spleen (Rinaldi *et al.*, 1965; Cho and Edgar, 1972; Tanimura *et al.*, 1995; Islam *et al.*, 1997; Hoque *et al.*, 2001; Rudd *et al.*, 2001), thymus (Islam *et al.*, 1997; Hoque *et al.*, 2001; Rudd *et al.*, 2001; Okoye and Uzoukwu, 2001), caecal tonsils (Islam *et al.*,1997;

Elankumaran *et a1.*, 2001) and other non lymphoid organs like kidneys (Cosgrove, 1962; van der Sluis, 1994), liver (Chowdhury *et al.*, 1996; Islam *et al.*, 1997) are also affected.

2.6.2. Gross pathology

2.6.2.1. Bursa of Fabricius

The pathognomonic lesions of IBD are found in bursa and is characterized by swollen (Mohanty *et al.*, 1971; Chowdhury *et al.*, 1996; Islam *et al.*, 2008), oedematous (Chowdhury *et al.*, 1996; Czifra and Jonson, 1999; Islam *et al.*, 2008; Goud, *et al.*, 2009), haemorrhagic (van der Sluis, 1994; Chowdhury *et al.*, 1996; Haque *et al.*, 2001; Islam *et al.*, 2008; Goud, *et al.*, 2009) bursa , cheesy mass within the bursal lumen (Chowdhury *et al.*, 1996; Islam *et al.*, 2008) and finally, atrophy of the bursa (Jhala *et al.*, 1990; Chowdhury *et al.*, 1996; Islam *et al.*, 2008). The bursa/body weight ratios are lower than normal (Rosales *et al.*, 1989c; Thangavelu *et al.*, 1998). The degree of virulence is assessed by the measurement of bursa/ body weight indices and bursal damage (Mazariegos *et al.*, 1990). Chickens vaccinated with intermediate strain exhibit low B/BW indices (Mazariegos *et al.*, 1990). Chickens inoculated with bursa derived and tissue culture attenuated classical or variant serotypes have significantly smaller bursa and larger spleen than the uninoculated control (Hassan *et al.*, 1996).

2.6.2.2. Spleen

Spleen becomes swollen (Chowdhury, *et al.*, 1996), enlarged (Rinaldi *et al.*, 1965) or may become atrophied (Chowdhury *et al.*, 1996), sometimes mottling and paler than normal in appearance (Chowdhury *et al.*, 1996). Hemorrhages are common (Cho and Edgar, 1972; Hoque *et al.*, 2001) and small gray and whitish foci may be present (Rinaldi *et al.*, 1965; Ley *et al.*, 1979).

2.6.2.3. Caecal tonsil

Haemorrhages (Chowdhury, *et al.*, 1996) and partially damaged caecal tonsils are found in some cases (lslam *et al.*, 1997).

2.6.2.4. Thymus

Necrosis (Chowdhury, *et al.,* 1996), haemorrhages (Hoque, *et al.,* 2001), and opaque boiled meat appearance with a thickened, gelatinous connective tissue capsule and hyperemia on the surface (Cosgrove, 1962; Dongaonkar *et al.,* 1979) are found.

2.6.2.5. Kidneys

The kidneys become swollen (Ley *et al.,*1979; van der Sluis, 1994; Chowdhury, *et al.,*1996 ; van den Berg, 2000), paler than normal (Chowdhury, *et al.,*1996), mottled (Ley *et al.,* 1979). Inflammatory swelling of the ureters is caused by retention of urine and hydronephrosis (Weis and Kaufer-Weis, 1994). Kidneys with pronounced tubules, ureters filled with urates (Cosgrove, 1962), hyperemia, subcapsular haemorrhages and pronounced hydronephrosis (Somvanshi *et al.,* 1992) are also reported.

2.6.2.6. Liver

Congestion (Chowdhury *et al.,* 1996; Islam *et al.,* 1997), paler than normal in appearance (Chowdhury *et al.,* 1996) and occasionally with focal necrosis (Nunoya *et al.,* 1992; Islam *et al.,* 1997), swollen and streak appearance (Hanson, 1967) are reported.

2.6.3. Histopathology

2.6.3.1. Bursa of Fabricius

Varying degrees of lymphocytic depletion from the follicles (Islam *et al.,* 1997; van Loon *et al.,* 2001; Rautenschlein *et al.,* 2001; Rudd *et al.,* 2001; Hoque *et al.,* 2001; Franciosini and Coletti, 2001; Islam *et al.,* 2008), interfollicular oedema (Czifra and Jonson, 1999; Hoque *et al.,* 2001; Franciosini and Coletti, 2001; Islam *et al.,* 2008), heterophilic infiltration in the interfollicular space (Tanimura *et al.,* 1995) and also in the follicles (Hoque *et al.,* 2001), formation of purple coloured necrotic cellular mass within the follicles (Tanimura *et al.,* 1995; Islam *et al.,* 1997), fibroplasia surrounding the follicles (Hoque *et al.,* 2001), formation of cystic spaces within the follicles (Hoque *et al.,* 2001; Franciosini and Coletti, 2001; Islam *et al.,* 2008) as well as in the bursal

epithelium, haemorrhages and congestion in the bursa, thickness and oedematous serosa and finally follicular atrophy (Franciosini and Coletti, 2001) have been reported. Infiltration of macrophages in the follicles (Tanimura *et al.,* 1995) necrosis of lymphocytes with pyknotic and karyorrhectic nuclei (Islam *et al.,* 1997) in the follicles and varying degree of follicular regeneration were also recorded.

The pathogenicity and the degree of lesions varies according to the strain involved (Ley *et al.,* 1983; Rosales *et al.,* 1989a; Sharma *et al.,* 1989; Nunoya *et al.,* 1992). Depending on the residual virulence of the attenuated virus, some vaccine strains can also cause bursal damage (Mazariegos *et al.,* 1990) and induce immunosuppression (Muskett *et al.,* 1979; Edward *et al.,* 1982; Reece *et al.,* 1982). Highest bursal lesions score occur in chickens vaccinated with intermediate strain, followed by mildly attenuated strain (Mazariogos *et al.,* 1990; Tsukamoto *et al.,* 1995a). The intermediate strain caused extensive bursal damage but follicular repopulation was detected, whereas, there was absence of repopulation in chickens inoculated with virulent strain (Rautenschlein *et al.,* 2001).

The intermediate vaccine strain of IBDV caused lymphocytic depletion (Mazariegos *et al.,* 1990; Franciosini and Coletti, 2001), acute necrosis (Mazariegos *et al.,* 1990; Tsukamoto *et al.,* 1995a; Franciosini and Coletti, 2001; Rautenschlein *et al.,* 2001), follicular atrophy (Mazariegos *et al.,* 1990; Franciosini and Coletti, 2001), inflammation (Mazariegos *et al.,* 1990) and bursal damage (Tsukamoto *et al.,* 1995a; Rautenschlein *et al.,* 2001), cyst formation (Tsukamoto *et al.,* 1995a; Rautenschlein *et al.,* 2001; Franciosini and Coletti, 2001), and increase of interstitial connective tissue (Franciosini and Coletti, 2001).

2.6.3.2. Spleen

Histopathological appearance of the spleen of the IBDV infected birds are characterized as lymphocytic depletion with marked haemorrhages (Chowdhury *et al.,* 1996; Islam *et al.,* 1997), thickening of the arterial wall with fibrinoid degeneration

(Chowdhury *et al.*, 1996), eosinophilic tissue debris containing karyorrhectic nuclei of necrotic lymphocytes (Henry *et al.* 1980; Islam *et al.*, 1997), hyaline degeneration of the arterioles (Dongaonkar *et al.*, 1979), pronounced heterophilic infiltration in the sinusoids as well as in the germinal centres, round aggregations of eosinophilic materials surrounding the germinal centres (Henry *et al.*, 1980), periarteriolar lymphoid and periellipsoid lymphoid sheaths (Tanimura *et al.*, 1995) and splenic hyperplasia of the white pulp with cell death (Rautenschlein *et al.*, 2001).

2.6.3.3. Caecal tonsils

Varying degrees of lymphocytic depletion (Nunoya *et al.*, 1992; Tanimura *et al.*, 1995; Chowdhury *et al.*, 1996; Islam *et al.*, 1997), associated with severe haemorrhages (Islam *et al.*, 1997), macrophage and heterophilic infiltration (Tanimura *et al.*, 1995), hyperemia and reticular cells proliferation (Dongaonkar *et al.*, 1979) are found in the caecal tonsil of IBDV infected birds. The devoid lymphocytic elements of the caecal tonsils are replaced by macrophages and heterophils (Nunoya *et al.*, 1992).

2.6.3.4. Thymus

Moderate to severe lymphocytic depletion (Cheville, 1967; Cho and Edgar, 1972; Chowdhury *et al.*, 1996; Islam *et al.*, 1997) with presence of tissue debris and interlobular oedema (Nunoya *et al.*, 1992; Islam *et al.*, 1997), hyperemia and reticular cells proliferation (Dongaonkar *et al.*, 1979), presence of empty spaces in the cortex, heterophilic infiltration especially in the medulla, numerous round aggregations of cell debris and karyorrhectic nuclei in the cortex and medulla (Henry *et al.*, 1980) of thymus are found in Gumboro disease affected birds.

2.6.3.5. Kidneys

Degeneration (Cosgrove, 1962; Chowdhury *et a1.*, 1996), dissociation or sloughing of (Henry *et al.*, 1980; Chowdhury *et al.*, 1996) and coagulation necrosis (Chowdhury *et al.*, 1996) of the tubular epithelium; heterophilic infiltration but a few mononuclear leukocytes and some eosinophilic materials and cellular debris in the tubules; a large

oedematous space between many tubules and collecting ducts (Henry *et al.*, 1980) are found in the kidneys of IBDV infected birds.

2.6.3.6. Liver

Congestion in the central vein (Chowdhury *et al.*, 1996), fatty changes, necrosis of hypatocytes (Nunoya *et al.*, 1992; Chowdhury *et al.*, 1996) and dilatation of the sinusoids of the liver (Nunoya *et al.*, 1992) are reported.

2.7. CLINICO-PATHOLOGICAL OBSERVATIONS

Blood calcium level is significantly lower than normal (Cosgrove, 1962) in IBDV infected birds. Marked increase in serum gamma globulin (van der Sluis, 1994), markedly increased lactic dehydrogenase (Kumar and Rao, 1991; Nunoya *et al.*, 1992; van der Sluis, 1994), decreased alkaline phosphatase (Nunoya *et al.*, 1992), raised chlolesterol, creatine (Kumar and Rao, 1991), creating phosphokinase, glutamic oxaloacetate transaminase level (Nunoya *et al.*, 1992), decreased serum levels of glucose, uric acid and urea (Kumar and Rao, 1991), decreased total cholesterol and phospholipid (Nuroya *et al.*, 1992), but no significant changes in the serum electrolytes levels (Cosgrove, 1962) are reported.

Panleukopenia (van der Sluis, 1994), lymphopenia (Cosgrove, 1962; Asdrubali and Mughetti, 1972), leukocytosis with heterophilia (Chineme, 1977; Kumar and Rao, 1991), eosinopenia, monocytosis, basophilic, decreased haemoglobin and PCV values (Kumar and Rao, 1991), prolonged clotting time (Chineme, 1977; Kumar and Rao, 1991), prolonged prothrombin time (Kumar and Rao, 1991) are also the haematological pictures in the IBDV infected birds.

2.8. MECHANISMS OF IMMUNOSUPPRESSION

Reduction in the number of B cells in the BF due to viral infection is the major cause of immunosuppression. Suppression of B cell function might be caused by damage to helper T cells or other cells involved in generating the immune responses (Sharma, *et*

al., 1989). Chickens infected with IBDV have suppressor cells in the spleen, which cause in vitro mitogenic hyporesponsiveness to concavalin A. These cells prevent normal spleen cells from responding to the mitogen (Sharma, 1987). The impairment of T cells and development of suppressor cells (Sharma, 1987) was demonstrated in vitro by using proliferation tests or by measuring; the cytokine release after mitogen activation of T cells (Lambrecht, *et al.,* 2000).

Besides lymphocyte lysis, apoptosis also plays a role in immunosuppression (Vasconcelos, *et al.,* 1995; Lam, 1997; Ojeda, *et al.,* 1997; Tanimura, *et al.,* 1997, Tanimura, *et al.,* 1998). Apoptosis could occur in a variety of organs like thymus (Inoue, *et al.,* 1994) BF and spleen (Vasconcelos, *et al.,* 1995; Lam, 1997).

2.9. IMMUNOSUPPRESSIVE EFFECTS

IBDV drew the attention of avian virologists mostly because of its severe immunosuppressive effects (Allan *et al.,* 1972). Actively dividing (Lasher and Shane, 1994; Lukert and Saif, 1997; Nagarajan and Kibenge, 1997) or growing (Lukert and Saif, 1997) or differentiating (Hirai, 1979) or IgM bearing (Hirai and Calnek, 1979; Rodenberg *et al.,* 1994) B lymphocytes are the target cells of IBDV. Alteration of immunoglobulin production (Ivanyi and Morris, 1976) and significant depression of serum IgM level (Hirai *et al.,* 1979) were observed after infection, regardless the time of infection.

IBDV alters hosts immunological capacity, affecting humoral or cellular immune responses or both by destruction of the lymphoid elements of the bursa of Fabricius and sometimes of spleen, thymus and caecal tonsils (Hirai *et al.,* 1974 and 1979).

IBDV multiplies in the lymphocytes, macrophages, heterophils and reticular epithelial cells of the bursa (Mandell *et al.,* 1972; Kaufer and Weiss, 1980). IBDV does not multiply in T lymphocytes or in peripheral B lymphocytes (Cursiefen, 1980). Depression of the humoral antibody response in IBDV infected chickens (Allan *et al.,* 1972; Faragher *et al.,* 1974 and 1979) and the suppression of cell mediated immune response, as determined by lymphocyte transformation assay (Sivanandan and

Maheswaran, 1981) have already been documented. IBDV affects the Harderian gland influencing the local immune system (Dohms *et al.*, 1981; Rosenberger, 1994) but IBDV infection leads to the accumulation of T cells in the bursa, concurrently to B cell depletion (Kim *et al.*, 2000). Thus, IBDV infection causes immunosuppression and the immunosuppression ultimately leads to increase the incidence of many diseases (Table-2).

Table 2: Concurrent infections occurring during the course of IBD

Causal agent	Disease or concurrent infection	Reference(s)
Bacteria	*E.coli* infection or colisepticemia	Ahmed *et al.*, 1993; Singh *et al.*, 1994; Binta *et al.*, 1995; Igbokwe *et al.*, 1996
	Salmonellosis	Wyeth 1975; Binta *et al.*, 1995
	Infectious coryza	Ahmed *et al.*, 1993
	Hemophilus gallinarum infection	van der Sluis, 1994
	Staphylococcus aureus infection	Binta, *et al.*, 1995
	Gangrenous dermatitis	Rosenberger *et al.*, 1975
Virus	Newcastle disease	Yachida *et al.*, 1975; Binta *et al.*, 1995
	Infectious laryngotracheitis	Rosenberger *and* Gelb, 1978
	Infectious bronchitis	Giambrone *et al.*, 1977
	Marek's disease	Cho, 1970
	Inclusion body hepatitis	LiWeijen and Cho, 1980
	Chicken infectious anaemia	Clould *et al.*, 1992a and 1992b
Protozoa	Coccidiosis	Ahmed *et al.*, 1993; Singh *et al.*, 1994; Chowdhury *et al.*, 1996
Fungus	Aspergillosis	Chowdhury *et al.*, 1996
	Aflatoxicosis	Chang and Hamilton, 1982; Somvanshi *et al.*, 1992
Mycoplasma	*Mycoplasma synoviae* infection or mycoplasmosis	Gimabrone *et al.*, 1977; Binta *et al.*, 1995
Other	Haemorrhagic aplastic anaemia	Rosenberger and Gelb, 1978

2.10. IMMUNIZATION STRATEGIES AGAINST IBDV

IBD can be controlled by vaccination (Hitchner, 1971; Rosales *et al.*, 1989b; Ismail and Saif, 1991; Lukert and Saif, 1997), but the outbreaks in the vaccinated flocks are also reported elsewhere (van den Berg *et al.*, 1991; Eterradossi *et al.*, 1992; Muhammad *et al.*, 1996; Hafez *et al.*, 2002). Various vaccines against IBD are commercially available. Some vaccines were tested their protection level experimentally giving challenge with vvIBDV and both significant and insignificant increase of antibody titre were reported (Islam *et al.*, 2005). A high level of maternal antibodies will protect most young chickens against challenge by vvIBDV for up to 3 weeks after hatching (van den Berg 2000). The apparent inability to control IBDV infections through vaccination sometimes may be due to improper administration of vaccine virus, antigenic differences among the viruses (Rosenberger *et al.*, 1987; Snyder, 1990; Jackwood and Jackwood, 1997), insufficient potency of the live-attenuated vaccine virus (Ismail and Saif, 1991), interference between the residual maternally derived antibodies and the vaccine virus (Wyeth and Cullen, 1978; Lukert and Saif, 1997; Eterradossi, 2001).
The vaccine prepared from classical strain did not give protection against variant IBDV strains (Snyder, 1990). Again, the immunogenicity of the virus my differ between strain to strain (Rosales *et al.*, 1989a, b, c; Mazariegos *et al.*, 1990; Tsukamoto *et al.*, 1995a; Thangavelu *et al.*, 1998; Abdel-Alim and Saif, 2001).

The invasive vaccine strains are able to break through higher maternal antibody levels (Kouwenhoven and van den Bos, 1994). Therefore, vaccination during low maternally derived antibody titre shows better immune response than high maternal antibody titre (Giasuddin *et al.*, 2003). Moreover, the better protection with more virulent strains of IBDV is due to more antigenic stimulation based on higher and longer replication in lymphoid tissues (Rautenschlein *et al.*, 2001). There is no evidence of antigenic variation between classical and vvIBDV strains: and they belong to classical serotype I (van der Marel *et al.*, 1991; van den Berg *et al.*, 1991; Eterradossi *et al.*, 1992). No vaccine based on vvIBDV is yet commercially available, although the research work on the

development of a vaccine with vvIBDV is still going on (van Loon *et al.*, 2001; Abdel-Alim and Saif, 2001).

Table 3: General characteristics of live and inactivated vaccines for poultry

Live vaccines	Inactivated vaccines
Smaller quantity of antigen. Vaccination response relies on multiplication within the bird	Large amount of antigen. No multiplication after administration
Easily killed by chemicals and heat	Easier to store
Relatively inexpensive, easy to administer, and can be mass administered: drinking water, spray	Expensive to produce and to apply, since almost always individually administered
Adjuvanting live vaccines is not common	Adjuvanting killed vaccines is frequently necessary
Susceptible to existing antibody present in birds (e.g. maternal immunity)	More capable of eliciting an immune response in the face of existing antibody
In immune birds, booster vaccination is ineffective	In immune birds, additional immune response is frequently seen
Local immunity stimulated (i.e. trachea or gut)	Local immunity may be restimulated if used as a booster but secondary response is poor or absent
Danger of vaccine contamination (e.g. EDS)	No danger of vaccine contamination
Tissue reactions (commonly referred to as a 'vaccine reaction') are possible and frequently visible in a variety of tissues	No microbe replication; therefore, no tissue reaction outside that which is adjuvant dependent
Relatively limited combinations, due to interference of multiple microbes given at the same time (e.g. IB, ND and LT)	Combinations are less likely to interfere
Rapid onset of immunity	Generally slower onset of immunity

IB: infectious bronchitis, LT: laryngotracheitis, ND: Newcastle disease, EDS: egg drop syndrome

Recently, vvIBDV strains have adopted to grow in CEF cell culture by genetic engineering (Lim *et al.,* 1999; Islam *et al.,* 2001b; van Loon *et al.,* 2001 and 2002) and residual pathogenicity of one of these has been tested in SPF chickens (van Loon *et al.,* 2001).The inactivated vaccine made from the vvIBDV provided full protection against challenge with classical virulent strain as indicated by the low bursa/body weight ratio (Abdel-Alim and Saif, 2001).

2.10.1. Factors Which Interfere With Immunization of Commercial Poultry Farm

Factors which interfere with immunization of commercial poultry can be divided into three main groups (PaulMcMullin, 1985). They are:

- Factors associated with the vaccine itself,
- Those of vaccine administration, and
- Those which are endogenous to the bird.

2.10.1.1. The vaccine itself

All of the factors associated with the vaccine itself tend to be closely inter-related. A deficiency in one can be partially compensated by another. A vaccine of moderate-to-poor titre may give satisfactory results if very carefully applied, while it may be a disaster if poorly applied.

2.10.1.1.1. Vaccine Quality

Vaccine quality is sometime blamed when antibody titers are insufficient or disease breaks out in a flock. However, evidence shows that in the great majority of cases, vaccines are of excellent quality and are not responsible for the failure. To allay concerns about vaccine quality, purchase only from reputable pharmaceutical companies whose products are manufactured under stringent quality control practices.

Table 4: Factors which interfere with vaccine efficacy in poultry

Type of factor	Impact on vaccine efficacy
Factors associated with the vaccine itself	
Virus serotype	Many infectious agents (e.g. infectious bronchitis virus) have different serotypes, and vaccine antigens do not provide protection against all field strains
Level of protection	Field strain of very high virulence, and/or highly attenuated vaccine strains
Factors associated with vaccine administration	
Handling	Certain live vaccines (e.g. live cell-mediated Marek's disease vaccines) are easily killed if mishandled
Diluent used	Viable vaccines administered in drinking water are destroyed if water sanitisers are not removed
Route	Vaccines administered by injection fail if vaccinators do not deliver the vaccine to the appropriate vaccination site
	Mass vaccination (drinking water and aerosol) tends towards lower uniformity than individual administration
Associations	Administration of certain combinations of live virus vaccines affects the single virus response if they have the same target tissues
Factors associated with the bird/flock	
Maternal immunity	In presence of high levels of maternal antibodies, live vaccines administered during the first two weeks of life may be neutralised
Immunosuppression	Stress, certain infectious agents (e.g. infectious bursal disease, infectious anaemia and Marek's disease in chickens, haemorrhagic enteritis in turkeys), mycotoxins (in particular aflatoxins) impair immune response
Sanitary status	The birds are already infected (incubation period) with the pathogen against which the vaccination is directed
Genetic factors	Different types of vaccine responses with respect to species or commercial hybrids
Management conditions	
Hygienic practices	Without clean-out and disinfection over successive flocks, the challenge dose might be too high or infection might occur too soon

2.10.1.1.2. Vaccine Modifications

Commercial poultry companies may try to reduce costs by eliminating vaccines or administering partial doses. The decision to vaccinate is based on a risk analysis assessment. If the disease is not present, do not vaccinate. If it is a risk, the vaccine must be administered according to the recommendations of the manufacturer. When partial doses are given, birds will not get enough vaccine to properly stimulate their immune system. The result will be decreased resistance to disease.

2.10.1.1.3. Titre and Stability

It is self-evident that the live-virus vaccines must have an adequate titre and this titre must have sufficient stability so that under normal conditions it can cause an infection of appropriate intensity. Stability of live-virus vaccines is affected by the success of lyophilization and the temperature under which it is stored. Periods of validity must be strictly followed, or the vaccine re-titrated.

2.10.1.1.4. Inactivation and Adjuvant

These factors have similar importance for inactivated vaccines as do lyophilization and titre for live vaccine. Type and quality of emulsion can influence the serological response to oil-adjuvant vaccines.

2.10.1.2. Administration of the Vaccine to the Bird

2.10.1.2.1. Administration and Handling of the Vaccine

A well designed vaccination program will not be effective if the vaccine is damaged by improper handling prior to administration. Live vaccines can be inactivated when exposed to adverse conditions. Once a vaccine is reconstituted, the "time clock is ticking" for it to be used.

2.10.1.2.2. Vaccine Administration Deficiencies

Improper vaccine administration of the vaccine is the most common cause of vaccine failure in poultry. Prior to application of the vaccine, the details of the whole process

must be well planned. This includes ensuring that the crew is trained in handling and applying the vaccine. The results of proper vaccination will be improved disease control and performance of the poultry. As one poultry grower commented, "Vaccines are no good if they do not get into the chicken."

2.10.1.2.3. Time of administration

Chickens may also already be incubating the disease at the time of vaccination. Despite proper administration, the birds become diseased because time is needed for antibody production to reach protective levels. Following first exposure to a live virus vaccine, antibody type G is detected approximately four to five days following exposure. Additional days are required for titres to reach protective levels.

2.10.1.2.4. Uniformity

In ultimate analysis it is essential that the antigen present in the vaccine is uniformly distributed within the flock. The use of "mass vaccination" (drinking water and aerosol) tends towards less uniformity in application than individual application, and need considerable operator care in order to control this tendency.

2.10.1.2.5. Association

Administration of certain combinations of live virus vaccines may affect the response to each virus, especially when they contain viruses which have the same target tissues. It should be remembered, however, that in industrial poultry production the aim is maximum productivity and not necessarily maximum protection against a given virus.

2.10.1.2.6. Vaccination Program

Each region typically has its own specific diseases. Thus it is not wise to try to develop a "one size fits all" or international vaccination program. In areas with a high density of poultry production, small flocks in close proximity to commercial flocks, or where

farms have poor biosecurity and management practices, more comprehensive and intensive vaccination programs may be necessary.

2.10.1.2.7. Diluent

The diluent used for live virus vaccines is very important to ensure that an adequate titre of virus actually reaches the birds. The classical problem of administering live-virus vaccine in chlorinated drinking water is well known, but less extreme.

2.10.1.3. Birds (endogenous factors)

The importance of adequate priming by prior exposure to the agent has been discussed above. It could be added that repeated exposure over too short a period may not be advantageous. Common practice dictates that the same vaccine should not be re-applied to a flock within 14 days.

2.10.1.3.1. Passive protection

Circulating antibody may affect the response to vaccination, even independently from the previous factor, i.e. when it is not produced by the bird itself. This may come about in two ways. Hyperimmune antiserum may be injected to provide passive protection but this is rarely used in commercial poultry today. The commonest source of passive protection is that transmitted from the breeder bird to her chick via the yolk. The baby chick has circulating antibodies in similar concentrations to those found in the breeder at 1-3 days of age. They fall to undetectable titres by 14 - 30 days (depending on the method of detection used). Aerosol vaccination of day-old chicks from immune parents against infectious bronchitis produces immunity as good as birds vaccinated at 15-20 days.

2.10.1.3.2. Immunosuppression

Stress of any sort is well known to reduce disease resistance and can also be expected to affect response to vaccination. Exceptionally poor environmental conditions could contribute to vaccination failure under some circumstances. The three infectious

agents most associated with the immunological system and most capable of producing immunosuppression are infectious bursal disease (Gumboro) virus, Chick Anaemia Virus and Marek's disease virus.

2.10.1.3.3. Maternal Antibodies

The immune status of the breeder flock can have an affect on the success of progeny vaccination. If the breeder flock has high levels of circulating antibodies which pass to the progeny through the egg, they may interfere with the replication of live vaccine viruses as they would for field challenge viruses. This will decrease the immune response to the vaccine because it is not stimulating the immune system as long and to as great an extent. For example, if a chick comes from a breeder hen with high levels of antibody against Infectious Bursal Disease (IBD), the chick will typically have high levels of maternal antibodies for several weeks. If vaccination is attempted in the presence of these antibodies, some of the vaccine virus will be neutralized and a decreased response to the vaccine results. On the other hand, delaying vaccination until maternal antibodies have been catabolized may leave the birds susceptible to field challenge.

2.10.1.4. Management Practices

Poor management practices in poultry flocks may contribute to vaccine failures. If infectious disease agents are allowed to build up in successive flocks without prior decontamination, it is possible that the challenge dose of a particular infectious agent will be large enough so that a normally effective vaccination program will be overwhelmed. In the long run, vaccines cannot replace a good management program.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. EXPERIMENTAL CHICKENS

500 unvaccinated Cobb-500 Day Old Chicks (DOC) received from the "Nourish poultry and Hatchery Ltd." by "Polash Poultry Farm" were considered as the experimental chickens.

A typically affected flock was also included in the present study to compare the results and the birds brought at the Laboratory of the Department of Pathology and Parasitology under Hazee Mohammad Danesh Science and Technology University for the diagnosis and treatment of diseases was also included.

3.2. RESEARCH AREA

Poultry farming and vaccination against IBDV was done in the above mentioned farm placed at Syedpur of Nilphamari district the chickens were collected following experimental schedule and laboratory examination was done at the Department of Pathology and Parasitology of Hajee Mohammed Danesh Science and Technology University, Basherhat, Dinajpur.

3.3. EXPERIMENTAL PERIOD

The duration of the experiment was one year from June 2009 to May 2010.

3.4. EXPERIMENTAL DESIGN

Table 5: Experimental design

Sampling occasion	Vaccination Status	No. of birds for Necropsy	Parameters studied
Primary vaccination with live freeze dried form of GM97 strain of IBDV (HIPRA GUMBORO®-GM97)			
Day 11	-	3	Clinical signs and symptoms
Day 13	2 (DPV)	3	Gross morbid lesions
Day 15	4 (DPV)	3	Bursa – body weight ratios
Day 17	6 (DPV)	3	Histopathology Bursa lesion scores
Day 17 Boosting with live freeze dried form of GM97 strain of IBDV (HIPRA GUMBORO®-GM97)			
Day 20	3 (DPB)	3	Clinical signs and symptoms
Day 23	6 DPB	3	Gross morbid lesions
Day 26	9 DPB	3	Bursa – body weight ratios
Day 23 (Affected flock)	-		Histopathology

3.5. MANAGEMENT OF CHICKENS

The birds were maintained in relative isolation. The shed was made by rice straw and floor was constructed with brick. The shed was "open sided" and East-west in position. The room was thoroughly cleaned by sweeping and then washing with tap water using hose pipe connected with a tap. The room was disinfected with a household phenolic disinfectant (Phenyl) and fumigates the room. Optimum temperature in the brooder house was maintained using electric bulbs in required number and at required distances. Rice husk was the litter material which was placed 2-3 inch depth and it was replaced following wetting either by faeces or water or by both. For the first week white paper was placed in the brooder which was replaced

regularly. Feeding and watering was adlibitum. For the first two days birds were maintained on suji (a coarse flour of wheat). Which was then replaced by commercial broiler starter and grower feed accordingly. In addition electrolyte and vitamin were given in water time to time. Entry to the house was restricted. Wearing rubber boots and dipping boots in disinfectant foot bath were compulsory for the visitors during entry and exit. The measurement was taken so that the wild animals & wild birds could not enter in to farm and spray the vehicles before entering in to the farm.

3.6. VACCINES AND VACCINATION

The vaccine used in this study was a commercial, manufactured modified live virus vaccine, obtained directly from the veterinary product saler and stored at -40ºC until used. The vaccine was administered according to the manufacturer's recommendations.

HIPRA GUMBORO®-GM97: A freeze-dried culture of the Live Gumboro virus, GM97 strain, containing minimum 10_2-10_3 EID_{50} (per dose) (Hipra Company).

Pack size: 100, 500, 1000 and 5000 dose.

Administration route

Administration route orally. 10 ml of drinking water was Injected to break the vacuum of the vial; shaked gently until complete resuspension of the freeze-dried tablet before administration.

Dosage

The optimum age for vaccination may be calculated using the level of maternal antibodies in the chicks at day old, but normally lies in the range of 12-18 days. At least 18 birds from the same batch was used.

The veterinary surgeon will establish the most suitable vaccination programme according to the health conditions of each farm and area.

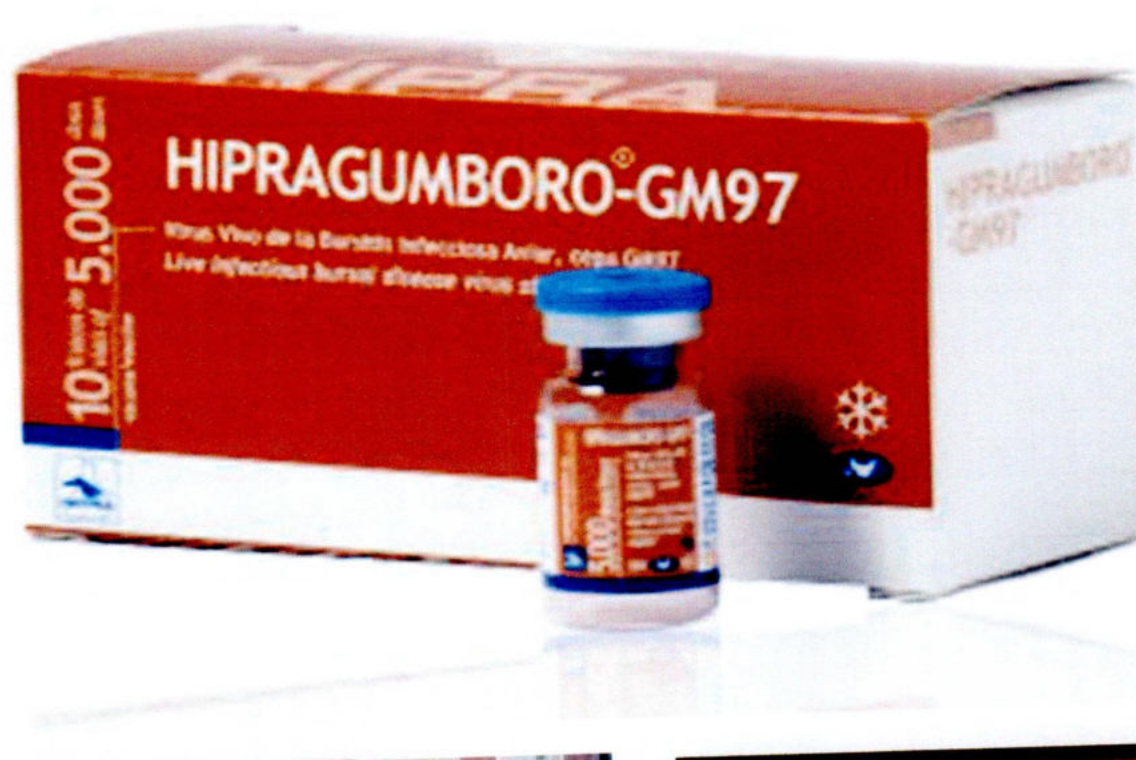
If the number of birds does not coincide with the number of doses of available vials, an overdose should be administered, never administer less than the recommended dosage.

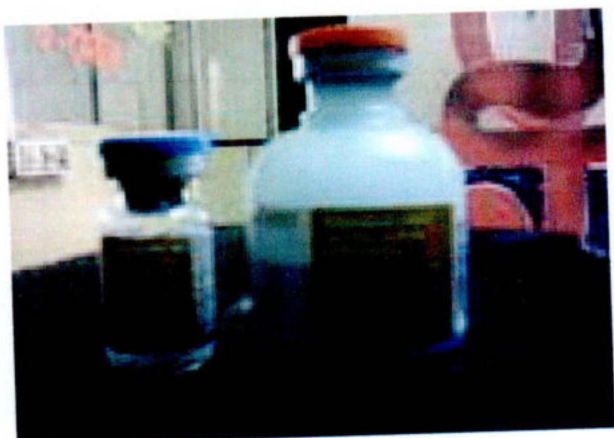
Special precautions

Due to residual pathogenicity in the bursa of Fabricius, the vaccine should only be used in areas already contaminated with vvIBDV, except in batches of infected chickens that show clinical symptomatology.

The vaccinal strain is transmitted to non-vaccinated chickens.

Store at +2 to +8 °C, protected from light.





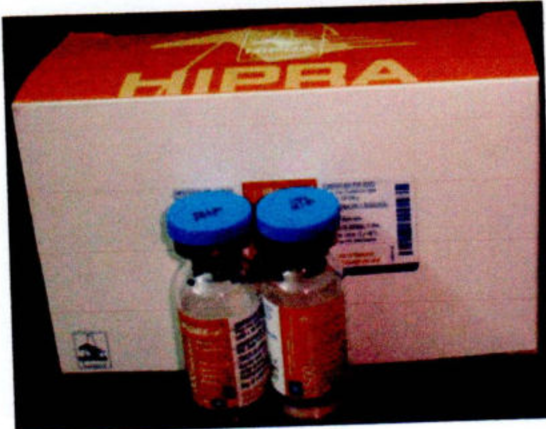


Figure 1: Vaccine vial.

3.7. SAMPLING OCCASION

The birds were collected from the flock for laboratory examination as per as experimental design.

3.8. NECROPSY

Necropsy of birds obtained from "Polash Poultry Farm". The necropsies of the experimental birds were done following a standard procedure (Charlton, 2000).

3.9. BURSA-BODY WEIGHT (B/BW) RATIO

Each bird was weighed before killing. The bursa of Fabricius was weighed and the average B/BW ratio was determined by the formula of Tanimura *et al.*, (1995) as following:

$$\text{B/BW ratio} = \frac{\text{Bursal weight in grams}}{\text{Body weight of individual bird in grams}} \times 1000$$

Here, B= Bursa; BW= Body weight.

3.10. HISTOPATHOLOGICAL STUDY

During necropsy, Bursa of Fabricius was collected, fixed in 10% buffered neutral formalin for histopathological studies. Formalin fixed tissue samples were processed and stained as per standard method (Luna, 1968).

3.10.1. Materials required for histopathology

Equipment and appliances:

- Samples (Bursa of Fabricius)
- 10% formalin
- Chloroform
- Paraffin
- Mounting media (DPX)
- Microscope
- Cover slips
- Alcohol
- Tape water
- Xylene
- Hematoxylin and Eosin stain
- Distilled water
- Clean slides

3.10.2. Processing of tissue for histopathology

3.10.2.1. Collection of tissue and Processing

During tissue collection the following point were taken into consideration-

The Bursa of Fabricius was collected in conditions as fresh as possible. The thickness of the tissues were as less as possible (5mm approximately).

The Bursa of Fabricius was collected from the experimental birds in the Histopathology Laboratory of Department of Pathology and Parasitology, HSTU, Dinajpur.

Fixation: 10% formalin was added in the plastic container. (10 folds of the tissue size and weight) and fixed for 3-5 days.

Washing: The tissues were trimmed into a thin section and washed over night in running tap water to remove formalin.

Dehydration: The tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per following schedule.

- 50% alcohol: one hour
- 70% alcohol: one hour
- 80% alcohol: one hour
- 95% alcohol: one hour
- Absolute alcohol: three changes (one hour for each changes)

Cleaning: the tissues were cleaned in chloroform for 3 hours to remove ethanol (1 and half hr in each, two changes).

Impregnation: Impregnation was done in melted paraffin (56- 60°C) for 3 hours.

Embedding: Paraffin blocks containing tissue pieces were made using templates and melted paraffin.

Sectioning: Then the tissues were sectioned with a microtome at 5-6μm thickness. The sections were allowed to spread on luke warm water bath (40-45 °C) and taken on a glass slide. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The slides containing sections were air dried and stored in cool place until staining.

3.10.2.2. Routine Hematoxylin and Eosin staining procedure

Preparation of Ehrlich's Hematoxylin solution

Components	Amount
Hematoxylin crystals	4.0 g
Alcohol, 95%	200.0 ml
Ammonium or potassium alum	6.0 g
Distilled water	200.0 ml
Glycerine	200.0 ml
Glacial acetic acid	20.0 ml

Hematoxylin is dissolved in the alcohol and the alum is dissolve in distilled water and mixed thoroughly. After these are in complete solution the glycerin and acetic acid are added.

Preparation of eosin solution (1% stock alcoholic eosin)

Components	Amount
Eosin Y, water soluble	1 g
Distilled water	20 ml
95% alcohol	80 ml

Eosin was dissolved in water and then 80 ml of 95% alcohol was added.

Working eosin solution

Components	Amount
Eosin stock solution	1part
Alcohol, 80%	3 parts

0.5ml of glacial acetic acid was added to 100 ml of working eosin solution just before use.

Staining protocol

The sectioned tissues were stained as described bellow:

- ❖ The sectioned tissues were deparaffinized in three changes of xylene (three minutes in each)
- ❖ Then the sectioned tissues were rehydrated through descending grades of alcohol as per following schedule-
    - o Absolute alcohol: three changes (three minutes for each)
    - o 95% alcohol: two minutes
    - o 80% alcohol: two minutes
    - o 70% alcohol: two minutes
- ❖ Dipping with distilled water for 10 minutes
- ❖ The tissues were stained with Ehrlich's Hematoxylin for 2-10 minutes
- ❖ Washed in running tap water for 10-15 minutes
- ❖ Then the tissues were dipped in ammonia water (few dips)
- ❖ Stained with eosin for one minute
- ❖ Differentiated and dehydrated in ascending grade of alcohol
- ❖ 95% alcohol – three changes (2-4 dips for each)
- ❖ Absolute alcohol – three changes (2-3 minutes for each)
- ❖ Cleaned in xylene - three changes (five minutes each)
- ❖ Tissues were mounted with cover slip by using DPX
- ❖ The slides were dried at room temperature and examined under a low (10X) and high (40X, 100X) power objectives.

CHAPTER IV

RESULTS

4.1. CLINICAL MANIFESTATIONS OF THE VACCINATED FLOCK

There was no remarkable clinical signs & symptoms in birds of the vaccinated flock.

4.2. NECROPSY/GROSS MORBID LESIONS

Necropsies of the birds were done thoroughly. The organs, such as- bursa of Fabricius, spleen, caecal tonsil, thymus, Kidneys, liver, thigh and breast muscle, junction of proventriculus and gizzard were examined properly, but there was no any pathological lesions. In case of bursa of Fabricius, the size and weight of the bursae were variable according to the birds and age of the birds (Table- 6).

A typically affected flock was also included in the present study to compare the results and the birds brought at the laboratory of the Department of Pathology and Parasitology for the diagnosis and treatment of diseases was also included.

The lesions that were found in affected flock includes- swollen, oedematous, haemorrhagic bursa , cheesy mass within the bursal lumen and finally, atrophy of the bursa; Spleen becames swollen and enlarged; Haemorrhages and partially damaged caecal tonsils; liver was congested and paler than normal in appearance; varying degrees of haemorrhages was found in the thigh and/or breast muscles, skeletal muscles was darkly discoloured and haemorrhages also found at the junction between the gizzard and proventriculus.

4.3. BURSA/BODY WEIGHT RATIOS

The Bursa/Body weight (B/BW) ratio were determined on D_{11}, D_{13}, D_{15}, D_{17}, D_{20}, D_{23} and D_{26}, including affected flock and the results were presented in Table- 6.

4.4. STATISTICAL ANALYSIS OF BURSA/BODY WEIGHT RATIO

Statistical analysis of Bursa/Body weight ratio is presented in Table- 7.

4.5. HISTOPATHOLOGY

The histopathological lesions were in general characterized by

- Most bursal follicles were apparently normal which were histologically characterized as unifomly cellular concentration in the follicles.
- Mild depletion of lymphoid cells were also found in some follicles in the same examined bird.
- Moderate depletion of lymphoid cells were found in few bursal follicles.
- Severe lymphoid depletion of lymphoid cells were also found in fewer follicles.
- Follicular atrophy without the development of follicular cysts were also observed, but this histopathological characteristics was greatly marked in the flocks showing typical outbreak of Gumboro.

Table 6: Bursa-Body weight ratio at day $_{11}$, day $_{13}$, day $_{15}$, day $_{17}$, day $_{20}$, day $_{23}$ and day $_{26}$

Sampling occasion	Birds No.	Live body weight (gm.)	Bursal weight (gm.)	Bursa-Body Weight ratio	Average
Day $_{11}$	1	272	0.6	2.21	1.99
	2	295.1	0.5	1.69	
	3	291	0.6	2.06	
Day $_{13}$	1	412.4	1.2	2.91	2.98
	2	348	1.1	3.16	
	3	383.1	1.1	2.87	
Day $_{15}$	1	472.7	1.5	3.17	3.19
	2	509.1	1.6	3.14	
	3	522.4	1.7	3.25	
Day $_{17}$	1	682.6	2.1	3.08	2.44
	2	570.7	1.1	1.93	
	3	520.2	1.2	2.31	
Day $_{20}$	1	826.7	2.1	2.54	2.45
	2	777.5	2.0	2.57	
	3	847.4	1.9	2.24	
Day $_{23}$	1	910.3	1.6	1.76	2.24
	2	949.5	1.8	1.89	
	3	851.2	2.6	3.06	
Day $_{26}$	1	930.1	1.2	1.29	2.38
	2	1201.1	3.7	3.08	
	3	1077.8	3.0	2.78	
Day $_{23}$ (Affected flock)	1	950	2.5	2.63	2.45
	2	900	2.1	2.33	
	3	875	2.1	2.4	

Table 7: Statistical analysis of Bursa/Body weight ratio of chickens at different age

Bursa body weight (B/BW) ratio								
Sampling occasion (Day)	**Day $_{11}$**	**Day $_{13}$**	**Day $_{15}$**	**Day $_{17}$**	**Day $_{20}$**	**Day $_{23}$**	**Day $_{26}$**	**Day $_{23}$ (Affected flock)**
Mean±SE	1.99±0.15	2.98±0.09	3.19±0.03	2.44±0.34	2.45±0.11	2.24±0.41	2.38±0.55	2.45±0.09
P Value	0.0044	0.0256	0.2144	0.0129	0.0051	0.0041	0.0036	0.1024
Level of significance	**	*	NS	**	**	**	**	NS

NS = Not Significant (P>0.05)

** = Significant (P<0.01)

* = Significant (P<0.05)

4.7. STATISTICAL ANALYSIS OF BURSAL LESION SCORING

Statistical analysis of Bursal lesion scoring is presented in Table-9.

Table 9: Statistical analysis of Bursal lesion scoring

Sampling occasion	Bursal lesion score	Mean
Day $_{11}$	0, 1, 1	1.33±0.33
Day $_{13}$	0, 1, 1	1.33±0.33
Day $_{15}$	1, 2, 3	2.00±0.58
Day $_{17}$	0, 1, 1	0.67±0.33
Day $_{20}$	1, 1, 1	1.33±0.33
Day $_{23}$	0, 1, 1	1.33±0.33
Day $_{26}$	0, 0, 1	1.33±0.33
Day $_{23}$ (Affected flock)	3, 3, 4	3.33±0.33
P Value	0.0003	
Level of significance	**	

NS = Not Significant (P>0.05)

** = Significant (P<0.01)

* = Significant (P<0.05)

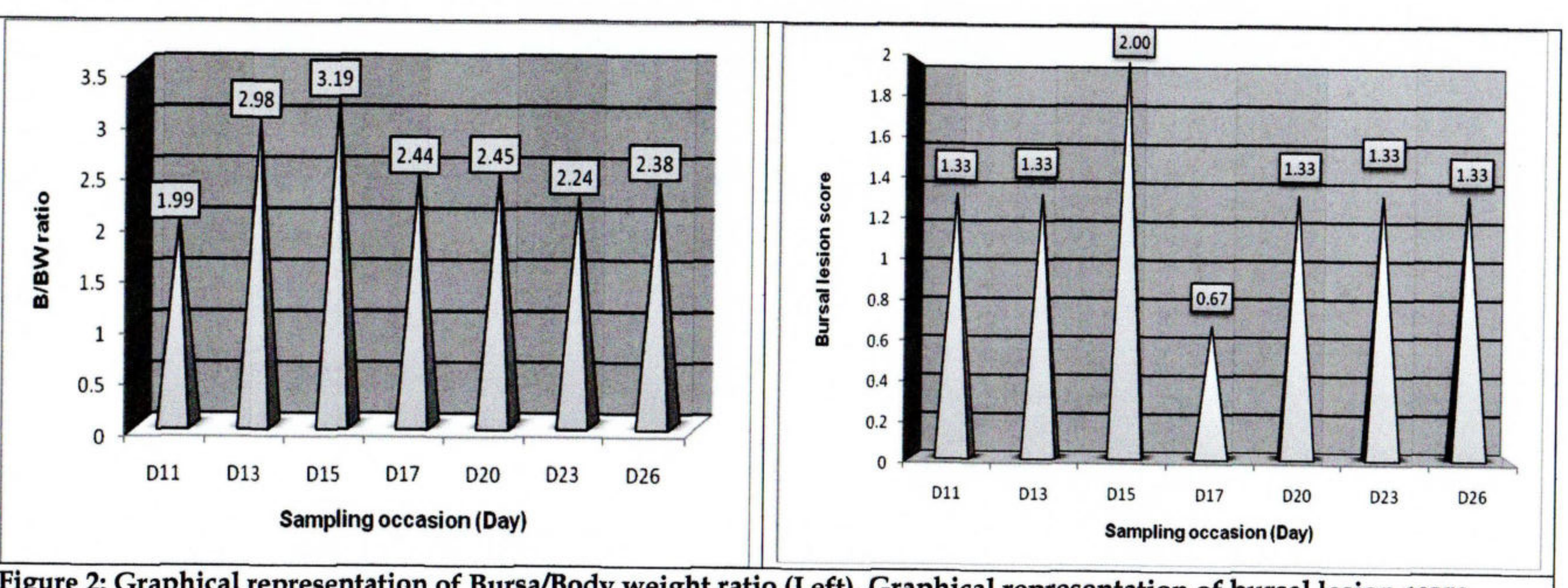


Figure 2: Graphical representation of Bursa/Body weight ratio (Left). Graphical representation of bursal lesion score (Right).

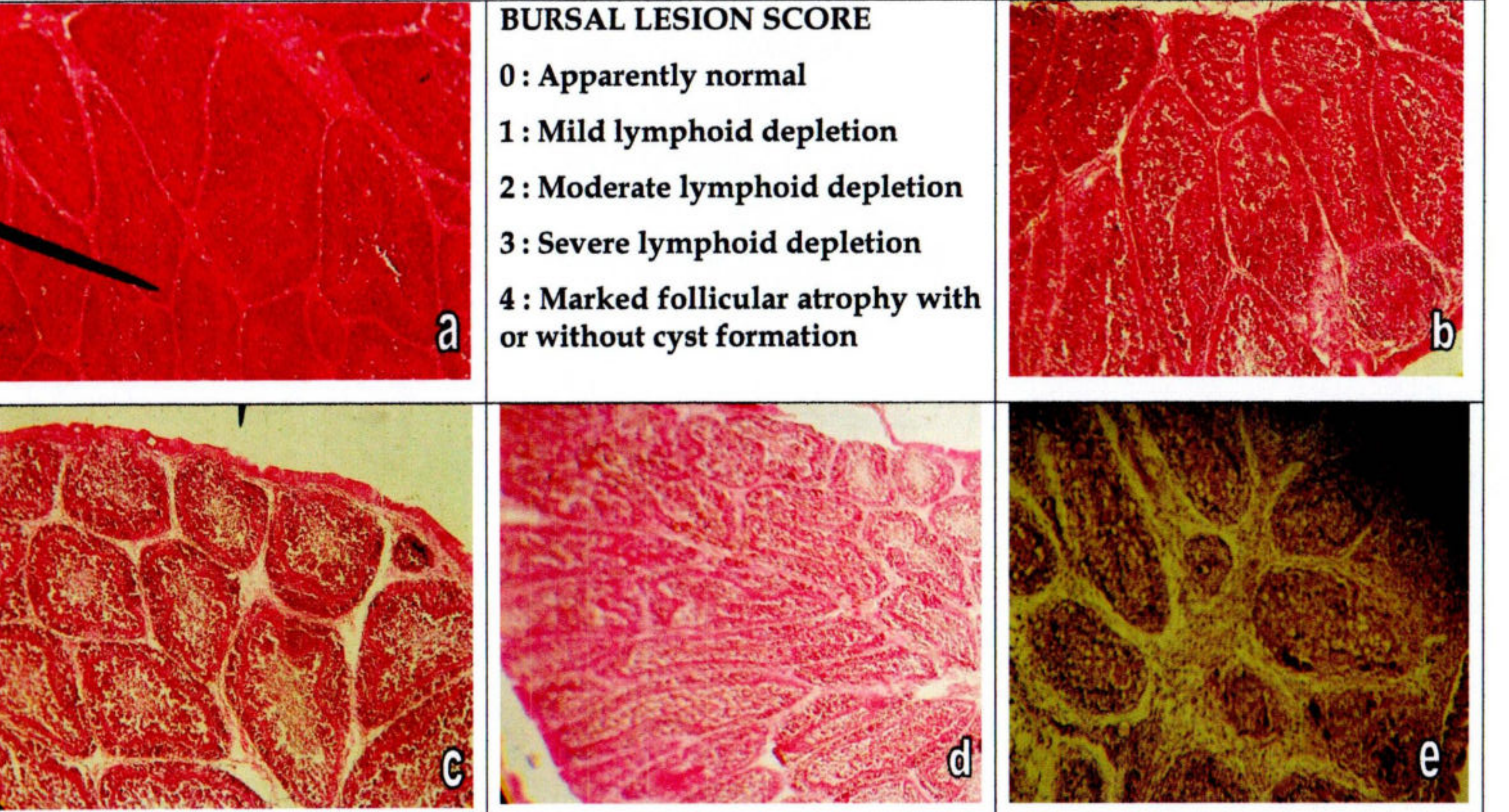


Figure 3 : Criteria of bursal lesion scores: a) apparently normal (score 0), b) Mild lymphoid depletion (score 1), c) Moderate lymphoid depletion (score 2), d) Severe lymphoid depletion (score 3), and e) Marked follicular atrophy with or without cyst formation (score 4).

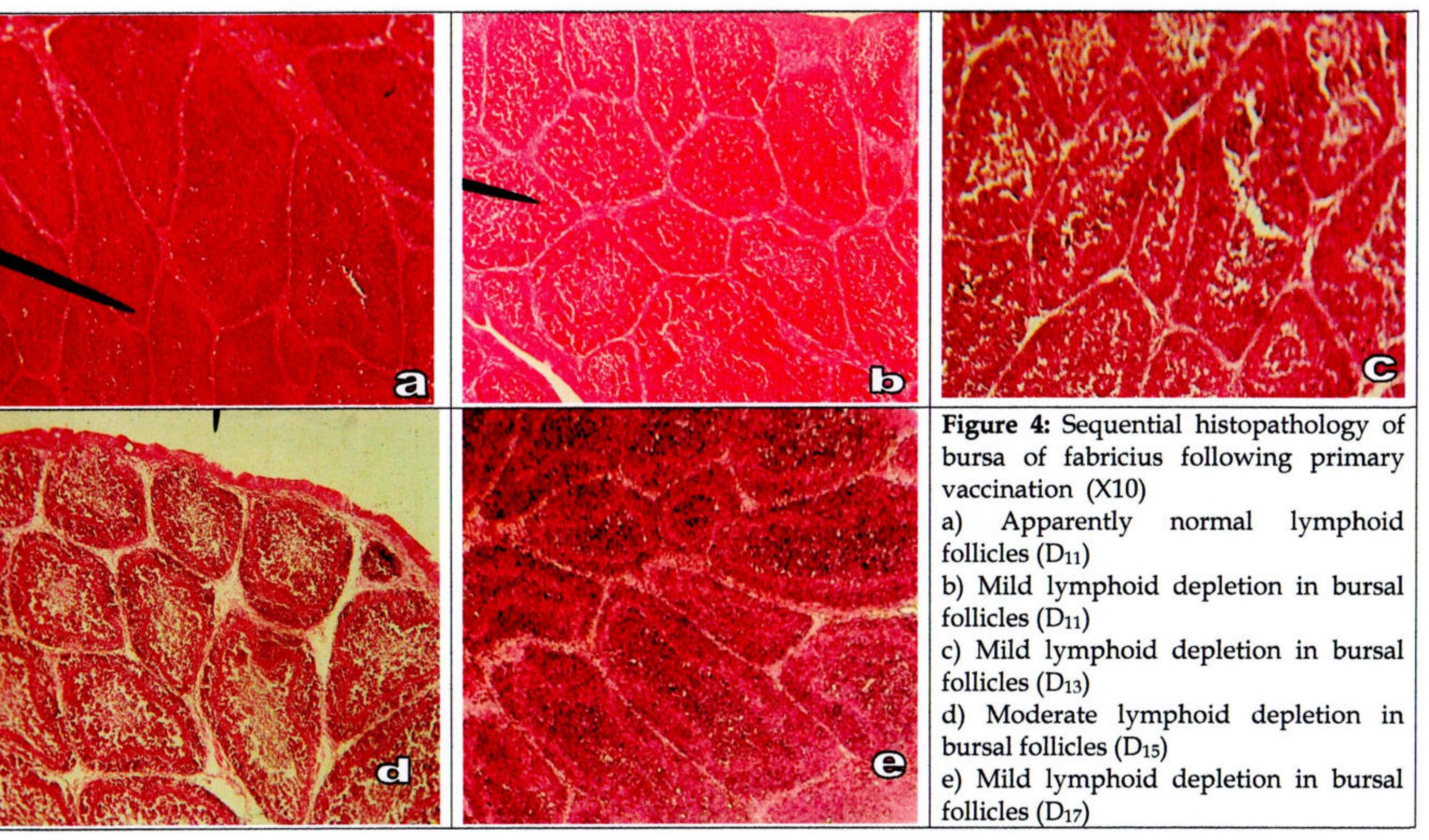


Figure 4: Sequential histopathology of bursa of fabricius following primary vaccination (X10)

a) Apparently normal lymphoid follicles (D_{11})

b) Mild lymphoid depletion in bursal follicles (D_{11})

c) Mild lymphoid depletion in bursal follicles (D_{13})

d) Moderate lymphoid depletion in bursal follicles (D_{15})

e) Mild lymphoid depletion in bursal follicles (D_{17})

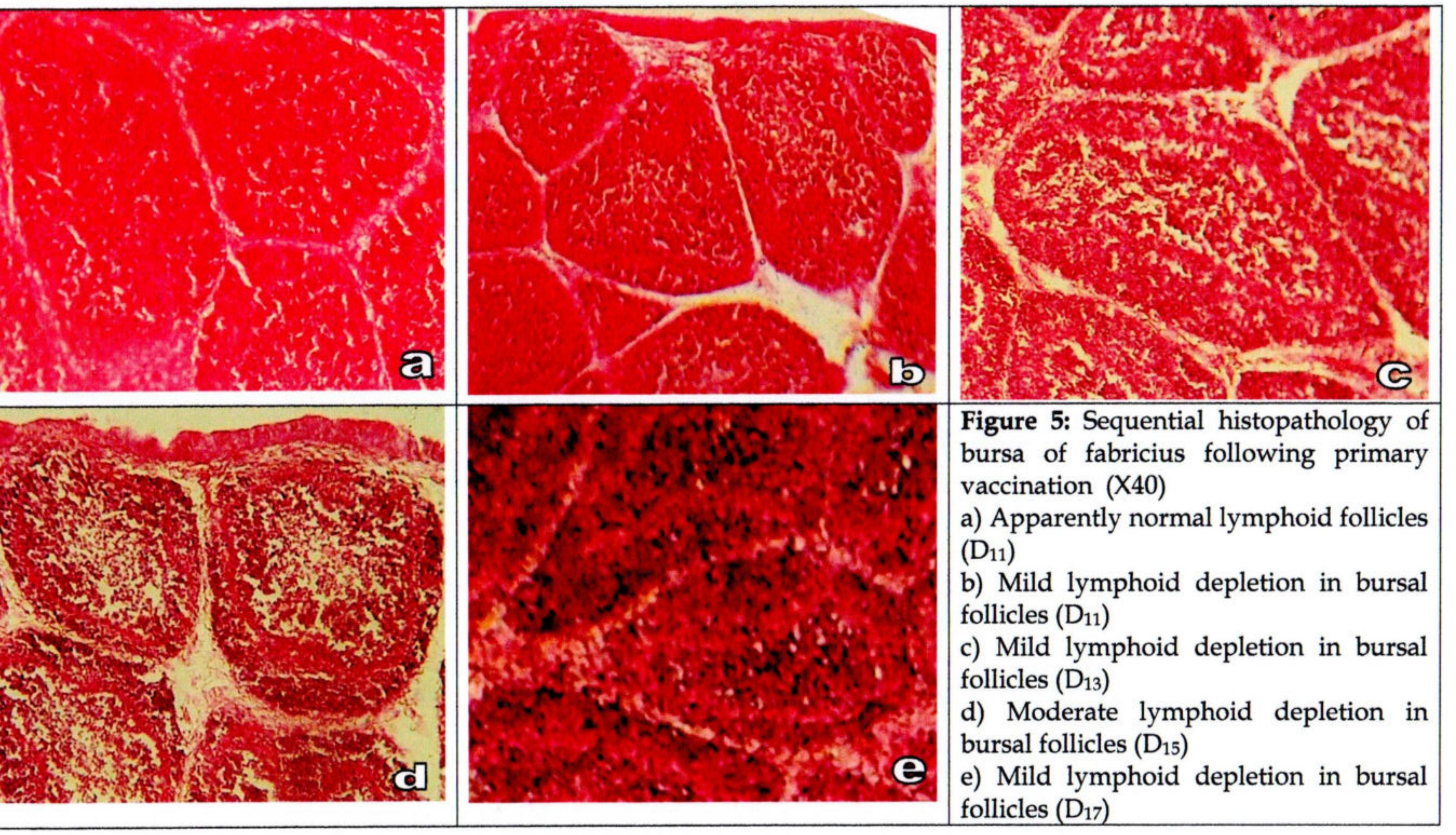


Figure 5: Sequential histopathology of bursa of fabricius following primary vaccination (X40)

a) Apparently normal lymphoid follicles (D_{11})

b) Mild lymphoid depletion in bursal follicles (D_{11})

c) Mild lymphoid depletion in bursal follicles (D_{13})

d) Moderate lymphoid depletion in bursal follicles (D_{15})

e) Mild lymphoid depletion in bursal follicles (D_{17})

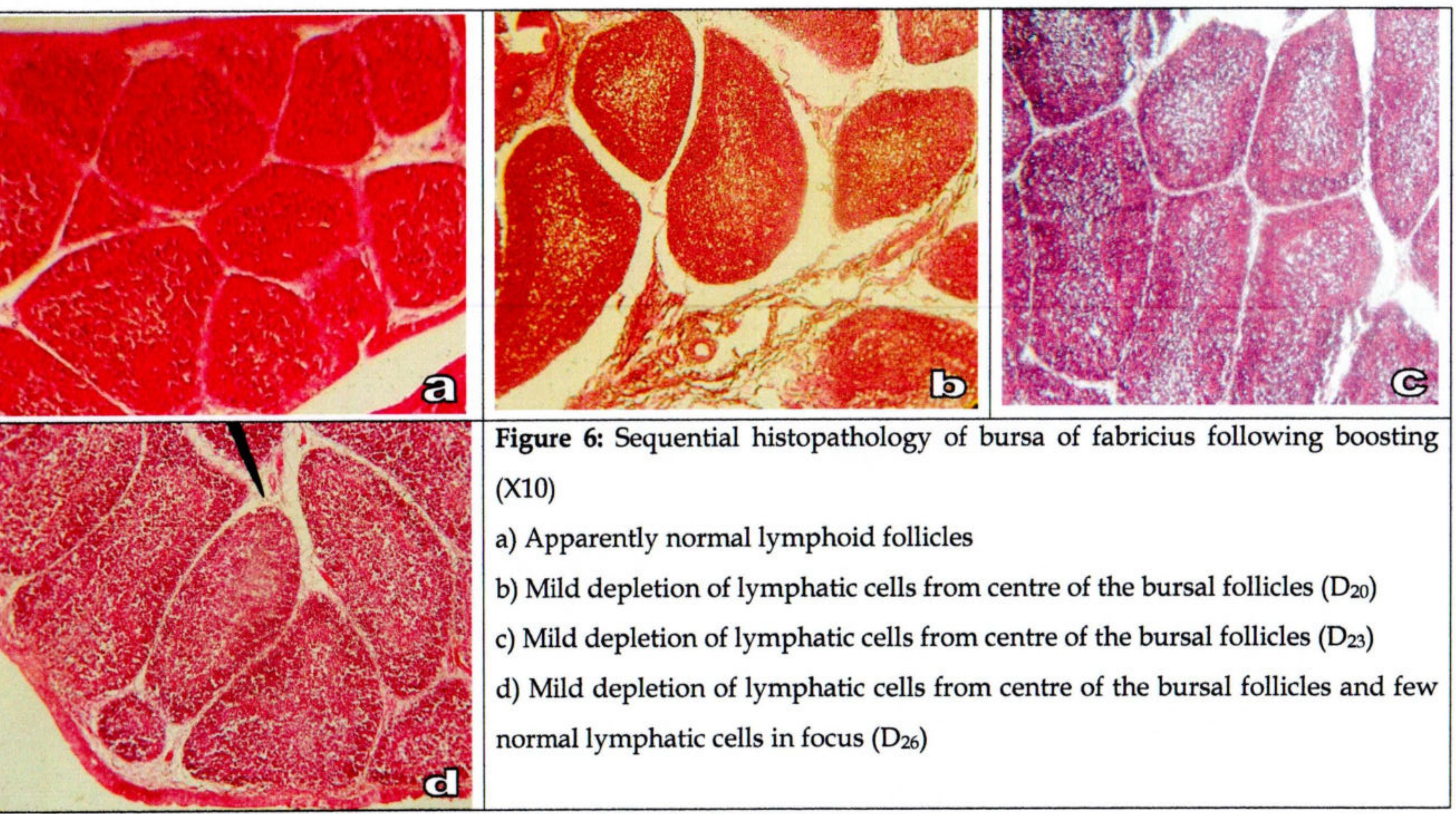


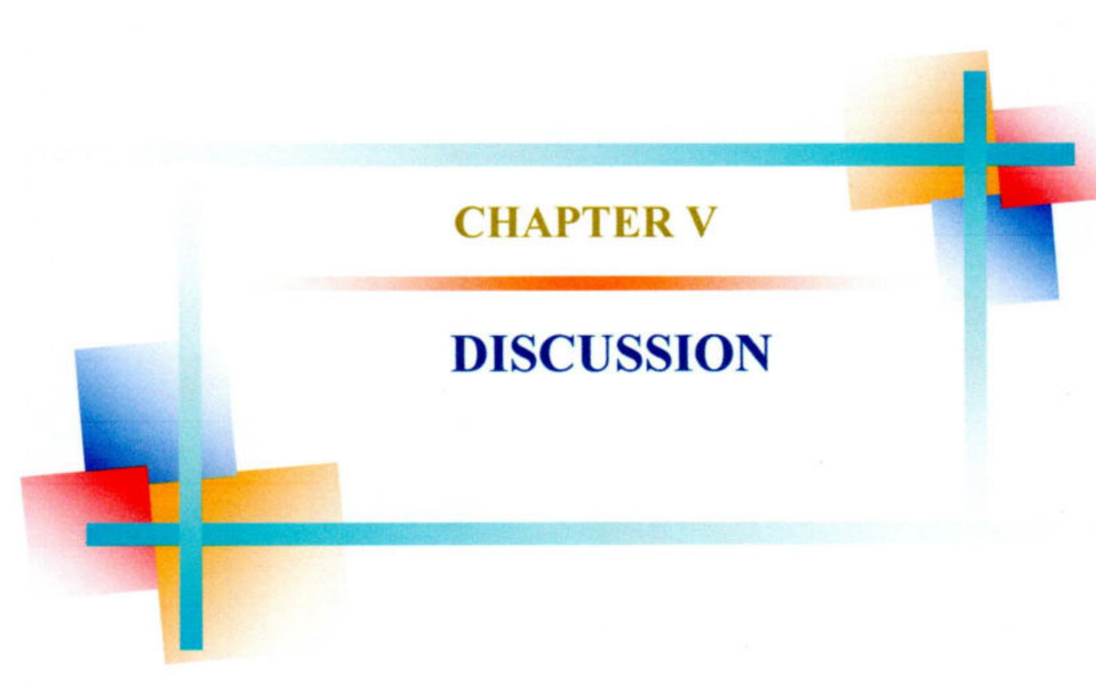
Figure 6: Sequential histopathology of bursa of fabricius following boosting (X10)

a) Apparently normal lymphoid follicles

b) Mild depletion of lymphatic cells from centre of the bursal follicles (D_{20})

c) Mild depletion of lymphatic cells from centre of the bursal follicles (D_{23})

d) Mild depletion of lymphatic cells from centre of the bursal follicles and few normal lymphatic cells in focus (D_{26})



CHAPTER V

DISCUSSION

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Pathogenicity of the Gumboro vaccine prepared from "GM97 strain" of HIPRA GUMBORO®-GM97 vaccine of Infectious Bursal Disease Virus (IBDV) was evaluated in commercial chickens (broiler) and showed relatively reduced pathogenicity in the broiler chickens under farm condition.

The present study was the reflection of the Infectious Bursal Disease Vaccine and determination of efficacy of vaccine after inoculation experimentally in commercial broiler flock. For this experimental study the following points were consider such as clinical signs and symptoms, necropsy or gross morbid lesions, bursal body weight ratios, histopathological lesions of bursa and bursal lesion score.

The vaccination schedule was strictly followed as per vaccine producer. Vaccination schedule is the first and fundamental factor to achieve expected immunogenic protection of the virus (Lukert and Saif, 1997). Faulty vaccination could play the important role to vaccine breaks and outbreaks are common in this context. However no apparent clue related to vaccine break was observed in the present study.

Maternally derived antibody (MDA) sustains in chickens for the first few days and this lasts for a variable times of age of chickens (Giasuddin *et al.,* 2003; Kouwenhoven and van den Bos, 1993). This antibody is an important factor causing inactivation of the vaccine virus and results vaccination failure (Hair-Bejo *et al.,* 2004). MDA could be capable for the innactivation of vaccine virus (Lucio, 1979). However , experimental flock of the present study was vaccinated at D_{11}, and boosted at D_{17} without determining the MDA level and the sampling occasion was done following D_{11} and D17 (Table 5).

Gumboro disease is a highly fatal disease where the morbidity rate was around 100% (Islam *et al.,* 2008) and mortality rate is variable and may up to 80% (van den Berg, *et*

al., 1991; Chowdhury, *et al.*, 1996; Hoque, *et al.*, 2001; Islam *et al.*, 2008). However, there was no apparent morbidity recorded in the present study and mortality rate was also zero following vaccination. This finding is agreed with the researchers (Babiker, *et al.*, 2004; Hasan, *et al.*, 2004).

The clinical manifestations of the typically affected Gumboro disease is characterized as high fever, off feed, reluctant to move, depression , drowsiness, watery diarrhoea, vent picking (Cosgrove, 1962; Islam *et al.*, 1997; van den Berg 2000 and Islam *et al.*, 2008).

However any of the signs stated above were not recorded in the vaccinated flock of the present study and similarly described by the many authors (Hasan *et al.*, 2004). Vaccinated flocks also show different typical clinical signs which certainly determine the failure of vaccination (van den Berg *et al.*, 1991; Hafez *et al.*, 2002) developed either by one or more factors of vaccine breaks (Rosenberger *et al.*, 1987; Islam and Saif, 1991; Eterradossi, 2001).

The routine necropsy was done following primary vaccination as well as boosting in the present study as per as experimental design (Table 5). There was no relevant gross morbid lesions recorded during the course of necropsy in the present experiment. But hemorrhage in the skeletal muscle, hemorrhage in the junction between proventriculus and gizzard, varying degrees of bursal lesions, enteritis etc. are common gross morbid lesions observed both in the vaccinated flock (Cosgrove, 1962; Lukert and Hitchner, 1984; Hoque, *et al.*, 2001; Islam *et al.*, 2008) and in the flock reared without Gumboro vaccination which indicate vaccination failure.

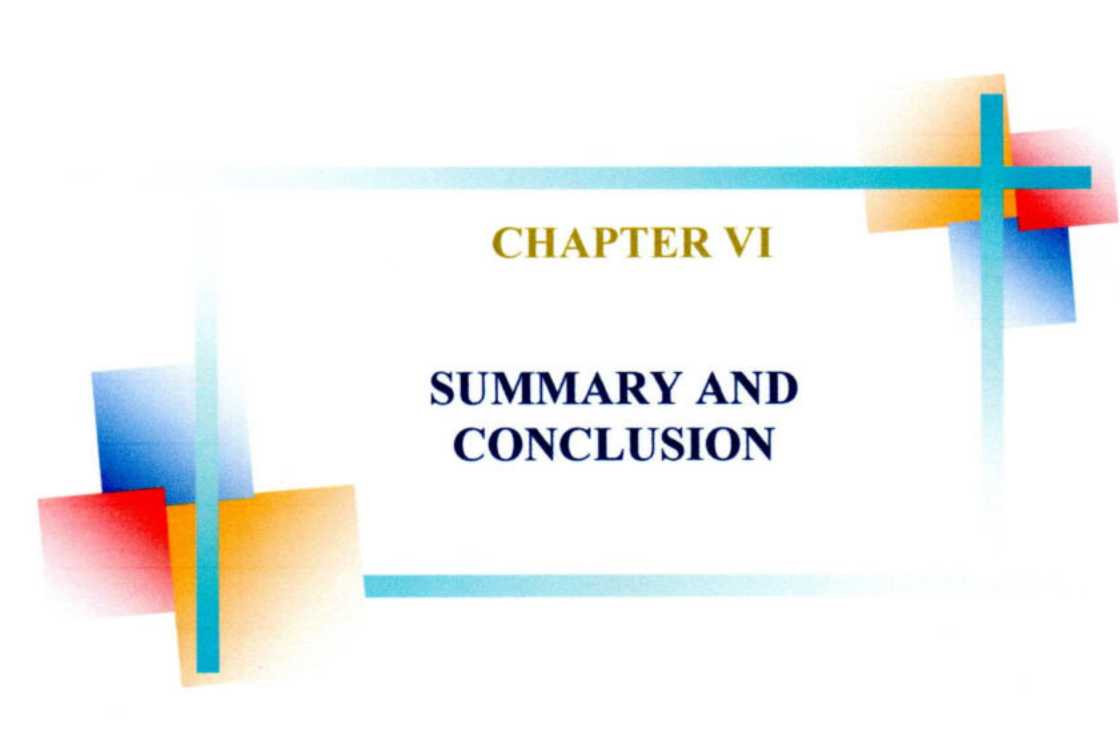
Bursa-body weight ratios are the vital factor in determining the pathogenicity of the respective viruses and there is a proportional relationship between bursa-body weight ratio and the pathogenicity of the respective virus (Mazariegos *et al.*, 1990). However, the bursa-body weight ratios were 1.99±0.15, 2.98±0.09, 3.19±0.03, 2.44±0.34, 2.45±0.11, 2.24±0.41 and 2.38±0.55 at Day $_{11}$, Day $_{13}$, Day $_{15}$, Day $_{17}$, Day $_{20}$, Day $_{23}$ and Day $_{26}$

respectively which differ significantly ($P<0.01$).

The bursa of typically affected flock histopathologically shows mild to severe lymphoid depletion, follicular atrophy, cystic formation of follicles, bursal hemorrhage (Rudd *et al.,* 2001; Hoque *et al.,* 2001; Islam *et al.,* 2008). The level of producing lesions in the bursa of Fabricius is also proportionally related with the degree of pathogenicity of the virus inoculated or infected naturally. In the present study the bursal lesion were histopathologically characterized as either normal follicles with or without mild to moderate lymphoid depletion without follicular atrophy or the development of cystic follicles. There was no indication of follicular regeneration in this study. However, the histopathological lesions observed in the present study did not mean the vaccine breaks, because the lesions stated here might be developed by the vaccine virus, and this agreed with many researchers (Rudd *et al.,* 2001; Alves *et al.,* 2007; Islam *et al.,* 2008) who characterized different bursal lesions produced by some vaccine strain. Bursal lesion scores was determined 1.33±0.33, 1.33±0.33, 2.00±0.58, 0.67±0.33, 1.33±0.33, 1.33±0.33 and 3.33±0.33 at Day $_{11}$, Day $_{13}$, Day $_{15}$, Day $_{17}$, Day $_{20}$, Day $_{23}$ and Day $_{26}$ respectively which differ significantly ($P<0.01$). Relatively low lesion score was observed in all sample occasion. These results are agreed with Raue *et al.,* (2004). Outbreaks in the vaccinated flock are common in experimental area (Islam *et al.,* 2008) but it was inevident in the present study.

From the above facts and findings it was concluded that the virus used in the vaccine HIPRA GUMBORO®-GM97 showed reduced pathogenicity and could be potential to prevent outbreaks in the flock which was characterized as-

- Sound health without development of any clinical signs of vaccinated flock
- Uniform bursal body weight ratios
- Uniform and reduced bursal lesion scores
- No remarkable gross morbid lesion on necropsy
- No outbreaks in vaccinated flock



CHAPTER VI

SUMMARY AND CONCLUSION

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A commercially available HIPRA GUMBORO®-GM97 containing live attenuated GM97 strain of infectious bursal disease virus in live, freeze dried form was tested for its pathogenicity in commercial chickens. Samples were collected from unvaccinated group at the age of D_{11}, then from primary vaccinated group at the age of D_{13}, D_{15}, D_{17} and at the age of D_{20}, D_{23} and D_{26} after boosting at D_{17}. After preservation and histological processing histopathological lesions were observed and bursal lesion scores were determined. Samples from affected flocks were collected, preserved and processed for the comparison of the study.

There was no any remarkable clinical signs found in experimental flocks, but in affected flock significant clinical signs (depletion, ruffled feathers, inappetance, slightly whitish diarrhoea, emaciation, dehydration) typical of IBD were observed. There was zero mortality in experimental groups. Variation in bursal weight was according to age and individual birds. Significant fluctuations in Bursal/Body weight ratios were observed in experimental flock exception with D_{15} (Primary vaccinated birds).

Average bursal lesion score of birds at D_{11} was 1.33, similar mean of bursal lesion score's were determined at D_{13}, D_{20}, D_{23} and D_{26}; there was remarkably high score's for the infected flocks. Apparently normal to mild lymphoid depletion was seen in experimental flock, exception with D_{15}, there was mild to moderate and few severe lymphoid depletion and few atrophied follicles were seen.

But in affected flock severe lymphoid depletion, interfollicular oedema, active lymphoid necrosis, formation of cyst was seen in histopathological examination.

Considering above facts it may be concluded that the live attenuated intermediate plus strain of HIPRA GUMBORO®-GM97 vaccine developed no any remarkable clinical signs and necropsy changes, but induce milder histopathological changes that is not sufficient for disease production. Therefore, the efficacy of vaccine HIPRA GUMBORO®-GM97 vaccine is significant. So the vaccine is efficient to protect IBD in commercial chickens.

It is therefore desirable to make continue of this of instituting the above programs yielding answers to many problems of IBD infection in poultry farms. From the research interest point of view following task may be scheduled for further study

- Evaluation the immunogenicity of HIPRA GUMBORO®-GM97 against field challenge
- Serological evaluation of this vaccine in commercial chickens

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